New Insights into Plant Amino Acid Transport and its Contribution to Nitrogen Nutrition

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Doctoral Thesis
Swedish University of Agricultural Sciences
Umeå 2015

Acta Universitatis agriculturae Sueciae
2015:33

Cover: Different experimental setups
(Photographs courtesy of Sandra Jämtgård and Iftikhar Ahmad)

ISSN 1652-6880
ISBN (print version) 978-91-576-8264-2
ISBN (electronic version) 978-91-576-8265-9
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Print: Arkitektkopia AB, Umeå 2015

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Abstract

Nitrogen (N) is a crucial element for plant growth and development, as it is a key constituent of numerous plant metabolites and structural components. However, most plant N is in the form of amino acids, which are building blocks of proteins, precursors of many secondary metabolites and the "currency" of N within plants (i.e. the form in which N is transported and transferred). Recent studies have shown that amino acids are prevalent in soils and may also serve as N sources for plants. This thesis (and four appended papers) focuses on these aspects of amino acids, particularly the molecular mechanisms of amino acid transport in plants and their potential contribution to plants N nutrition.

In *Arabidopsis thaliana* (Arabidopsis) at least 67 genes are annotated as putative amino acid transporters. In this thesis, results are presented suggesting that two amino acid transporters, LHT1 and AAP5, in Arabidopsis play important roles in amino acid uptake from soil at field-relevant concentrations (Paper I). This conclusion is based on the uptake characteristics of mutant plants (*lht1*, *aap5* and *lht1xaap5*), displaying very little residual root uptake of amino acids. Furthermore, these two transporters have complementary affinity spectra: LHT1 for acidic and neutral amino acids; and AAP5 for basic amino acids. To probe the disputed contribution of organic N to plant N nutrition, mutants with both suppressed and enhanced expression of LHT1 were grown in agricultural soil. The results indicate that plants can take up significant amounts of amino N, despite microbial competition, suggesting that uptake of amino acids contributes to plant N nutrition (Paper II). Moreover it was shown that disrupted N cycling in leaf mesophyll cell affects leaves of different developmental status differently with respect to N and C status. Thus amino acid transport is critical for the maintenance of C and N status and leaf metabolism and LHT1 plays a key role in the process (Paper III).

To broaden the studies from herbaceous plants to trees, a *Populus* orthologue of LHT1 (PtrLHT1.2) was identified. The *PtrLHT1.2* gene from *Populus tremula* L. x *tremuloides Michx*. was cloned and expressed in a *LHT1* uptake mutant of Arabidopsis. Results of its heterologous expression in Arabidopsis suggest that PtrLHT1.2 is a functional orthologue of AtLHT1 (Paper IV), indicating that PtrLHT1.2 could be involved in root amino acid uptake in Poplar tree.

Keywords: Amino acids, organic N transport, amino acid transporters, Arabidopsis, Poplar, amino acid cycling.

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Dedication

To my parents

Contents

List of Publications

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

I Svennerstam H, Jämtgård S, Ahmad I, Huss-Danell K, Näsholm T, Ganeteg U (2011). Transporters in Arabidopsis roots mediating uptake of amino acids at field relevant concentrations *New Phytol* 191: 459-467

II Ganeteg U, Ahmad I, Jämtgård S, Cambui CA, Inselsbacher E, Svennerstam H, Schmidt S, Näsholm T (2015) Amino acid transporter mutants of Arabidopsis suggest a role of soil organic nitrogen for plant nutrition. *Submitted manuscript.*

III Ahmad I, Matrosova A, Svennerstam H, Holmlund M, Ninkovic V, Israelsson-Nordström M, Ganeteg U. The Lysine Histidine Transporter 1 regulates leaf C/N balance in Arabidopsis. *Manuscript.*

IV Ahmad I, Gratz R, Svennerstam H, Jämtgård S, Love J, Holmlund M, Ivanov R. Ganeteg U. The early-senescence-like and root-uptake phenotypes caused by mutation of *Arabidopsis* LHT1 can be alleviated by heterologous expression of the *Populus* amino acid transporter PtrLHT1.2 *Manuscript*

Paper I is reproduced with the permission of the publisher.

The contribution of Iftikhar Ahmad to the papers included in this thesis was as follows:

I Performance of the work, contributing to the writing and preparation of the manuscript.

1 Contributing to the ideas, performance of the work, contributing to the writing and preparation of the manuscript.

2 Planning, performance of the work, analysis and summary of the results, writing and preparation of the manuscript.

3 Planning, performance of the work, analysis and summary of the results, writing and preparation of the manuscript.

Abbreviations

All abbreviations are explained when they first appear in the text.

1 Introduction

1.1 Nitrogen

Nitrogen (N) is a key element for life on earth. It is an essential constituent of myriads of components of all living organisms, including chlorophyll, nucleic acids, amino acids and proteins. Consequently, it also plays major roles in virtually all physiological processes. In addition, most (78%) of the atmosphere consists of N. However, this is largely in the inert gaseous form N_2, which is not generally available for plants because only a few organisms can break its triple bond, thereby converting it to reactive N. It also plays key biogeochemical roles through the so-called N cycle, referring to the flows of diverse forms of N in the biosphere. A major process in the N cycle is the conversion of N_2 to reactive N species, “nitrogen fixation", which is mediated by both natural and synthetic processes. The natural processes include lightning, photochemical reactions and biological N fixation. During lightning discharges the strong triple bond of the N_2 molecule is broken and some N atoms react with free oxygen and hydrogen, forming HNO_3. This HNO_3 can fall to earth with rain and become available for plants as NO_3^-.

The main form of natural N fixation, accounting for about 90% of the total, is biological (Oldroyd and Dixon, 2014). In this process N is fixed by N-fixing microorganisms via a reaction, catalysed by the enzyme nitrogenase and energized by ATP hydrolysis, which converts inert N_2 gas into readily available NH_3 (Oldroyd and Dixon, 2014). N-fixing microorganisms can exist both as free-living bacteria and in symbiotic relationships with various host plants. The free-living N-fixing bacteria, e.g. Cyanobacteria and *Rhodospirillum*, are generally independent of other organisms. Other N-fixers, such as *Rhizobium*, *Frankia* and *Acetobacter* form symbiotic relationships with plant roots. The N-fixing *Rhizobium* forms root nodules with roots of leguminous plants. Leguminous plants are important contributors to biological

N fixation in the global N cycle since there are 17,000 legume species, many of which have high economic and nutritional values (O'Rourke et al. 2014). In this symbiotic relationship the plant acquires NH_3 as an N source while *Rhizobium* obtains carbohydrates required for its growth (Bernhard, 2010; O'Rourke et al. 2014). In addition to natural N fixation, N can be fixed industrially by the Haber-Bosch process, which generates NH_3 from N_2 under high atmospheric pressure and temperatures in the presence of an iron catalyst.

The use of fertilizers generated by the Haber-Bosch process has significantly enhanced the productivity of many agricultural systems (Smil, 1999) and forests. Thus, N fertilizers have played key roles in providing the growing human population with food and other resources. In addition, agriculture and forestry are under intense pressure to increase production further, from limited areas, resulting in increasing use of commercial N fertilizers. However, excessive use of fertilizers has resulted in environmental pollution and anthropogenic activities have dramatically altered the biogeochemical N cycle. Indeed, according to Gruber and Galloway (2008), excessive use of inorganic N (IN) fertilizers is the biggest cause of N cycle perturbation. The applied N fertilizers have only 30-50 % efficiency, hence large amounts of N fertilizers are leached out (Tilman et al. 2002), and the increasing pollution resulting from these activities is affecting human health as well as the environment. Thus, the environmental effects of N fertilizers are alarming and there are urgent needs to identify ways to use N more efficiently and reduce its impact on both the environment and human health. Hence there are equally urgent needs for research regarding all aspects of plant N nutrition and nitrogen use efficiency (NUE). NUE depends on several processes, including N uptake, translocation, assimilation and remobilization (Good et al. 2007; Masclaux-Daubresse et al. 2010). It can also be defined in several ways, with respect (for instance) to plant biomass or grain yields, e.g. as the yield produced per unit of N supplied to plants in soil (Lea and Azevedo, 2006), or the ratio between amounts of N removed from the field by harvesting plants and amounts of N fertilizer supplied, as reviewed in Avice and Etienne (2014). Modern agricultural practices and research can substantially increase NUE and reduce adverse effects of fertilization on human health and the environment. For example, Chen et al. (2014) found that grain yields of three staple crops could be increased (while substantially reducing N fertilizer applications, N leaching and greenhouse gases emissions) by using integrated soil/crop system management practices in China. Nevertheless, increasing NUE and decreasing the use and cost of synthetic fertilizers remain major challenges for N nutrition researchers (McAllister et al. 2012).

1.2 Soil nitrogen

Since plants require large amounts of N, thus N deficiency can severely inhibit their growth and development. In the soil, plant-available N can be divided into two main categories: inorganic (mainly NO_3^- and NH_4^+) and organic (mainly amino acids, peptides and proteins). Plant roots can take up N of both categories (Lipson and Näsholm, 2001; Näsholm et al. 2009). Therefore, to optimize plant N nutrition it is essential to understand both the absolute and relative concentrations of different N forms in soil solutions, and their availability to plants.

In the soil, dead organic matter is decomposed by activities of soil microbes that release complex organic N forms such as proteins and peptides, from which organic N-containing monomers (amino acids) are released by proteolytic enzymes. Amino acids can be taken up directly by plant roots (Näsholm et al. 1998; Näsholm and Persson, 2001) or be mineralized by microorganisms into inorganic N forms: NH_4^+ and NO_3^- (Figure 1).

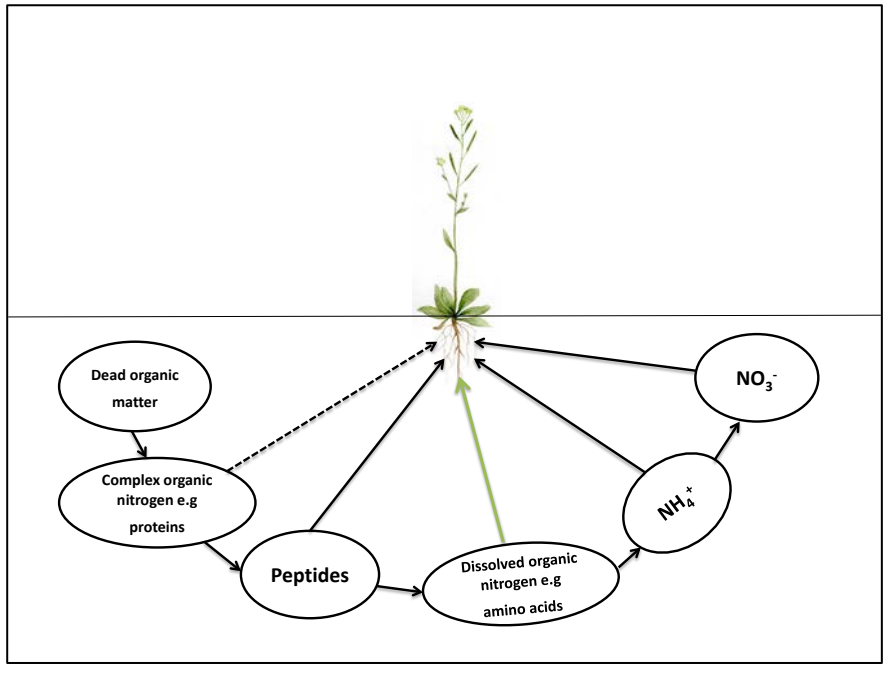

Figure 1. The transformation of complex N forms in the soil, schematically illustrated by the degradation of proteins in dead organic matter into plant-accessible amino acids (green arrow), and further conversion into inorganic N compounds by bacterial and fungal activities.

It has long been known that plants take up inorganic N, but there is strong and growing evidence that amino acids are also important direct N sources,

particularly in cold ecosystems such as boreal forests (Näsholm et al. 1998; Read and Perez-Moreno, 2003) and taiga forests (Kielland et al. 2007), where mineralization rates are slow and soils contain abundant organic N about five times larger than inorganic N pool. Moreover, the concentration and turnover of free amino acid is substantially higher in seasonally frozen soils of Taiga forests (Kielland et al. 2007). The importance of organic N for plants in natural ecosystems can be described by the abundance of plant-available organic N in soil and rates of its uptake (relative to rates of inorganic N uptake) by plant roots. In soils of many regions the organic N pool is larger than the inorganic N pool, e.g. boreal and taiga soils frequently have five-fold higher organic N (ON) contents than inorganic N contents, as reviewed by (Näsholm et al. 2009; Warren, 2013). Furthermore, Kielland et al. (2006) demonstrated that in some soils amino acids are more abundant than inorganic N. Similarly, Yu et al. (2002) described the distribution of hydrolysable proteins and free amino acids in total dissolved organic N (DON) pool of soils. It was reported that, in some of the soils, the total dissolved N pool comprised of 77 to 99 % of DON, of which free amino acid constitute about 1.5-10%. In addition, proteins, peptides and bound amino acids are major contributors of the total organic N pool. In fact beside inorganic N, the soil contains large quantities of organic N but those forms of N are not always available for plant uptake. For this reason, Schimel and Bennett (2004) suggested a new model of the N cycle, and proposed that depolymerisation of complex N compounds, e.g. proteins/peptides, is the rate-limiting step, and thus the bottleneck for production of accessible organic N compounds, e.g. amino acids. A further indication of the importance of organic N in soils is the recent finding by Inselsbacher and Näsholm (2012) that amino acids account for ca. 80% of diffusive fluxes of N in boreal forest soil, and inorganic forms just ca. 20%. Agricultural soils have also been shown to contain significant amounts of soluble amino acids (Jämtgård et al. 2008; Holst et al. 2012).

Furthermore, Jämtgård et al. (2010) found ca. 50-fold higher concentrations of bound amino acids than free amino acids (0.1-12.7 µM), and that the bound organic N pool was larger than the total inorganic N pool, in solutions of agricultural soils of mid-Sweden. Higher rates of production and concentrations of amino acids than inorganic N forms have also been found in soils of temperate forests (Berthrong and Finzi, 2006). Thus, several studies have shown that forms of N other than mineral N are present and available in various terrestrial ecosystems (reviewed in Näsholm et al. 2009).

1.3 Plant N sources

Plants are capable of using a wide range of N forms as N sources, for instance the inorganic forms NO_3^- and NH_4^+, and organic forms such as proteins, peptides, amino acids and chitin. In several species, NH_4^+ was absorbed at highest rates followed by amino acids and NO_3^- (Ohlund and Näsholm, 2001; Näsholm et al. 2009). Amino acids are also the major transport form of N in plants (Tegeder and Ward, 2012; Tegeder, 2014), and can thus be regarded as the "currency" of N exchange (Bush, 1999; Chen et al. 2001; Pratelli and Pilot, 2014). Furthermore, in contrast to IN, amino acids are sources of both C and N for plants. Thus, they are highly valuable as the assimilation and metabolism of C and N is strongly inter-dependent: photosynthesis and growth are dependent on N supply, while C-skeletons are required for N uptake, assimilation and utilization (Stitt and Krapp, 1999).

Traditionally, most research on plants N nutrition has focused on inorganic N forms, because of beliefs that plants exclusively take up NO_3^- and NH_4^+ (Näsholm et al. 2009; Paungfoo-Lonhienne et al. 2012) and only depend indirectly on organic N via its mineralization to NO_3^- and NH_4^+. However, recent research has challenged this paradigm by showing that organic N is an important part of soil total N contents and the overall N cycle, although its quantitative importance as a direct component of plants N budgets is still debated.

1.3.1 Organic N sources

Recent plant ecophysiology and biogeochemistry studies have supported the notion that plants annual N requirements exceed annual mineral N production. This clearly implies that plants must also acquire N from other sources, and likely possibilities are organic forms (Schmidt and Stewart, 1999; Näsholm et al. 2009). Accordingly, Näsholm et al. (1998) demonstrated that diverse plants can directly take up dually-labelled amino acids from boreal forest soils, i.e. without mineralization, and either with or without mycorrhizal associations (Chapin et al. 1993; Näsholm et al. 1998; Näsholm et al. 2001). For example, Persson and Näsholm (2001a) examined uptake rates of 15 universally labelled amino acids by 31 plant species from six contrasting boreal forest habitats, and concluded both that plants can generally take up amino acids and that they may play a significant role in plant N nutrition. Moreover, amino acids have been shown to be taken up and used as N sources by plants of temperate, arctic, tundra, Mediterranean and alpine ecosystems (Schimel and Chapin, 1996; Schmidt and Stewart, 1999; Näsholm et al. 2009).

In addition to amino acids, Paungfoo-Lonhienne et al. (2008) have shown that two non-mycorrhizal species, *Hakea actites* and Arabidopsis, can take up

and assimilate proteins without assistance of any mycorrhiza. They suggested that proteins may not promote plant growth as strongly as inorganic N if they are the sole N source, but may provide significant complementary N supplies. Thus, amino acids, peptides and even proteins can all be used as N sources by plants, and may play significant roles in their nutrition. However, the roles of amino acids in this context have been most extensively studied because of their small size, rapid diffusion and uptake rates, and importance for plant growth and metabolism (Näsholm et al. 2009; Inselsbacher and Näsholm 2012).

1.3.2 Objections to the significance of organic N uptake

More than 100 years ago Hutchinson and Miller (1911) proposed that organic N promotes root and shoot growth of plants, but for many years this possibility and associated phenomena were largely neglected. Organic N research, particularly physiological and molecular aspects of amino acid uptake and metabolism, have received increasing attention recently. However, there is ongoing debate about the true significance of these processes, and the degree to which reported observations of direct uptake of intact amino acids in the field may be artefacts arising from mineralization of NH_4^+ by soil microbes prior to plant uptake.

The main objections to the hypothesis that organic N may play an important role in plants' nutrition are as follows:

1. The direct uptake and contribution of organic N to plant N nutrition is negligible compared to the uptake and contribution of inorganic N sources.
2. Following the application of dually-labelled amino acids to soil, the ^{15}N and ^{13}C are separated by microbes, e.g. by transamination, prior to plant N uptake (Jones et al. 2005). Plants then take up the ^{15}N as $^{15}NH_4^+$ while the ^{13}C label is lost as $^{13}CO_2$, or taken up as carbon back-bones released by the microbes (Kuzyakov and Xu, 2013).
3. Microbes outcompete plants for N because of their large surface areas for absorption and higher substrate affinities (Hodge et al. 2000), hence plants only take up N that is left over from the microbial mass.
4. Amino acids have minor importance since they constitute a small component of the whole N pool of soil. Most studies supporting amino acid uptake have used higher amino acid concentrations than natural occurring soil concentrations, so the results may give biased indications of their importance.
5. Only small percentages of dual-labelled amino acids are recovered as intact amino acids.

The competition between plant and soil microorganisms for N uptake is a complex process and the presence of mycorrhizal associations with some plant

roots adds further complexities (Hodge et al. 2000). There are also gaps in our knowledge of plant organic N nutrition. Therefore, the uptake, transport and contribution of amino acids to plant total N budgets need further investigation.

However, current knowledge of these phenomena is summarized in the following sections.

1.4 Nitrogen transporters

In plants, the transport of molecules and ions into or between cells and translocation between source and sink tissues is regulated by cellular membrane processes. Transport of any solute or molecule against a concentration or electrochemical gradient (from high to low potential) requires an energy input and is thus defined as active transport, whereas movement of molecules down the gradient does not require any direct energy input and is thus defined as passive transport (Sondergaard et al. 2004), although energy may be required to construct and maintain machinery (transporters) that permits solutes to cross membranes.

There are also two types of active transport: primary and secondary. In primary active transport the chemical energy source (e.g. ATP-hydrolysis, light, or red-ox reactions) is directly coupled to the transporter. In secondary active transport the energy stored in an electrochemical gradient over the membrane is used: one solute moves down the electrochemical gradient while another solute is moved against the gradient, a process called “co-transport” (Sondergaard et al. 2004). Transport proteins are classified as pumps (primary active), carriers (passive or secondary active) and channels (passive). Pumps utilize chemical energy (ATP), light or red-ox reactions to drive transport processes. The carriers, e.g. amino acid transporters, bind a specific solute and undergo a conformational change that transfers the solute to the other side of the membrane. Channels form water-filled selective pores in membranes and mediate passive transport of inorganic ions such as K^+, Ca^+ and Cl^- across membranes.

The concentration dependence of substrate uptake rates can be used to assess the transport mechanism of a given solute. Carrier transport involves binding and dissociation of molecules in active sites of transporter proteins, so transport rates increase with increasing substrate concentration until the binding-sites are fully occupied. In contrast, simple diffusion through open channels is not restricted by substrate binding/dissociation. Therefore the transport rate is directly proportional to the difference in concentration between the two sides of the membrane (assuming that adsorption of the solute to other substances and other potentially interfering processes can be neglected).

Saturated uptake patterns resemble enzyme kinetics, and carrier transport can thus be analysed using the Michaelis-Menten parameters K_m and V_{max} (Michaelis and Menten, 1913; Johnson and Goody, 2011). V_{max} is defined as the maximum uptake rate at saturating concentrations while K_m is defined as the substrate concentration at which half of V_{max} is achieved. The Michaelis-Menten parameters were initially developed to characterize enzymatic kinetics, but they can also be applied to substrate uptake rates, concentration dependency, and substrate affinities of transporters. The kinetic parameters (K_m and V_{max}) of transport rates can be used to distinguish transporters of different classes; a low K_m value indicates activity of a high affinity transporter while a high K_m value indicates activity of a low affinity transporter.

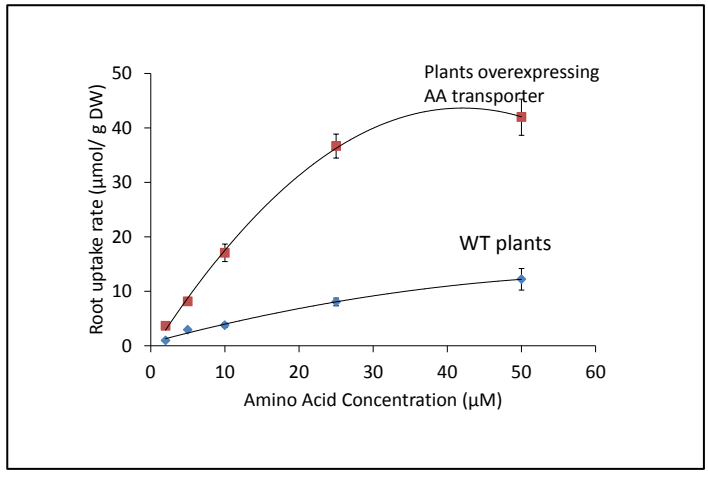

Figure 2. Root uptake of L-Gln by WT and 35S::AtLHT1 Arabidopsis plants. The Km for L-Gln uptake is similar in both genotypes but the LHT1 overexpressors have higher L-Gln uptake rates and hence higher Vmax values.

The concentrations of NH_4^+, NO_3^- and organic N (e.g. amino acids, nucleotides and peptides) vary widely among different soil types, tissues and cell compartments (Glass et al. 2002; Näsholm et al. 2009). To cope with this diversity of N forms and concentrations, plants have evolved separate uptake systems for different N forms. In higher plants several transporters for N compounds have been identified and characterized. These transporters differ in tissue expression patterns, specificity, affinity and capacity for different N compounds (Williams and Miller, 2001; Glass et al. 2002; Miller and Cramer, 2005). Transport systems are categorized on the basis of their affinities towards the substrate (Glass et al. 2002) and knowledge of the known classes of plant N transporters is briefly reviewed below.

1.4.1 Inorganic N transporters

In Arabidopsis four families of NO_3^- transporters have been identified: the Nitrate Transporter 1 (NRT1), Nitrate Transporter 2 (NRT2), Chloride Channel (CLC) and slow anion-channel associated homologue (SLAH) families (Krapp et al. 2014). Moreover, besides being an important nutrient for plant growth, NO_3^- acts as a signalling molecule that regulates gene expression, NO_3^- uptake and metabolism (Krapp et al. 2014). Little et al. (2005) showed that the NRT2.1 transporter is not only involved in uptake but can also act as a NO_3^- sensor when external concentrations of NO_3^- are low and coordinate responses that increase its availability.

There are two general classes of NO_3^- uptake systems in plants: high affinity transporters (HATS), which operate at concentrations lower than 0.5-1.0 mM and low affinity transporters (LATS), which take up NO_3^- at higher concentrations (Williams and Miller, 2001; Nacry et al. 2013; Krapp et al. 2014). The HATS are further categorized into cHATS (constitutively active high affinity transporters) and iHATS (inducible high affinity transporter). The cHATS are constitutively expressed, even in the absence of NO_3^-, while iHATS are only expressed after exposure to NO_3^- concentrations lower than 0.5 mM (Glass et al. 2002). In addition, NO_3^- uptake is energized by the maintenance of an electrochemical gradient across membranes mediated by ATP hydrolysis and proton co-transport (Williams and Miller, 2001; Glass et al. 2002; Miller and Cramer, 2005; Krapp et al. 2014). In Arabidopsis the NRT1 and NRT2 families of NO_3^- transporters are well characterized and NO_3^- transport is proton-coupled (Miller and Cramer, 2005; Nacry et al. 2013; Sun et al. 2014).

Like their NO_3^- counterparts, NH_4^+ uptake systems are divided into HATS and LATS: the HATS are uniport carriers that mediate active transport (Ludewig et al. 2002) while the LATS provide channels that allow passive transport at higher substrate concentrations, with a threshold of ca. 200 µM between them (Williams and Miller, 2001). Molecular studies have revealed that Arabidopsis possesses six HATS for NH_4^+ transport, belonging to the ammonium transport (AMT) gene family (reviewed by Nacry et al. 2013; Williams and Miller, 2001).

1.4.2 Amino acid transporters

The uptake, transport and distribution of amino acids are facilitated by membrane proteins called amino acid transporters. Amino acid transport is a secondary active process mediated by proton-coupled symport across membrane barriers (Bush, 1993). Amino acid transporters are thus gatekeepers for the influx and efflux of amino acids between and within cells. Hence, they

are positioned at interfaces of complex metabolic networks that regulate intra- and extra-cellular transport as well as translocation between sources and sink tissues, as reviewed in Tegeder (2012).

As already mentioned, in Arabidopsis more than 60 genes encode known or putative amino acid transporters (Rentsch et al. 2007; Tegeder and Rentsch, 2010; Tegeder, 2012). These amino acid transporters are differentiated on the basis of tissue expression patterns, substrate specificity and environmental and developmental control of their expression and activity (Liu and Bush, 2006). Plant amino acid transporters are classified into two super families: the ATF (amino acid transporter) and APC (amino acid polyamine choline facilitator) families. The ATF family is also called the amino acid/auxin permease (AAAP) family. The ATF family is comprised of six subfamilies consisting of: amino acid permeases (AAPs), lysine histidine transporters (LHTs), proline transporters (ProTs), ϒ-aminobutyric transporters (ϒ-GATs, which transport GABA and GABA-related compounds), aromatic and neutral amino acid transporters (ANTs) and the auxin-resistant family (AUX) (Fischer et al. 1998; Rentsch et al. 2007; Pratelli and Pilot, 2014).

The APC family of amino acid transporters consists of two subfamilies: cationic amino acid transporters (CATs) and L-type amino acid transporters (LATs) (reviewed in Rentsch et al. 2007; Pratelli and Pilot, 2014). Important functions of amino acid transporters include root amino acid uptake, intra- and extra-cellular transport, long-distance translocation and remobilization of amino acids, and since transporters regulate plant metabolism they are essential for, and profoundly affect, plant growth and development (Sondergaard et al. 2004; Liu and Bush, 2006; Rentsch et al. 2007; Tegeder, 2012).

1.4.3 Identification and characterization of amino acid transporters

In order to understand N transport regulation and N allocation in plants it is essential to identify and characterize amino acid transporters (Rentsch et al. 2007). In efforts to identify these agents both forward and reverse genetic techniques have been applied. Forward genetics techniques are used to identify the gene(s) responsible for particular phenotypic characteristic(s), whereas reverse genetics techniques are used to identify the phenotypic characteristics associated with a known gene. Forward genetic studies typically involve screening randomly generated mutants for a particular phenotypic trait, followed by genetic analyses to identify the mutated gene(s). Reverse genetic studies commonly start with repression or overexpression of a known gene, followed by phenotyping to unravel its function. Thus, both approaches are used to study gene functions and have been used to identify various amino acid transport proteins in plants.

Another widely used technique, generally applied to verify a gene's function, is genetic complementation. Notably, genetic complementation of yeast mutants is an effective approach for identifying and isolating proteins responsible for the transport of diverse compounds, such as sugars and amino acids (Bush, 1993; Chen and Bush, 1997). In this technique a mutant yeast strain lacking a targeted capacity is transformed with a library of cDNAs then subjected to conditions in which the only yeast cells that should be able to grow are those that have been transformed with a gene that "complements" the mutation. Such genes are functional analogues of the gene impaired in the mutant, thus the inserted cDNA "rescues" the mutant phenotype.

Frommer et al. (1993) identified and isolated the amino acid permease transporter 1 (AAP1) from an Arabidopsis cDNA library by genetic complementation of a yeast transporter mutant. This yeast mutant was defective in L-Pro uptake, and hence unable to grow on medium containing L-Pro as the sole source of N. Transformants were screened on medium with L-Pro as the sole N source, and the AAP1 transporter was isolated from those that rescued the uptake phenotype of the yeast mutant. It was found to be a hydrophobic, 53 kDa protein, with 12 membrane-spanning regions and no homology to previously known transporters. In the same year, Hsu et al. (1993) also reported isolation of the AAP1 transporter by functional complementation of a yeast histidine (L-His) transporter mutant. Following the discovery of AAP1 many other plant amino acid transporters were identified by molecular cloning and genetic complementation in yeast.

In addition to amino acid transporters that are involved in amino acid uptake, plants also seem to possess amino acid exporters, which mediate effluxes of amino acids from cells. The *in planta* functions of these proteins are not well understood, and indications that they may act as exporters are indirect, mostly based on sequence homologies to proteins with such functions in bacteria and other organisms (Rentsch et al. 2007; Tegeder, 2012). However, the Arabidopsis BAT1 (bi-directional amino acid transporter) reportedly mediates uptake of alanine (L-Ala) and arginine (L-Arg), but may also export glutamine (L-Gln) and lysine (L-Lys) when expressed in yeast (Dundar and Bush, 2009). Similarly, Pilot et al. (2004) showed that the glutamine dumper 1 (GDU1) gene encodes a putative membrane protein that could be involved in regulation of glutamine secretion from cells, and Pratelli et al. (2010) subsequently showed that seven glutamine dumper proteins are involved in amino acid export in Arabidopsis cells.

The studies this thesis is based upon included experiments with members of the LHT and AAP families, therefore these transporters are discussed in more detail in the following sections.

1.4.4 The LHT amino acid transporter family

In Arabidopsis, the LHT amino acid transporter family includes 10 members, designated LHT1-10 (Tegeder and Ward, 2012). Chen and Bush (1997) isolated Lysine Histidine Transporter 1 (LHT1) from an Arabidopsis Expressed Sequence Tag (EST) cDNA library by functional complementation of a yeast amino acid transporter mutant. Results of the yeast studies suggested that LHT1 is a member of a new class of transporters, which was named in accordance with the substrate specificity observed by Chen and Bush (1997). It was identified as a hydrophobic membrane protein of 446 amino acids with 9-10 transmembrane domains. Analysis of its tissue expression pattern suggested that LHT1 is preferentially expressed in leaves, siliques, flowers and roots. Chen and Bush (1997) analysed the substrate specificities of LHT1 for 13 amino acids and suggested that LHT1 has high affinity for L-Lys, L-His and L-Glu but low affinity for L-Leu, L-Ala, and L-Ser.

To identify transporters involved in root uptake of amino acids, Hirner et al. (2006) screened six Arabidopsis amino acid transporters mutants (*LHT1*, *AAP3*, *AAP6*, *AAP8*, *AAP2* and *AAP4*) on media with amino acids as their sole N source. Of these, only *LHT1* mutants showed growth inhibition, which was accompanied by reduced amino acid uptake rates, showing that LHT1 plays important roles in not only amino acid uptake but also plant growth and development. In addition, Hirner et al. (2006) showed that the LHT1 amino acid transporter is expressed in root epidermis and leaf mesophyll cells of Arabidopsis. Hence, beside root uptake, LHT1 has another very important role in leaf uptake and cycling of amino acids in the mesophyll. In this cycling process, amino acids released from cells (efflux), either passively or actively, are resorbed from the apoplasm into the cytosol (influx). Any disruption in this cycling will disturb N homeostasis within the cells. This was corroborated by the finding that amino acids accumulated in the apoplasm of *lht1* plants (Hirner et al. 2006), indicating that their leaf mesophyll cells cannot take up these amino acids from the apoplasm.

In order to determine if the root and leaf uptake phenotypes were independent of each other, Hirner et al. (2006) re-expressed *LHT1* under the control of a leaf specific promoter in *lht1* knock-out mutants. When these plants were grown on fertilized soil the leaf phenotype was rescued, showing that the leaf phenotype was not caused by the root uptake deficiency. Moreover, several other kinds of N cycling in plants have been detected, in addition to amino acid cycling in mesophyll cells. The initial distribution of N is followed by metabolic re-cycling and finally remobilization of N from senescing tissues to actively growing tissues (Cooke and Weih, 2005). Moreover, Cooper and Clarkson (1989) hypothesized that N is cycled between

roots and shoots, and thus about 60% of amino N is cycled within the xylem. In addition, N atoms are generally cycled at least once before being incorporated into plants' structural components. Thus, the cell-to-cell transport and cycling of amino acids in mesophyll cells play crucial roles in plant's N dynamics, physiology and growth, and LHT1 plays a key role in the retrieval of amino acids and their cycling in leaf mesophyll cells (Hirner et al. 2006).

In contrast to the findings of Chen and Bush (1997) in yeast complementation assays, Hirner et al. (2006) found indications that LHT1 in Arabidopsis has low affinity for basic amino acids, intermediate affinity for acidic amino acids, such as L-Asp and L-Glu, and high affinity for Gly, L-Ala, L-Pro and L-Ser. Svennerstam et al. (2007) also identified LHT1 as an important transporter for root uptake of amino acids by screening seeds from ethyl methanesulfonate (EMS)-treated plants and a collection of 69 T-DNA knock out transporter mutants of Arabidopsis (with insertions in 39 genes). Both the forward (EMS) and reverse genetic (T-DNA insertion) analyses indicated that the LHT1 transporter plays an essential role in uptake of amino acids in Arabidopsis roots. The uptake affinities of LHT1 were tested using a solution containing seven amino acids at 25 µM. In accordance with Hirner et al. (2006) LHT1 was found to be involved in root uptake of acidic and neutral amino acids. The *in planta* results of Svennerstam et al. (2007) conflicted with the conclusions of Chen and Bush (1997), but verified those of Hirner et al. (2006), that LHT1 has high affinity for L-Ala, Gly and L-Ser, but lower affinity for L-His, L-lys and L-Glu in Arabidopsis.

Taken together, available evidence shows that the LHT1 transporter is involved in root uptake of acidic and neutral amino acids, as well as uptake and cycling of amino acids in mesophyll cells (Hirner et al. 2006; Svennerstam et al. 2007). In addition, LHT1 reportedly negatively regulate disease resistance by modifying Gln availability through its interaction with a salicylic acid-dependent pathway (Liu et al. 2010).

Besides LHT1, other members of the LHT family also have important functions in plants. Lee and Tegeder (2004) reported that AtLHT2 is a high affinity transporter for the uptake of acidic and neutral amino acids into tapetum cells of anthers, and subsequently showed that it plays an important role in early stages of flower development. Other LHTs (LHT4/5/6) have different spatial and temporal expression patterns in floral organs, and hence apparent roles in plant reproduction (Foster et al. 2008). Perchlik et al. (2014) proposed that LHT6 is also involved in root uptake of amino acids. It was shown to have affinity for acidic amino acids as well as L-Gln, L-Ala and L-Phe, but not basic amino acids. Thus, the LHT subfamily generally seems to

have high affinity for acidic and neutral amino acids (Lee and Tegeder, 2004; Hirner et al. 2006).

1.4.5 The AAP amino acid transporter family

In Arabidopsis, the AAP subfamily consists of eight members, designated AAP1-8, which transport different amino acids (Rentsch et al. 2007; Svennerstam et al. 2008; Lee and Tegeder, 2004; Tegeder, 2012), and are involved in various processes such as root uptake (Lee et al. 2007; Hirner et al. 2006; Svennerstam et al. 2008), phloem loading and seed loading (Tegeder and Rentsch, 2010; Tegeder 2012; Tegeder 2014) of amino acids.

Lee et al. (2007) identified AAP1 as a root transporter that takes up neutral amino acids, L-His and L-Glu at concentrations of 150 µM or more. However, Perchilk et al. (2014) proposed that the AAP1 transporter can take up L-Glu and neutral amino acids at naturally occurring soil concentrations (30 µM and 150 µM). Like LHT1, AAP1 reportedly has weak affinity for basic amino acids such as L-Lys and L-Arg. Since all members of the LHT transporter family show affinity for acidic and neutral amino acids, but not basic amino acids, plants presumably have separate transport systems for taking up basic amino acids (Svennerstam et al. 2007). Hence, Svennerstam et al. (2008) applied two screening strategies to identify low- and high-affinity transporters for these substances. In the first strategy 23 amino acid transporter T-DNA mutant lines were grown on media containing growth-inhibiting concentrations of L-Arg (3 mM nitrate + 1 mM L-Arg), assuming that mutants with impairments in a low affinity transporter would grow most strongly. In the second strategy the mutant plants were grown on media with very low concentrations of ^{15}N-labelled L-Arg (3 mM nitrate + 0.03 mM L-Arg), assuming that any mutant line with a significant reduction in ^{15}N uptake would have deficiencies in a high affinity transporter. Both strategies identified the AAP5 transporter as being essential for the uptake of cationic amino acids (L-Arg and L-Lys). Moreover the double mutants (*lht1*aap5*) showed ca. 78% reduction of amino acid uptake, indicating that LHT1 and AAP5 transporters have complementary affinity spectra for amino acids (Paper I).

The AAP2 amino acid transporter is localized in phloem cells of Arabidopsis and participates in phloem loading (Hirner et al. 1998). In addition, Zhang et al. (2010) showed that the AAP2 transporter in Arabidopsis is localized in the phloem and plays an important role in the transfer of amino acids from xylem to phloem, and subsequently to the embryo. Finally, for this brief review, histochemical analysis has shown that the AAP3 transporter is expressed in root phloem, suggesting that it is involved in amino acid uptake,

but no phenotypic perturbations have been observed in AAP3 knockout mutants (Okumoto et al. 2004).

2 Objectives

This thesis focuses on two major aspects of amino acid transport in plants: root uptake and internal N cycling.

When the project started it was known that plant roots have the capability to take up amino acids from the soil. The amino acid transporters AtLHT1, AtAAP1, and AtAAP5 had been identified and shown to be involved in this process in Arabidopsis. The aim of the first study was to investigate the contributions of these transporters to the uptake of different amino acids at field-relevant concentrations (Paper I).

Direct evidence that organic N makes significant contributions to plant N nutrition is still lacking and whether or not uptake of amino acids occurs in the field is still debated. In an attempt to clarify this issue, plants uptake of amino acids from agricultural soil was studied by applying a molecular biology approach, using Arabidopsis mutants with altered amino acid uptake capacities (Paper II).

As discussed above, in addition to root-uptake, AtLHT1 has been shown to play a major role in uptake and cycling of amino acids in leaf mesophyll cells. To increase our understanding of the importance of amino acid transport for plant growth and development, effects of N cycling disruptions in *AtLHT1*-mutants on leaves representing a range of developmental states were analysed (Paper III).

Lastly, attempts were made to apply knowledge gained from the annual herbaceous plant Arabidopsis to a model system for trees. For this purpose, possible *Populus* orthologues of AtLHT1 were identified and one of them (PtrLHT1.2) was chosen for further characterization (Paper IV). Results of *in planta* complementation of an Arabidopsis knock-out mutant (*lht1*aap5)* with PtrLHT1.2 may indicate that it is a functional orthologue of AtLHT1.

3 Materials and methods

In the studies underlying this thesis, outlined above, amino acid transporter mutants of model organism were used as tools to disentangle focal aspects of amino acid transport, such as root uptake and internal N cycling, and the importance of these processes for plant N nutrition.

3.1 Model organisms

In biological research, model organisms that have advantageous traits, and sufficient supposed representativeness of broader taxonomic or ecologically functional groups, are often used to study selected processes, assuming that findings may be at least partly extended to other species. In the research this thesis is based upon *Arabidopsis thaliana* and *Populus tremula x tremuloides* (hybrid aspen) were used as model plants for annual herbaceous plants and trees, respectively.

3.1.1 Arabidopsis thaliana

Arabidopsis thaliana is an important model plant for plant scientists because it has several highly advantageous characteristics. It is a small plant, and easy to grow under controlled conditions in both petri dishes and soil. It produces abundant seeds and completes its life cycle within 6 to 8 weeks. Its genome is one of the smallest of any plants (27000 genes on five pairs of chromosomes), and was the first to be sequenced (when 114.5 Mb of 125 Mb was sequenced: (Arabidopsis Genome Initiative, 2000). Furthermore, Arabidopsis is amenable to diverse physiological, biochemical and molecular treatments, it can be easily transformed using *Agrobacterium tumefaciens*, and a wide spectrum of genetic resources of the species are publicly available (http://www.arabidopsis.org/; Weigel and Glazebrook, 2002).

However, using Arabidopsis as a model system also has some drawbacks. Notably, it is a herbaceous plant, so it is impossible to study certain processes of trees, e.g. wood formation, in it. Similarly, Arabidopsis is non-mycorrhizal, so results regarding (for example) some aspects of its nutrition cannot be generalized to mycorrhizal plants. Nevertheless, it was used in Studies I-III partly because it can be quickly and easily handled, treated and analysed in laboratory-based N uptake studies. Moreover, Arabidopsis produces small, fibrous root systems that facilitate tightly controlled treatments in petri plates and extraction of whole root systems for amino acid uptake studies.

3.1.2 *Populus*

Populus is an economically important genus of trees for wood production, and several species (and hybrids) are used as model plants to study various tree-specific processes, such as long-term perennial growth and secondary wood formation (Jansson and Douglas, 2007), for which the herbaceous plant Arabidopsis is unsuitable. It has a small genome compared to most tree species, with an estimated size of 485 ± 10 MB (±SD) and 19 chromosomes (Tuskan et al. 2006), about four times larger than the Arabidopsis genome. *Populus trichocarpa* (black cottonwood) was the first woody plant whose genome was sequenced (Tuskan et al. 2006). The hybrid aspen (*Populus tremula x Populus tremuloides*) is a good candidate for a model tree system since it grows quickly, can be efficiently propagated through tissue culture, a large collection of EST sequences is available and it can be easily genetically transformed (Yu et al. 2001; Sterky et al. 2004). In addition, *Populus* is much more closely related to Arabidopsis than most tree taxa, which facilitates comparative and functional genomic studies (Tuskan et al. 2006; Jansson and Douglas, 2007; Sterky et al. 2004).

3.2 Amino acid uptake conditions

Amino acid uptake has been explored in both sterile (axenic cultures) and non-sterile (soil) conditions during the course of the studies, as outlined below.

3.2.1 Sterile uptake conditions

In non-sterile conditions it is difficult to separate microbial N uptake from plant N uptake. Plant roots or rhizosphere microorganisms could take up supplied amino acids, since both have been shown to possess amino acid transporters. Thus, there is competition for the uptake of amino N (Hodge et al. 2000; Jones et al. 2005 a), and it is important to perform uptake studies in sterile (axenic) conditions to avoid supplied amino acids being taken up and/or

degraded by microbes. The amino acid uptake by plant roots can then be examined in isolation, greatly simplifying interpretation of the results.

However, performing uptake studies in axenic conditions has some drawbacks, as they are not consistent with natural soil conditions in terms of microbial uptake, concentrations of available amino acids and their adsorption to soil particles (Jones et al. 2005; Jones et al. 2005 b). A further complication, if nutrient solutions are used, is that diffusion will be much faster than in soil. In rhizospheres, the microbes have advantages over plant roots because of their faster growth, rapid turnover and larger specific surface areas for absorption (Kuzyakov and Xu, 2013). Furthermore, if incubation timings are too long the uptake solution will be significantly depleted of amino acids, resulting in under-estimation of uptake rates, and labelled C will be lost through respiration. Hence a short incubation time (60 minutes) was applied in the uptake experiment (Paper I) to avoid these effects.

3.2.2 Non-sterile uptake conditions

As already mentioned, the contribution of organic N to plant N nutrition has been a matter of debate for several decades. To address this issue a molecular biology approach was applied, in which amino acid transporter mutants with altered amino acid uptake capacities were used to investigate plant uptake of intact amino acids directly from soil solution. Universally labelled glutamine ($^{13}C_5$, $^{15}N_2$-glutamine) and $^{15}NH_4Cl$ solutions were introduced directly into the soil of pots with growing plants and the plants were subsequently harvested. As much of the root systems as possible were excavated from the soil, then the leaves and roots were washed with flowing tap water (Figure 3) and thrice in 0.5 mM $CaCl_2$ solution to remove any attached tracer.

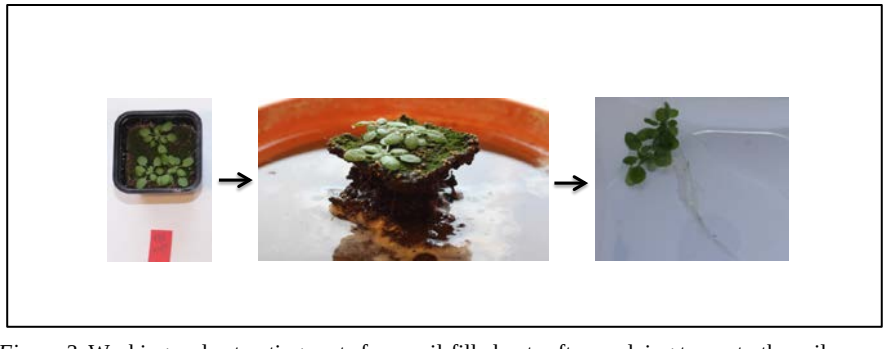

Figure 3. Washing and extracting roots from soil-filled pots after applying tracer to the soil.

This approach may provide more ecologically relevant results than sterile techniques because it gives estimates of plant roots uptake of supplied amino

acids directly from the soil solution in the presence of competing soil microbes. Hence the experimental system is closely related to natural soil conditions. A drawback of such experiments is that the ^{13}C may be respired as $^{13}CO_2$ if the incubation times are too long, resulting in underestimations of the uptake of intact amino acids from the relationships between ^{13}C and ^{15} N measurements in the tissues. Generally, amino acid uptake studies performed with dually-labelled amino acids in soil or uptake solution measure gross uptake (Näsholm et al. 1998; 2001) and do not take effluxes of amino acids into consideration. The molecular mechanisms underlying such effluxes in plants are not well known (as reviewed in Näsholm et al. 2009). However, comparisons of the gross uptake of labelled amino acids from soil and net uptake in depletion solution indicate that efflux rates are negligible or insignificant in barley and *Pinus sylvestris* (Persson and Näsholm, 2001a, 2001b; Sterky et al. 2004; Jämtgård et al. 2008).

3.3 Studying root-amino acid uptake

Plant roots both take up and exude nutrients. Thus, the net uptake of any substrate is the total influx (gross uptake) minus the total efflux (Szczerba et al. 2006).

Two methodologies were used to measure amino acid uptake in the studies this thesis is based upon.

3.3.1 Solution-depletion experiments

In the solution depletion method, plant roots are submerged in a solution containing a mixture of amino acids of known volume and concentrations. After a specified time plants are removed from the solution and the concentration of each amino acid in the solution is measured. The differences between initial and final concentrations of the amino acids gives the net amounts taken up (total amino acid uptake – total amino acids efflux). The advantage of this method is that uptake of multiple amino acids can be measured simultaneously. Conditions in depletion experiments resemble natural soil conditions in that more than one amino acid is available for uptake. The concentrations of amino acids in the solution should be sufficient enough to be taken up by plants, because lower concentrations can underestimate the uptake by over-expressor plants. Moreover, to compare uptake rates of amino acids it is important to consider the natural concentrations of amino acids in soil, otherwise in vivo uptake rates could be overestimated. It should also be noted that in depletion experiment with long incubation times the C from

amino acids may be respired as CO_2 and the solution may evaporate, which may bias the uptake results.

3.3.2 Isotope labelling technique

Another approach for studying amino acid uptake is to use amino acids labelled with C and N isotopes, such as ^{13}C, ^{14}C and ^{15}N (Figure 4). Some amino acids are universally labelled, i.e. all C and N atoms are isotopically labelled (as ^{13}C or ^{14}C and ^{15}N). The labelled amino acids can be used for uptake studies in either sterile conditions (for instance in sterile petri plates or amino acid solutions; Figure 2) or non-sterile conditions (e.g. in unsterilized soil; Figure 3).

A common objection regarding uptake experiments performed using dually-labelled amino acids and non-sterile conditions is that the plants may take up NH_4^+ mineralized from the supplied tracers (amino acid) by microorganisms rather than intact amino acids. Critics also claim that the root uptake of HCO_3^- and its utilization by plants, as well as losses of labelled C via CO_2 respiration, are not considered in dually-labelled amino acid uptake studies (Rasmussen and Kuzyakov, 2009).

In addition, Rasmussen et al. (2010) hypothesized that labelled inorganic C may be generated by mineralization of supplied dually-labelled amino acids and subsequent uptake of this inorganic C (HCO_3^-) may bias the uptake results.

In order to distinguish whether the plants take up intact amino acids, or their constituent N and C after mineralization by soil microbes, it is important to use amino acids in which both C and N atoms are labelled. This was done by supplying plant roots with a pulse of amino acids dually-labelled with ^{13}C and ^{15}N. These isotopes were subsequently analysed in plant tissues (Paper II) and the relationship between acquired ^{13}C and ^{15}N was used to determine if the amino acids were taken up in an intact or mineralized form (Näsholm et al. 1998; 2001). Detection of ^{15}N, but not ^{13}C in the plant parts would indicate that the dually-labelled amino acids are mineralized by microbes before being taken up by plants, but a strong correlation between ^{13}C and ^{15}N would indicate that amino acid are taken up as intact amino acids.

A problem with the dual-labelling technique is the difficulty in detecting very low levels of ^{13}C within plants. This is because ^{13}C is diluted ca. 60-150 times more strongly in plants than ^{15}N due to both higher ^{13}C natural abundance (1.08 %) and the higher total carbon contents (45-50% DW) in plants (Näsholm and Persson, 2001). To counter this problem higher amounts of tracers (amino acids) could be used in the uptake experiment. However, that would result in the substrate levels being higher than those of natural habitats,

and hence raise risks of over- or under-estimating amino acid uptake (Jones et al. 2005a).

In addition to dually-labelled amino acids (Paper II), ^{14}C-labeled amino acids were also used in uptake studies (Paper I) at typical concentrations for agricultural soils. In these cases the plant roots were submerged in amino acid solutions of known volumes and concentrations (Figure 4).

Figure 4. In uptake experiment using ^{14}C labelled amino acids Arabidopsis plants were grown on sterile vertical agar plates for 18 days in short day growth conditions (left panel), then their roots were immersed in amino acid solutions with field-relevant concentrations (right panel). Photo: Sandra Jämtgård.

For this uptake experiment (Paper I), roots of intact plants that had been grown on sterile media were submerged in solutions of ^{14}C-labelled amino acids in semi-sterile conditions. Thus, incubation times were short to minimize the growth of micro-organisms and evapo-transpiration of the uptake solutions. Since the supplied amino acids were only ^{14}C labelled this technique cannot differentiate between take-up of intact amino acids and take-up of their mineralized constituents. However, it provides indications of gross uptake of amino acids by plants at relatively low concentrations (although it does not account for effluxes).

3.4 Measurement of soil N

The traditional techniques to study the composition of soil N are soil extraction and centrifugation. (Jones and Willett, 2006; Kielland et al. 2007; Rousk and Jones, 2010). They involve removing soil from its natural environment (disturb natural soil structure), extraction with aqueous solutions of selected salts, centrifugation and finally analysis of N pools in the extracts (Jones and Willett, 2006; Inselsbacher, 2014). Moreover, other treatments are also often applied, such as homogenization, sieving, filtration and pH buffering before the chemical analysis (Jones and Willet, 2006; Inselsbacher, 2014). Clearly, mineralization and/or transformation of various N compounds may occur

during these sampling procedures, hence there are clear risks of under- or over-estimating some of the N pools (Jämtgård et al. 2010; Rousk and Jones, 2010; Inselsbacher and Näsholm, 2011; Inselsbacher et al. 2011; Inselsbacher, 2014). It is challenging to determine the diffusive fluxes of plant-available N forms in undisturbed soil microsites. Thus, most uptake studies were based on theoretical models to estimate these fluxes, so the results could be misleading.

Thus, a recently developed technique (microdialysis) was used to estimate *in situ* diffusion of amino acids, NH_4^+ and NO_3^- in soil microsites (Figure 5). This method is more efficient than use of lysimeters and soil extraction techniques because it allows for continuous sampling of plant-available N from soil solutions (Inselsbacher et al. 2011). It can detect even small changes in diffusive fluxes of N in soil solution, and thus is suitable for estimating qualitative and quantitative changes in soil solutions N contents with minimum disturbance of the soil structure (Inselsbacher et al. 2011). In Study III, probes of a microdialysis instrument were inserted at different positions at ca. 1.5 cm under the soil surface, dialysate samples were collected at selected time intervals and then analysed for NH_4^+, NO_3 and total amino acids.

*Figure 5.*Arabidopsis plants grown in agricultural soil with microdialysis probes installed in the root zone to analyse the N fluxes during the labelling experiment. Photo Erich Inselsbacher.

4 Results and Discussion

4.1 AtLHT1 and AtAAP5 are crucial for amino acid uptake at field-relevant concentrations

The amino acid transporters LHT1 (Hirner et al. 2006; Svennerstam et al. 2007), AAP1 (Lee et al. 2007) and AAP5 (Svennerstam et al. 2008) have been identified as participants in root uptake of amino acids. Previous studies on AtAAP1, AtLHT1 and AtAAP5 transporters have been performed using several organisms, e.g. yeast and Arabidopsis, and varying concentrations of supplied amino acids (Forsum et al. 2008; Svennerstam et al. 2008; Tan et al. 2008; Lee et al. 2007; Svennerstam et al. 2007; Hirner et al. 2006; Boorer & Fischer, 1997; Chen & Bush, 1997; Fischer et al. 1995; Frommer et al. 1993). However, none of the previous studies provided information about plant uptake of amino acids at naturally occurring soil solution concentrations, which is clearly important to avoid potentially misleading interpretations of results. Thus the goal of the study reported in Paper I was to investigate the role of these amino acid transporters for uptake at field-relevant concentrations.

Typically, amino acid concentrations in soils of agricultural systems, temperate forests and boreal forests are in the low µM range (Jämtgård et al. 2010; Jämtgård et al. 2008; Jones et al. 2005; Henry & Jefferies, 2002; Yu et al. 2002; Raab et al. 1999; Raab et al. 1996; Kielland, 1994). Therefore, in this study the uptake of six amino acids: two neutral (L-Gln, L-Ala), two acidic (L-Glu, L-Asp) and two basic (L-Lys, L-Arg) at concentrations ranging from 2 to 50 µM was analysed. Arabidopsis plants lacking expression of functional *LHT1* (*lht1.4*, *lht1.5*), *AAP1* (*aap1*), *AAP5* (*aap5*) and double mutants (*lht1*aap5)* as well as plants overexpressing *AtLHT1* (*35S::AtLHT1)* were used to disentangle the role of each of these transporters in amino acid uptake.

The estimated root amino acid uptake rates were then plotted as a function of substrate concentrations. To calculate the K_m and V_{max} of any substrate it is essential for its uptake to be saturated at the highest applied substrate

concentrations (Figure 2). L-Gln, L-Lys and L-Arg uptake met this criterion, showing saturating kinetics at the highest substrate concentrations within the applied range of 2-50 µM, but the uptake kinetics of L-Glu, L-Asp and L-Ala remained linear across the concentration range (Paper I, Figure 2). Thus, Michaelis-Menten parameters could not be calculated for L-Glu, L-Asp and L-Ala uptake. Moreover, uptake rates of L-Asp and L-Glu were lower than those of the neutral and basic amino acids. The lack of saturating kinetics for L-Glu, L-Asp and L-Ala uptake in this experiment could have been due to the maximum substrate concentration being too low, hence V_{max} may not have been reached either. Analysis of these amino acids uptake at higher concentrations could resolve this issue. Nevertheless, the results strongly indicate that the AtLHT1 transporter mediates uptake of L-Glu, L-Asp and L-Ala amino acids, since their uptake rates were significantly reduced in *Atlht1* plants.

We also investigated effects of increasing uptake capacities on the transport kinetics, by comparing uptake rates of amino acids of *AtLHT*-overexpressing (*35S::LHT1*) and control plants. The overexpressors displayed both higher uptake rates of L-Gln than the controls and saturating kinetics for L-Gln (Figure 2; Paper I, Figure 3), but linear kinetics for the other three amino acids tested (L-Glu, L-Asp and L-Ala). Furthermore, the K_m of their L-Gln uptake kinetics was not changed (unsurprisingly as there was no change in the binding site), but the V_{max} was significantly higher than the WT value (Figure 2), due to the higher abundance of the LHT1 transporter protein in the plasma membrane. Overexpression of *AtLHT1* did not change uptake kinetics of the basic amino acids, L-Lys and L-Arg.

Some previously reported attempts to increase root uptake of N by genetic manipulations had been unsuccessful. For example, Fraisier et al. (2000) stated that overexpression of the NO_3^- transporter NRT2.1 in *Nicotiana plumbaginifolia* did not increase acquisition of NO_3^-. The lack of success led to speculation that increasing IN uptake by overexpressing NO_3^- and NH_4^+ transporters induced increases in efflux rates, thus reducing or abolishing any effect on plants net N uptake (Britto & Kronzucker, 2004). However, Forsum et al. (2008) also successfully increased N uptake rates using the *35S::LHT1* construct and found that plants overexpressing *AtLHT1* exhibited significantly higher growth rates than WT controls, when amino acids (L-Gln, L-Asn and L-Glu) were supplied at concentrations of 0.5 mM and 1.5 mM. Thus, increases in amino acid influxes are not necessarily balanced by increased effluxes of amino acids or nitrogen.

In Study I the uptake rates of amino acid transporter mutants were compared to those of WT plants. In *lht1* plants the uptake of L-Gln, L-Ala and

L-Glu was 61 to 85% lower than WT rates, but the uptake of L-Lys and L-Arg remained unaffected. Similarly, in *aap5* plants uptake of L-Lys and L-Arg was 68 to 88% lower than WT rates, while the acidic and neutral amino acids uptake rates remained unchanged (Paper I, Figure 4). Furthermore, *LHT1*-overexpressing plants showed 219-456% higher uptake rates of L-Gln., L-Ala, L-Glu and L-Asp than WT plants. However, the *aap1* plants did not display any reduction in uptake rates of any amino acid. The results strongly indicate that LHT1 and AAP5 (but not AAP1) are important for root uptake of amino acids at field-relevant concentrations. This conflicts with conclusions by Lee et al. (2007), however these authors investigated uptake rates of plants with altered expression of *AAP1* at substantially higher concentrations (150 µM and 10 mM) than those used in Study I.

Recently Perchilk et al. (2014) reported that another member of the LHT family, LHT6, may also be involved in root uptake of acidic and neutral amino acids at low concentrations (30 and 150 µM in the cited study), but LHT6 showed no affinity for basic amino acids. LHT6 also showed affinity for acidic amino acids and for L-Ala at higher concentrations (2 mM). Moreover, in contrast to the previous study regarding AAP1 (Lee et al. 2007), Perchilk et al. (2014) found that the AAP1 transporter can take up neutral amino acids and L-Glu at naturally occurring (30 µM and 150 µM) soil concentrations. This also conflicts with results of our study (Paper I), in which no uptake of amino acid by AAP1 was recorded in the 2-50 µM concentration range. The reason for this discrepancy in results is not clear.

4.2 Uptake of intact amino acids from soil inferred from molecular biology experiments

After characterizing the LHT1 and AAP5 transporters and their potential roles in amino acid uptake in laboratory conditions, we applied a novel molecular approach using genetically modified Arabidopsis plants with differing amino acid uptake capacities to enhance understanding of their roles in amino acids uptake from soil. In the rhizosphere, plants roots are in direct and intense competition with soil microbes for amino acids. Indeed, Kuzyakov and Xu (2013) asserted that plants are weaker competitors than soil microorganisms for amino acids in soil. It has also been claimed that the dual (^{13}C, ^{15}N) labelling approach provides inconclusive results and potentially misleading indications of roots amino acid uptake from soil (Rasmussen et al. 2010).

Our novel experimental approach, of growing Arabidopsis mutants with known differences in genotypic and phenotypic capacities for taking up amino acids from soil and analysing their uptake, provided new possibilities to

determine whether or not roots directly take up intact amino acids from soil (Paper II). For that purpose, Arabidopsis WT, *AtLHT1* knockout mutants and plants overexpressing *AtLHT1* were grown in agricultural soil and exposed to a minute pulse of dual-labelled $^{13}C^{15}N$-L-Gln. The rationale was that if plants are insignificant competitors for amino acids in soil and results of previous dual-labelling studies could be explained by passive uptake and/or uptake of mineralized amino acid N and C (Rasmussen et al. 2010), the labelling patterns of roots (and shoots) in the three genotypes should be similar. Further, any increase in ^{13}C or significant regression between ^{13}C and ^{15}N in *LHT1* knock-out plants following addition of a dually labelled amino acid would confirm objections that the dual-labelling approach provides inconclusive results since the ^{13}C must have been acquired from sources other than the dually-labelled amino acid.

The results (Figure 6) showed some increase in ^{15}N in *Atlht1* plants, but only marginal increases in ^{13}C and no correlation between the isotopes. The lack of relationship between isotopes in the *LHT1* mutant indicates that *Atlht1* knock-out plants only acquired ^{15}N in mineralized form. In contrast, both WT and *35S::LHT1* plants showed significant accumulations and correlations of ^{13}C and ^{15}N contents, indicating that these two genotypes absorbed intact amino acids from soil. The higher uptake of L-Gln by *35S::LHT1* than *lht1* plants also indicates that the root amino acid uptake capacity is an important trait for plant N nutrition, enabling plants to compete against microbes for soil amino acids.

To summarize, the three genotypes showed clear differences in uptake of labelled L-Gln suggesting that WT and *35S::LHT1* plants can access amino acids from agricultural soil. Furthermore, it can be concluded that use of dually-labelled amino acids provides robust results, and can be validly used to assess plant amino acid uptake from soil. Our success in these respects shows the potential for using Arabidopsis mutants to obtain insights into plant-microbial competition for amino acids.

An additional line of criticism regarding the dual-labelling approach, expressed by Jones et al. (2005a), is that the typically high concentrations of amino acids resulting from injections of dually-labelled amino acids may favour plants and disfavour soil microbes. However, we injected a small, 5 ml pulse of 100 µM universally labelled L-Gln (^{13}C,^{15}N L-Gln) and monitored diffusive fluxes of L-Gln, other amino acids, NH_4^+ and NO_3^- during the experiment by microdialysis (Figure 5). Following the injection of labelled L-Gln the diffusive fluxes of L-Gln increased from 1.7 to 4.1 nmol m^{-2} s^{-1} (Paper II, Figure 1), approximately corresponding to soil solution concentrations of

just 1.0 to 2.4 µM. Thus, at least in the dual-labelling study reported in Paper I, plant-microbe competition was assessed under close to natural conditions.

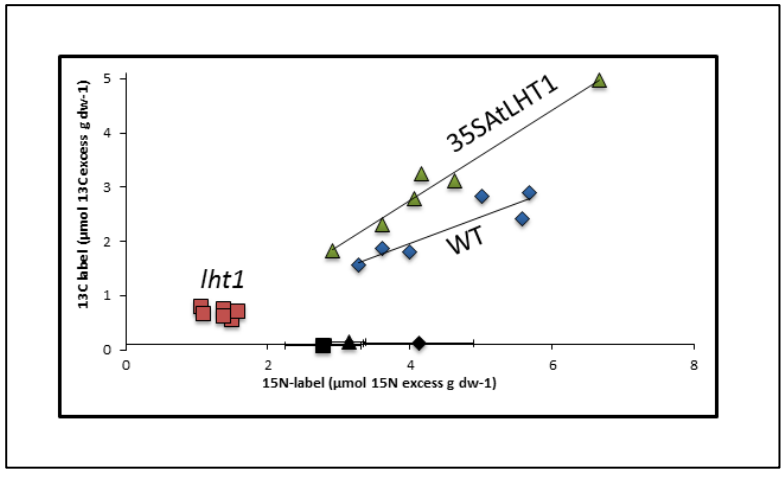

Figure 6. ^{13}C and ^{15}N contents in roots of indicated Arabidopsis genotypes an hour after application of universally labelled glutamine and $^{15}NH_4Cl$.

We also speculated that differences in ^{15}N natural abundance between genotypes could provide insights into the potential role of amino acids in plant N nutrition. The transformation of N in soil discriminates against the heavier ^{15}N isotope (Högberg, 1997). This means that natural ^{15}N abundance should be higher for organic N in soil than for the mineral forms such as NH_4^+ and NO_3^-. Consequently, plants acquiring relatively high amounts of organic N should display higher natural ^{15}N abundance than plants relying more on mineral N.

Therefore, we grew the three LHT1 lines in agricultural soil under the same experimental condition as used for the L-Gln experiment and harvested shoots for measurements of natural ^{15}N abundance after three weeks of cultivation. The results showed that *35S::LHT1* plants displayed substantially higher natural ^{15}N abundance than WT and *lht1* plants, while differences between the two latter genotypes were small (Paper II, Figure 4). A further difference between genotypes detected in this experiment pertained to the C status of the plants; *35S::LHT1* plants displayed a higher C/N ratio than the other two genotypes. Differences in neither natural ^{15}N abundance nor C/N ratios can conclusively demonstrate that plants acquire amino acids from soil. However, in combination with the results from the labelling study, and the observation that amino acids account for significant proportions of total diffusive soil N fluxes in this type of agricultural soil, our results are consistent with the notion that amino acids contribute to plant N nutrition also in agricultural soil.

4.3 Role of amino acid transport in maintaining C and N balance

As described in Papers I and II, AtLHT1 plays an essential role in root uptake of amino acids. *AtLHT1* is also expressed in leaf mesophyll cells, mediating amino acid uptake from the apoplast (Hirner et al. 2006; Liu et al. 2010). *AtLHT1* mutants with reduced amino acid uptake in mesophyll cells display an early senescence-like phenotype. Internal N cycling in the mesophyll is thus essential for proper plant growth and metabolism, and LHT1 plays an important role in this process (Hirner et al. 2006; Svennerstam et al. 2007; Liu et al. 2010).

In plants, N is needed to assimilate C and conversely C skeletons are needed to assimilate N (Nunes-Nesi et al. 2010; Stitt & Krapp, 1999). Because of the tight connection between C and N, we hypothesized that the disrupted N cycling in *lht1* plants would change not only their N metabolism, but also their C metabolism. Therefore, to complement the studies of Hirner et al. (2006) and Liu et al. (2010) we investigated how disruption of N cycling affects both C and N metabolism. In this study (described in Paper III) the Arabidopsis leaf rosette was used as a model to study how perturbation of N cycling changes the biochemistry, molecular biology and physiology of leaves across a range of developmental stages.

In order to identify the time point when biochemical changes occur, whole rosettes were sampled at six time points from 21 to 49 days after sowing (DAS). The whole-plant analysis showed that changes in most of the studied parameters occurred at 42 DAS. Therefore, to further investigate effects of the disruption of N cycling in *AtLHT1* mutants on biochemical processes and gene expression at different developmental stages, we examined changes in individual leaves. Six leaves (ranging from leaf 10 to leaf 20) from 42-day-old plants were sampled, as shown in Figure 7. Leaves of *lht1* plants had lower biomass and smaller areas, and their older leaves showed senescence-like symptoms (Figure 7).

The N concentration was higher in leaves of mutants than WT leaves (except leaf 10, the oldest), while the C concentration was higher in WT than *lht1* plant leaves (all leaves) as shown in Paper III, Figure 7. In accordance with whole plant data, the total free amino acid (TFAA) concentration was similar in all genotypes in all leaves, but the composition of amino acids differed. Notably, older mutant leaves showed lower Glu+Asp/Gln+Asn ratios than WT counterparts, indicating increased remobilization of N during senescence (Cabello et al. 2006). In addition, starch contents were substantially lower in older mutant leaves than in WT counterparts (Paper III, Figure 9).

Since *lht1* plants showed signs of C starvation, including in middle-aged leaves, we hypothesized that the photosynthetic machinery of the mutants

might be disturbed, even in leaves with no visible senescence. Accordingly, we found that *lht* plants had significantly lower chlorophyll and RuBisCo contents than wild-type plants. To further investigate these perturbations, photosynthetic parameters were analysed. Measurements of CO_2 assimilation in response to light (light-response curves) of middle-aged leaves (leaf 14) revealed that CO_2 fixation was affected, as the maximum photosynthesis rate was lower in the mutants (Paper III, Figure 10). This suggests that the photosynthetic machinery was already degrading at this stage (leaf 14) in mutant plants, possibly due to the induced senescence-like process (Liu et al. 2010). However, since the stomatal conductance was also lower in the mutants, the reduction in CO_2 fixation could have been due to a restriction in CO_2 supply. Therefore, CO_2 fixation was measured at different internal CO_2 concentrations [C_i]. Both mutants assimilated less CO_2 at similar [C_i], indicating that the reduction in CO_2 fixation was due to photosynthetic restrictions rather than physical limitations of CO_2 supply imposed by stomata.

In conclusion, processes related to both photosynthetic electron transport and carboxylation were affected, which could explain the lower CO_2 fixation in *LHT1* mutants.

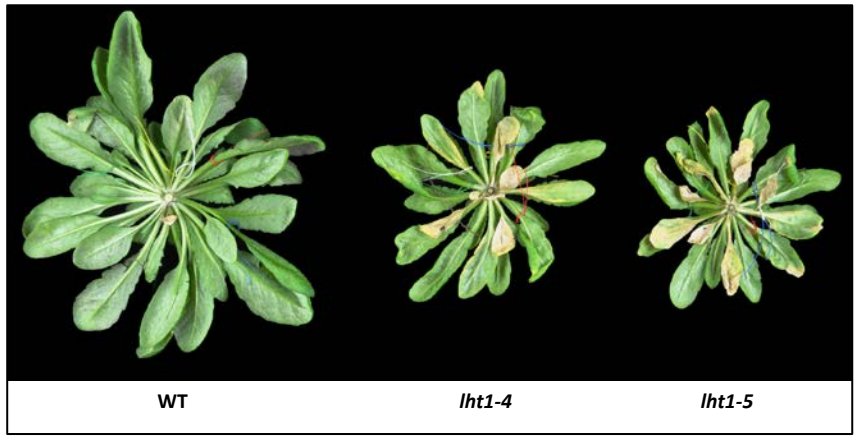

Figure 7. Experimental setup for assessing effects of perturbations in N cycling in individual leaves using WT and *AtLHT1* (*lht1-4*, *lht1-5*) mutant plants. In each rosette six leaves were selected, tagged with string of different colours, and analysed after harvesting at 42 Days After Sowing.

To further disentangle how the perturbation of N cycling in mutant plants affects leaves of different developmental stages, we analysed the expression of genes involved in C/N metabolism and leaf senescence (Paper III, Figure 11). The early senescence marker *WRKY53* was up-regulated in young and middle-aged leaves, whereas the late senescence marker *SAG12* was up-regulated in

middle-aged and old leaves. The N assimilation enzyme glutamine synthetase (GS) has been identified as a marker for leaf senescence (Diaz et al. 2008; Masclaux-Daubresse et al. 2005). Two GS isoforms are present in most plants: GS1 (cytosolic; encoded by *GLN1.1*) and GS2 (chloroplastic; *GLN2*). GS1 is mainly present in roots, but is also expressed in leaf mesophyll and companion cells during leaf senescence (Brugière et al. 2000). Accordingly, in *lht1* plants levels of *GLN1.1* and *GLN2* transcripts were higher and lower than in WT plants, respectively.

The *lht1* plants had lower photosynthetic activity than wild type plants, and thus lower C contents. *ASN1* (dark-induced-6 gene; *DIN6/ASN1*) is induced by darkness, low sugar levels (Lam et al. 2003; Thum et al. 2003; Baena-González et al. 2007) and during dark-induced senescence, but not natural senescence (Buchanan-Wollaston et al. 2005). *ASN1* was induced in *lht1* plants, resulting in increased levels of Asn. Dark-induced senescence involves cytosolic pyruvate orthophosphate dikinase (PPDK) supplying oxaloacetate (OAA) for Asn synthesis (Parsley et al. 2006; Lin and Wu, 2004). Accordingly, cytosolic *PPDK2* expression was induced in *lht1* leaves, whereas chloroplastic *PPDK1* expression was unaffected. The levels of Asn were higher in all *lht1* leaves, and the highest observed levels were in young leaves, indicative of increased export of Asn to younger leaves.

The metabolic system and its regulatory networks are extremely complex. Therefore, any conclusions regarding the chain of events leading to a mutant phenotype have to be drawn with caution. However, the results from this study have provided some insights into the importance of maintaining N homeostasis, and the role of amino acid transport in this elaborate system. A hypothetical model, based on this study and data from the literature, of the effects of disrupting N cycling on C and N metabolism in middle-aged source leaves of *Arabidopsis* is shown in Paper III, Figure 12.

The early senescence-like phenotype has been previously attributed to Gln deficiency inducing ROS and SA-dependent programmed cell death (PCD) (Liu et al. 2010). Results of Study III show that *lht1* plants also displayed C starvation symptoms, resulting in up-regulation of genes and processes not associated with SA-dependent PCD (i.e. Asn remobilization; Buchanan-Wollaston et al. 2005). Speculatively, Rubisco synthesis, which depends on an adequate N supply, is reduced due to local Gln (and thus N) deficiency. The resulting reductions in photosynthetic activity, and thus levels of C-skeletons available for NH_4^+ assimilation, down-regulate chloroplastic GS2 (Oliveira and Coruzzi, 1999). The local Gln deficiency and C starvation causes downstream effects on red-ox balance inducing SA dependent PCD (Liu et al. 2010). The result is not only local N deficiency, but also lower C status, and the *LHT1*

mutants respond as having relatively high levels of N versus C, e.g. by accumulation of Arg, which has high N:C ratio. However, it cannot be excluded that the reduced photosynthetic activity, and thus the C starvation symptoms, are downstream of SA-dependent programmed cell death.

The *LHT1* mutation confers enhanced pathogen resistance, but the disruption of N cycling also results in reductions in biomass production and seed yields, illustrating that manipulating single pathways to obtain desirable effects for a crop plant are also likely to have deleterious effects on other processes. Thus, this study emphasizes the importance of amino acid transport for plant growth and development, and highlights the need for further characterization of single amino acid transporters to increase our understanding of processes underlying NUE.

4.4 Amino acid transporters in trees

Many amino acid transporters have been functionally characterized in the herbaceous plant Arabidopsis (Pratelli & Pilot, 2014; Tegeder, 2014; Tegeder, 2012; Tegeder & Rentsch, 2010). However, only a couple have been identified in the model tree *Populus*. Couturier et al. (2010 a) identified and characterized the PtAAP11 transporter by heterologously expressing it in a yeast mutant. The functional characterization of PtAAP11 transporter showed that it is a high affinity proline (L-Pro) transporter and plays a role in providing L-Pro during xylem cell wall formation. Another transporter, PtCAT11, was also identified by genetic complementation of a yeast mutant, and shown to participate in L-Gln remobilization from source to sink tissues during the senescence process in *Populus* (Couturier et al. 2010 b). However, little is known about amino acid transporters in tree species. Thus, the aim of Study IV was to apply knowledge gained from analyses of the herbaceous plant Arabidopsis to increase understanding and the tools available for studying amino acid transport and NUE in perennial trees, particularly the model genus *Populus*.

In silico analysis of the *Populus trichocarpa* (Pt) genome (Tuskan et al. 2006) revealed 12 members of the PtLHT family. A phylogenetic tree revealed that of these gene models PtrLHT1.2 showed closest phylogenetic relationship (91.7% similarity) with Arabidopsis LHT1 (Paper IV, Table 1). The relative expression of the corresponding gene (*PtrLHT1.2*) in young hybrid aspen plants (*Populus tremula* L. x *tremuloides* Michx.) was highest in leaves followed by roots and stems (Paper IV, Figure 3). Hence we wanted to identify and characterize potential orthologue(s) of AtLHT1 in the model tree *Populus* using an *in planta* system for functional complementation studies.

As mentioned earlier, several transport proteins in Arabidopsis have been identified and characterized by genetic complementation of yeast mutants. However, results from amino acid transporter characterization in single-cell systems, e.g. yeast and *Xenopus* oocytes, have been shown to differ from results of *in planta* studies. For instance, AtLHT1 was first discovered by Chen and Bush (1997) and its characterization in yeast revealed apparent uptake affinities for L-Lys, L-His and L-Glu. However, later studies have shown that AtLHT1 has high affinities for neutral and acidic amino acids, but not basic amino acids (Hirner et al. 2006; Svennerstam et al. 2007). This highlights the value of using *in planta* systems for genetic complementation studies. Therefore, we developed such a system based on Arabidopsis mutants with disrupted amino acid transport. The Arabidopsis double mutant (*lht1*aap5*) shows early senescence-like symptoms and growth inhibition caused by the *lht1* mutation (Hirner et al. 2006; Svennerstam et al. 2007; Liu et al. 2010; Paper III) accompanied by reduced root uptake of neutral and acidic amino acids, due to the *LHT1* mutation, and basic amino acids, due to the *AAP5* mutation (Svennerstam et al. 2008; Svennerstam et al. 2007; Hirner et al. 2006). The leaf phenotype caused by the *lht1* mutation has been shown to be unrelated to the amino acid uptake process (Hirner et al. 2006). This mutant is thus excellent for complementation studies, to assess whether a transporter can rescue both the leaf phenotype and the root uptake phenotype. Use of *lht1*aap5* also allows us to determine the affinity and kinetic properties of amino acid transporters, since LHT1 and AAP5 have complementary uptake profiles (Paper I).

Thus, to verify that PtrLHT1.2 is a transporter and determine its uptake capacity and affinity for different amino acids, it was cloned and expressed in the double mutant *lht1*aap5* under control of the CaMV 35S promoter. Phenotyping of positive transformants revealed that heterologous expression of PtrLHT1.2 in *lht1*aap5* plants rescued the leaf phenotype (Figure 8). No signs of yellowing leaves or growth inhibition were observed in three individual transformants. These findings suggest that PtrLHT1.2 is an amino acid transporter that is targeted to the plasma membrane of leaf mesophyll cells.

A L-^{14}C-Gln root uptake experiment confirmed that all three transformants took up L-^{14}C-Gln at 5-15 times higher rates than *lht1*aap5* plants (Paper IV, Figure 6). Moreover, the relative expression pattern of *PtrLHT1.2* was similar to the L-^{14}C-Gln uptake pattern, confirming that expression of PtrLHT1.2 restores the L-Gln uptake phenotype.

To further investigate the amino acid affinity of PtrLHT1.2 an uptake experiment was performed with a mixture of 12 amino acids including acidic, basic and neutral amino acids, each at 10 µM concentration (Paper IV, Figure

7). Both *T1:3* and *T4:4* plants showed higher uptake of all amino acids than *lht1*aap5* and WT plants, with high affinity for neutral and acidic amino acids and low affinity for basic amino acids.

Figure 8. Phenotype of Arabidopsis *lht1*aap5* plants expressing PtrLHT1.2, at 42 Days After Sowing. Transformants resembled the WT, showing no signs of senescence or growth inhibition. Thus, the early senescence-like phenotype caused by the *LHT1* mutation is rescued by heterologous expression of PtrLHT1.2.

This was confirmed in a kinetic study of L-Gln and L-Arg uptake, as PtrLHT1.2 restored the saturated uptake pattern for L-Gln, but not for L-Arg (Paper IV, Figure 8). Thus, PtrLHT1.2 rescued the root-uptake phenotype associated with the *LHT1* mutation, but not the *AAP5* mutation. These uptake results are in accordance with AtLHT1's high affinity for neutral and acidic amino acids, and very low affinity for basic amino acids (Svennerstam et al. 2011, Paper I).

Taken together, the results suggest that PtrLHT1.2 is a functional orthologue of AtLHT1 that is involved in both mesophyll and root uptake of amino acids. Moreover, PtrLHT1.2 appears to be involved in uptake of neutral and acidic amino acid, like its AtLHT1 counterpart.

5 Conclusions and future perspectives

Amino acids are the major transport forms of N within plants and are hence the "currency" for N exchange between cells, tissues and organs. Accordingly, plants have evolved several classes of amino acid transport proteins with different substrate specificities and tissue expression patterns. They play vital roles in several processes that influence NUE, and thus are crucial for plant growth and development.

The results presented in this thesis suggest that two amino acid transporters, LHT1 and AAP5, are crucial for root amino acid uptake at field-relevant concentrations in Arabidopsis, and have complementary affinity spectra; LHT1 takes up neutral and acidic amino acids while AAP5 takes up basic amino acids. The importance of LHT1 was corroborated by results of analyses of its putative orthologue in *Populus* (PtrLHT1.2), suggesting that PtrLHT1.2 could also be involved in root uptake of amino acids. *In planta* complementation of the Arabidopsis mutant (*lht1*aap5*) with PtrLHT1.2 suggests that PtrLHT1.2 (like AtLHT1) is a high affinity transporter for neutral and acidic amino acids. Expressing *PtrLHT1.2* under control of the CaMV-35S promoter in Arabidopsis transporter mutants rescued both growth and uptake phenotypes of the *lht1*aap5* mutant, showing that PtrLHT1.2 is an amino acid transporter and potentially a functional orthologue of AtLHT1. The next step is to analyse the tissue expression pattern of PtrLHT1.2 through GFP and/or GUS reporter systems. It will also be of great interest to create *PtrLHT1.2* RNAi-silenced and overexpressing *Populus* lines to further characterize its roles in amino acid uptake and transport. Future work should also include the discovery of additional amino acid transporters in species other than Arabidopsis, including conifers and various crop plants. Identification and characterization of more amino acid transporters will increase the toolbox for studying N transport and NUE in herbaceous species, grasses and trees.

The identification of the molecular processes underlying root uptake of amino acids has opened new avenues for studies of organic N nutrition. A new molecular biology approach, analysing amino acid uptake at field-relevant concentrations in *AtLHT1* mutants grown in agricultural soil, provided new insights into organic N uptake. As stated in the Introduction section, authors have criticised the notion that plants may access organic N in the field. The new approach is highly relevant in this context, providing answers to most of the issues that have been raised.

1. The direct uptake and contribution of organic N in plant N nutrition is negligible compared to inorganic N sources. *Our results show that Arabidposis is equally efficient in acquiring N from L-Gln as from* NH_4^+.
2. The dually labelled amino acids in soil are separated into ^{15}N and ^{13}C prior to plant N uptake. *We show that only plants with a functional expression of LHT1 displays a relationship between the two isotopes* ^{15}N *and* ^{13}C*. This implies that any uptake of* ^{13}C *from other sources than from the labelled amino acid is negligible.*
3. Microbes outcompete plants for N uptake and plants only take up N that is left over from the microbial mass. *In our experiment, plants were equally efficient in acquiring organic (L-Gln) and inorganic N (*NH_4^+*).*
4. Amino acids are of minor importance since they constitute a small component of the whole N pool of soil. *We show, using a low-invasive microdialysis technique, that amino acids are abundant in agricultural soil. Our results also show that the addition of labelled organic N had only a marginal effect on soil solution concentrations.*
5. Only small percentages of dual labelled amino acids are recovered as intact amino acids. *Plants acquire only small amounts of N, irrespective of chemical form, in the short term. Long term experiments show plants gradually acquire larger quantities. Unfortunately, in long-term experiments, organic and inorganic N uptake cannot be distinguished.*

The significant regression observed between ^{13}C and ^{15}N suggests that Arabidopsis can access intact amino acids before they are mineralized into NH_4^+ and NO_3^- and that LHT1 plays a key role in this process. Plants can apparently take up these intact amino acids despite intense competition from microbial uptake in agricultural soil. This suggests that amino acids may make important contributions to plant N nutrition in agricultural soils, as they are believed to do in boreal forest soils. A major future challenge is to determine

the quantitative importance of root uptake of amino acids for plants total N budgets. One way to quantify this may be to grow the *AtLHT1* mutants in soil and providing a continuous supply of labelled L-Gln (or amino acids) in soil at regular intervals (as in nature) and then quantify the recovered labelled N in plant tissues. In pulse experiments the added N gets depleted with time while a regular supply would enable quantification of competitive abilities of plants.

Apart from root uptake of amino acids, LHT1 plays an important role in amino acid uptake from the apoplasm to leaf mesophyll cells. This cycling of amino acids is decisive for maintaining the N balance in mesophyll cells. We found that disrupted N cycling in leaf mesophyll cells of leaves of different developmental status, changes the C:N balance and expression of several genes involved in metabolism which ultimately force mutant plants to enter senescence earlier than control plants. Therefore, it was concluded that the LHT1 transporter plays an important role in cycling amino acids in mesophyll cells and thus maintaining plants C:N balance and metabolism. It would be interesting to examine the transcriptomic, proteomics and metabolomic profiles of leaves of different developmental stages to further elucidate the amino acid cycling in mesophyll cells.

It has been shown that a mutation in a single amino acid transporter can profoundly affect plant growth; hence future work may involve the identification and functional characterization of additional amino acid transporters in different plant model systems to investigate how they cooperatively regulate N fluxes within plants.

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7 Acknowledgements

It is time to acknowledge all people who have helped me during these years. If I have forgotten to mention anyone I apologize for this, it is unintentional.

Ulrika Ganeteg, thanks a lot for giving me the opportunity to carry out this research in your lab and for encouragement to complete this PhD. You are an awesome supervisor and I am honoured to be your first PhD student. Thanks, for being available to help me whenever I needed and even sometimes helping me in the experimental work.

Torgny Näsholm, I am really inspired by your scientific knowledge and optimistic and positive attitude. I am particularly indebted to you for all encouragement, for your help with manuscripts, reading my thesis and spending so much time on discussing science with me.

Mattias Holmlund, thanks for helping me in the lab, especially with the qPCR, and spending so much time on reading my thesis and manuscripts and for your valuable suggestion to improve them.

I would like to say humble thanks to the Opponent and the examining committee for reading my thesis.

Reference group members; **Anita** and **Leszek** for your valuable suggestions and discussions to improve my projects.

Our co-authors; **Regina, Rumen, Anastasia, Velemir, Maria** and **Jonathan** for their contribution and help with different experiments.

Sandra, it is always fun working with you in lab, your planning for the experiments was impressive.

Henrik, thanks for being co-author and performing the awesome uptake experiments for the Poplar manuscript.

The members of the N-group, **Olusegun, Hyungwoo, Fredrik, Nils, Linda, Johanna, Lasse, Margareta, Ann, John D** and former members **Mathieu, Erich** and **Camila** for your help, rewarding discussion and support.

Thanks to the administrative staff of UPSC particularly **Inga-Lis** and **Britt Marie** for their help and support.

Thanks to colleagues and friends in the lab: **Kristofer, Prashant, Abdellah**, **Sonali, Amir and Weiya** for your help and support in one way or the other.

Pali (for your help with western blotting and for your kind and supportive behaviour), **Melis** (it is always fun talking to you), **Edward** (thanks for being a good friend and for always willing to help), **Ogonna** (it is so easy to laugh with you) **Anastasia** (it's a pleasure to be your friend and thanks for helping me with the photosynthetic measurements), **Amir** (thanks for your inspiring discussions), **Sofie** (it was a pleasure working with you in the SE lab) **Alfonso** (for your guidance and advice).

Former and present office-mates; **Anastasia, Alfonso, Maria, Pierre, Emma, Shashank,** and **Jihua** for nice discussions and creating a friendly environment.

Thanks to **Azeez, Wali, Sultan, Abdul Sattar Nizami, Akhtar, Dr Basharat, Dr Munawar** and **Dr Nadeem** for their advice, support and encouragement.

Thanks to all Pakistani friends and families in Umeå for delicious food. I truly appreciate the time spent with you.

Last but not the least special thanks to my family back home for believing in me and for their endless love and support.