Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2008:50

Plants are known to have the ability to take up amino acids from the soil solution and use them as a source of nitrogen. In this thesis, two transporters involved in *Arabidopsis* amino acid uptake were identified, LHT1 and AAP5, each responsible for the uptake of a specific spectrum of amino acids. Mutant plants, either lacking or over-expressing the identified transporters were found to be affected in both the uptake of- and in the case of LHT1, growth on amino acids.

Henrik Svennerstam the author of this thesis received his graduate education at the Department of Forest Genetics and Plant Physiology, SLU, Umeå. He has a Master of Forest Science from SLU, Umeå.

Acta Universitatis Agriculturae Sueciae presents doctoral theses from the Swedish University of Agricultural Sciences (SLU).

SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension, as well as environmental monitoring and assessment are used to achieve this goal.

Online publication of thesis summary: http://epsilon.slu.se/eng

ISSN 1652-6880
ISBN 978-91-85913-83-1

Doctoral Thesis No. 2008:50 • Amino Acid Uptake in *Arabidopsis* • Henrik Svennerstam

Doctoral Thesis No. 2008:50
Faculty of Forest Sciences

Amino Acid Uptake in *Arabidopsis*

-the Transporters Involved, Kinetics of Uptake and Growth on Amino Acids

Henrik Svennerstam

Amino Acid Uptake in *Arabidopsis*

-the Transporters Involved, Kinetics of Uptake and Growth on Amino Acids

Henrik Svennerstam
Faculty of Forest Science
Department of Forest Genetics and Plant Physiology
Umeå

Doctoral Thesis
Swedish University of Agricultural Sciences
Umeå 2008

Acta Universitatis Agriculturae Sueciae
2008:50

ISSN 1652-6880
ISBN 978-91-85913-83-1

Tryck: Arkitektkopia, Umeå, Sweden, 2008

Abstract

Svennerstam, H. 2008. Amino Acid Uptake in *Arabidopsis*
-the Transporters Involved, Kinetics of Uptake and Growth on Amino Acids.
Doctoral thesis, ISSN 1652-6880, ISBN 978-91-85913-83-1

Nitrogen (N) is essential for all living organisms and is considered to be the limiting factor for plant growth in many ecosystems. Although generally believed to rely on mineral N to fulfill their N needs, plants have also been found to access organic N such as amino acids.

Despite extensive research, the importance of amino acids as N sources for plants still remains unclear. The work presented in this thesis has focused on identifying the transporters responsible for amino acid uptake in plants and to characterize mutants lacking these transporters. Two transporters important for *Arabidopsis thaliana* amino acid uptake were identified, the lysine histidine transporter 1 (LHT1) and amino acid permease 5 (AAP5). These two transporters were found to have complementary, non-overlapping affinity spectra, i.e. LHT1 displayed affinity for neutral- and acidic amino acids and for L-Histidine, whereas AAP5 exhibited affinity for L-Arginine and L-Lysine only. Mutants lacking both LHT1 and AAP5 were found to have little residual uptake of the amino acids tested, suggesting these transporters to be the most important for *Arabidopsis* root amino acid uptake. Mutants lacking LHT1 or AAP5 displayed much reduced uptake rates in the low µM range suggesting these transporters mediate efficient uptake at field relevant concentrations. LHT1 mutants did not only have impaired uptake capacity, but also grew less than wild type when grown on for example L-Glutamine as the sole N source. In contrast, by over-expressing LHT1, plants grew larger on amino acids, suggesting a connection between uptake capacity and growth. Growth experiments using labeled amino acids in a mixture with nitrate revealed that a substantial amount of plant N was amino acid derived, suggesting that *Arabidopsis* has the ability to efficiently use amino acids as a source of N.

The results presented in this thesis provide a mechanistic understanding to the process of root amino acid uptake in plants. This knowledge is important for future research within the field of plant organic N nutrition and *Arabidopsis* genotypes with altered amino acid uptake capacities can be used as tools to further elucidate the ecological benefit plants may have by taking up amino acids.

Keywords: Arabidopsis thaliana, LHT1, AAP5, nitrogen, amino acid, uptake, knockout, transporter.

Author's address: Henrik, Svennerstam, Department of Forest Genetics and Plant Physiology, SLU, Box 901 87, Umeå, Sweden
E-mail: Henrik.Svennerstam@genfys.slu.se

Till farfar Rune

Trying is the first step towards failure.

Homer Simpson

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., Ganeteg, U., Bellini, C. & Näsholm, T. (2007). Comprehensive Screening of *Arabidopsis* Mutants Suggests the Lysine Histidine Transporter 1 to Be Involved in Plant Uptake of Amino Acids. *Plant Physiology* 143: 1853-1860.

II Svennerstam, H., Ganeteg, U. & Näsholm, T. (2008). Root uptake of cationic amino acids by *Arabidopsis* depends on functional expression of Amino Acid Permease 5. *New Phytologist,* published online.

III Svennerstam, H., Jämtgård, S., Huss-Danell, K., Ganeteg, U. & Näsholm, T. Root amino acid uptake in *Arabidopsis* (manuscript).

IV Forsum, O., Svennerstam, H., Ganeteg, U. & Näsholm T. (2008). Capacities and constratinsts of amino acid utilization in *Arabidopsis*. *New Phytologist,* published online.

Papers I, II and IV are reproduced with the permission of the publishers.

Preface

Nitrogen is essential for life; it is an indispensable component of proteins and DNA. Nitrogen is the fourth most abundant element in plants, only surpassed by hydrogen, carbon and oxygen. Thus, nitrogen is the element taken up in the greatest amounts from the soil. Soils around the world contain a diverse array of nitrogenous compounds; to date only some of these have been regarded as plant available.

Plant nutrition has been of great interest for decades, both for understanding general plant physiology but also because of its potential for increasing the growth of crop plants and other managed or domesticated plants. Special attention has been paid to nitrogen since it has been found to be a limiting factor for plants in managed- and unmanaged terrestrial ecosystems; both organic- and mineral nitrogen-containing fertilizers have been widely used to increase plant growth. The traditional view is that plants primarily rely on ammonium and nitrate to fulfil their nitrogen demands (if plants with nitrogen fixing symbionts are excluded); this is reflected in the use of ammonium nitrate and urea as the default mineral fertilizers around the world. Although not considered to be a nitrogen source of significant magnitude, amino acids were found, in the early 20^{th} century, to be taken up by plants. Since then, extensive research on soil amino acid availability has been conducted, worldwide, and a number of aspects of plant amino acid nutrition have been studied, including the capacity for amino acid uptake by various species, the transporters involved, the kinetics of uptake and the ecological significance of amino acid uptake.

This thesis focuses on the identification of the molecular mechanisms associated with root amino acid acquisition; the goal was to contribute to our understanding of plant amino acid uptake and its significance for plant N nutrition.

Introduction

Amino acid uptake, a historical view

Nitrogen (N) is a component of many compounds that occur in soils; of these, only a few are considered to be available to plants. The general view is that plants rely on the mineralization of organic nitrogenous compounds for the release of ammonium and the subsequent production of nitrate (Figure 1), the primary inorganic N sources (Tamm 1991, Schimel & Bennett 2004). In the early 20th century the first evidence that amino acids were taken up by plants started to appear (Hutchinson & Miller 1911 and references therein). Since then, plant scientists have investigated a wide array of amino acid-related research areas to determine whether plant uptake of amino acids is of ecological importance. There is also evidence that plants can acquire more complex N sources such as peptides (Salonen & Simola 1977, Schmidt et al. 2006) and intact proteins (Paungfoo-Lonhienne et al. 2008).

Amino acids in soils

Soils around the world, both in managed- and unmanaged systems, have been found to contain amino acids (Raab et al. 1996, 1999, Nordin et al. 2001, Öhlund 2004, Jones et al. 2005b, Kielland et al. 2006, 2007, Jämtgård et al. 2007, Kranabetter et al. 2007). The natural occurrence and concentrations of amino acids in soils vary considerably between ecosystems, but, in addition, the methods for quantifying soil amino acid concentration or content vary, making comparisons difficult. Although extraction methods vary (Öhlund 2004), amino acids are usually found in the lower μmolar range (0.01-10 μM) (Raab et al. 1996, 1999, Öhlund 2004, Jones et al. 2005b), although concentrations up to 100 μM have been recorded (Raab et al. 1996). Nordin et al. (2001) showed that the relative amount of amino acids increased along a production gradient, from high to low productivity, indicating that amino acids might be of greater importance for plant N nutrition in low productivity systems. A higher proportion of dissolved organic N (DON) has also been shown to be present in late successional forest soils (Kielland et al. 2006). DON is sometimes the largest component of soil N, of which amino acids may constitute 10-20% (Jones & Kielland 2002). Therefore, the abundance of DON has led to an alternative suggestion that, rather than being dependent on microbial mineralization of amino acids, plants rely on the breakdown of proteins into amino acids (Jones & Kielland 2002, Schimel & Bennet 2004). Furthermore, it is believed that although amino acid concentrations are low, their high turnover rate suggests that they are an important N source in some ecosystems (Kielland 1995, McFarland et al. 2002, Jones & Kielland 2002).

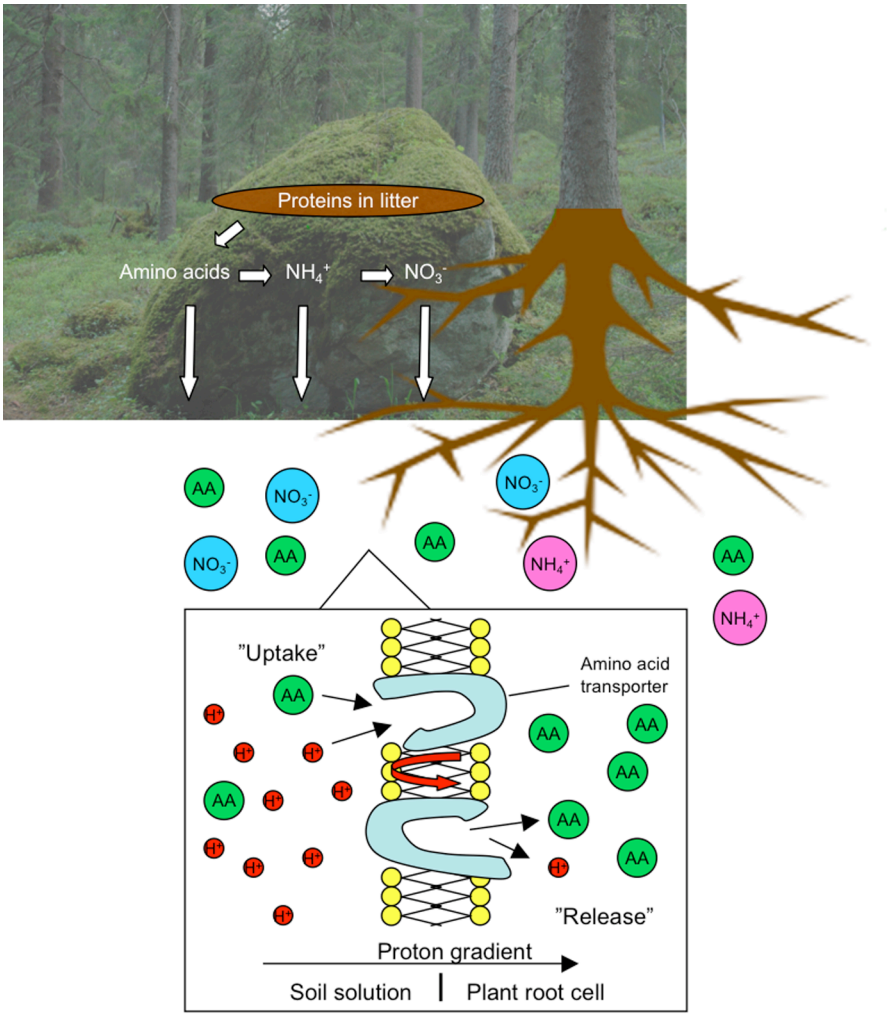

Figure 1. Simplified scheme of the breakdown of proteins to plant available nitrogenous compounds and an illustration of the carrier facilitated amino acid uptake in plants.

The soil content of amino acids is often related to those of nitrate and ammonium. Although, in general, the concentration of single amino acids is lower than that of inorganic N, the total water-extractable free amino acid concentration can be substantially higher than that of ammonium or nitrate (Kielland 1994, Henry & Jeffries 2002, Öhlund 2004). Plant amino acid uptake studies must, therefore, take account of the fact that, in the field, concentrations are in the lower µmolar range.

Amino acid uptake

The capacity to absorb organic N seems to be a common characteristic of plants and has been known of for some time now; for example Hutchinson and Miller demonstrated glycine uptake by plants as early as 1911. Amino acid uptake experiments have been carried out both in the laboratory (Jones & Darrah 1994, Raab et al. 1996, Falkengren-Grerup et al. 2000, Persson et al. 2001, Weigelt et al. 2005) and in the field (Näsholm et al. 1998, Nordin et al. 2001, Näsholm & Persson 2001, Persson et al. 2003, Xu et al. 2004), using either intact plants or excised roots; the diversity of data makes comparisons difficult. It can, however, be argued that the different approaches to research are useful, resulting in a broad knowledge of plant amino acid uptake. Moreover, due to the possibility that amino acids break down during uptake investigations, some experiments have used dual labelled amino acids to verify the uptake of intact amino acids (Näsholm et al. 1998, 2000, Streeter et al. 2000).

Kinetics of amino acid uptake

Like other nutrients that are taken up actively by plants, amino acid uptake rates are highly dependent on substrate concentration**.** This was demonstrated by Wright (1962), who tested the uptake rates of Glycine in Chinese mustard in the concentration range 0.5-8 mM and found that it became saturated at higher concentrations. There are now several studies of this phenomenon in an range of other plant species, microbes and mycorrhizal root tips **(**Soldal & Nissen 1978, Borstlap et al. 1986, Chapin et al. 1993, Jones & Darrah 1993, Kielland 1994, Jones & Hodge 1999, Wallenda & Read 1999, Vinolas et al. 2001, Sokolovski et al. 2002**)**.

The process of substrate transport by proteins and the relationship between substrate concentration and transport rate belongs to the field of enzyme kinetics, a subject area that originally focused on enzymatic reactions. When studying the influence of concentration (and other factors such as pH) on active uptake of different substrates, two kinetic parameters are calculated, K_m and V_{max}. A prerequisite for the calculation of these parameters is that saturation must occur when measuring uptake at increasingly higher substrate concentrations. V_{max} is the calculated maximum uptake rate and K_m is the half saturation constant, i.e. the concentration at which 50% of V_{max} is reached (Figure 2). It is mainly the K_m parameter that reflects whether or not a transporter or uptake is of, so- called, low- or high affinity. High affinity is a term that describes an uptake system that is adapted to low substrate concentrations, whilst low affinity describes the opposite phenomenon. The distinction between transporters with high- or low affinity is rather

subjective but the cut-off point can be considered to be around a substrate concentration of 100 µM.

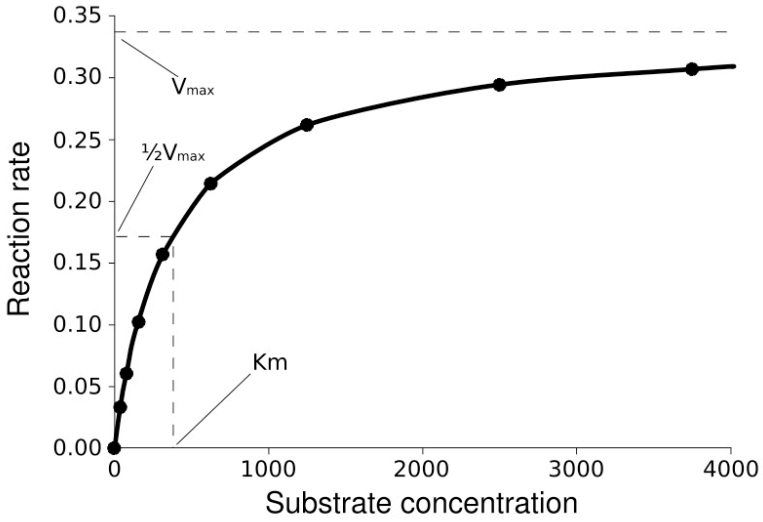

Figure 2. Illustration of enzyme kinetics with the kinetic parameters K_m and V_{max} explained (reproduced from wikipedia.org).

A complicating factor when studying uptake kinetics in plants is that they sometimes display bi- or multiphasic uptake, suggesting multiple uptake mechanisms acting in different or overlapping concentration ranges (Soldal & Nissen 1978, Borstlap et al. 1986, Schobert & Komor 1987). The kinetic constants calculated for amino acid uptake in plants vary considerably, both for the different amino acids taken up by a single species and between plant species. These differences can, possibly, be explained by the conditions in ecosystems and the life strategies of plants themselves (evolutionary pressure). Kinetic studies have shown forest and crop plants to have K_m values for root amino acid uptake ranging from single digit µmolar concentrations up to mM concentrations and V_{max} values up to 67 µmol g^{-1} dw root h^{-1} (Soldal & Nissen 1978, Borstlap et al. 1986, Schobert & Komor 1987, Kielland 1994 , Jämtgård et al. 2008). The K_m values found, both *in vitro* and *in vivo*, are also influenced by the pH of the substrate solution (Soldal & Nissen 1978, Borstlap et al. 1986, Boorer et al. 1996, 1997, Fischer et al. 2002), consequently, experiments should be carried out within relevant pH ranges.

In comparison with the kinetic parameters found for the uptake on amino acids, the uptake characteristics of ammonium and nitrate are highly variable between species and experiments. The kinetic

parameters found for ammonium and nitrate uptake can overlap those for amino acids but sometimes indicate a lower affinity (higher K_m values) and potentially higher V_{max} (Schobert & Komor 1987, Chapin et al. 1993, Kielland 1994, Wallenda & Read 1999 Hangs et al. 2003); this might reflect the higher concentration of ammonium and nitrate found in soils compared to amino acids.

Plant uptake of mineral N

As already mentioned, amino acid uptake and its effect on plant N nutrition, is usually compared to nitrate and ammonium. Unlike amino acids, the uptake systems for ammonium and nitrate are well characterised. Studies of nitrate suggest that three uptake systems exist. These systems are divided into two groups, the high (HATS) and low (LATS) affinity transport systems. The HATS transport system is further divided into the constitutively expressed (cHATS) and the substrate induced (iHATS) systems (for review see Williams & Miller 2001, Glass et al. 2002). The threshold K_m between LATS and HATS is generally thought to be around 0.5 mM (Williams & Miller 2001). Several nitrate transporters in *Arabidopsis* have been identified and belong to the NRT transporter family; all are thought to be proton symporters (Williams & Miller 2001). The systems for ammonium uptake are, like nitrate, divided into high and low affinity (Williams & Miller 2001); in this case high affinity is considered to occur up to 200 μM. The transport of ammonium is different from that of nitrate since it is not necessarily associated with proton symport, but but can also be driven by electrical attraction, i.e. ammonium is attracted by the negatively charged interior of plant cells. Low affinity transport of ammonium can, therefore, be mediated by channels (Williams & Miller 2001). The nature of the high affinity ammonium transport is not entirely clear, but it has been suggested that it is carried out either by uniport transport (Ludewig et al. 2002) or that it is proton coupled (Williams & Miller 2001).

Ecological relevance of amino acid uptake

The question of the ecological relevance of amino acid uptake, often considered in relation to the mineral N sources of ammonium and nitrate, has been a matter of debate for years. Amino acid uptake, compared to that of mineral N, has been studied extensively, with experiments conducted in both the field and the laboratory. Virtanen and Linkola (1946) found evidence for plant amino acid uptake and showed that, in relation to ammonium and nitrate uptake, this was quite low; however, they argued that this uptake could be important. Chapin et al. (1993) performed uptake studies in the laboratory on field collected roots of *Eriophorum vaginatum* and, based on their data, suggested that 60% of the N absorbed by this species in the field could be

amino acid-derived. It also seems likely that the relative importance of amino acid uptake could be greater under N-limited conditions (Jones & Darrah 1994, Nordin et al. 2001, Bardgett et al. 2003, Harrison et al. 2008).

On the other hand, there are studies indicating the limited significance of amino acid uptake, suggesting that plants are, instead, dependent on microbial mineralization of amino acids (Hodge et al. 2000). In addition, Owen & Jones (2001) claim that amino acids are of limited importance in the agricultural system they studied, since, compared to other pools, a relatively small fraction of the amino acids supplied experimentally were found in plants. Another view of plants' capacity to take up amino acids is that it could be more relevant for the re-capture of DON lost during root exudation (Jones et al. 2005a).

There is also debate about whether plants compete efficiently against micro organisms for amino acids. In experiments using isotopically labelled amino acids, isotopes are found preferentially in micro organisms, especially when plants and microorganisms are harvested shortly after tracer addition (Bardgett et al. 2003). Jones et al. (2005b) suggest that plants are better competitors at high amino acid concentrations than at low ones, where microbes are thought to have the competitive edge. There are also experiments suggesting that plants compete well for amino acids in comparison to micro organisms (Schobert et al. 1988, Schimel & Chapin 1996). Rather than being competitors, plants can also benefit from micro organisms, and their amino acid-derived N uptake can be enhanced by mycorrhizal symbiosis (Turnbull et al. 1995, Wallenda & Read 1999, Sokolovski et al. 2002, Schmidt et al. 2006). Due to the contradictory nature of the findings and suggestions regarding the relevance of soil amino acids for plants, as described above, it is currently impossible to give a conclusive answer to the question of whether amino acids have a significant impact on the N nutrition of plants. Therefore, this subject requires further investigation.

Mechanisms of plant amino acid uptake

Amino acids are considered to be the "N currency" of plants, being transported to and between organs (Bush 1999). The physical and chemical properties of amino acids do not allow for efficient passive transport across plant membranes, therefore evolution has equipped plants with transport proteins that can facilitate the movement of amino acids either into- or within plants. Plant amino acid transporters are generally believed to be proton-coupled symporters (Bush 1993), meaning that they are secondary active, utilizing the proton gradient over the plasma membrane to energize the transport of amino acids into the cell (Figure 1). Although generally thought to be taken up like

all other amino acids, it has been suggested that the uptake of basic amino acids can be facilitated by a uniport system, driven by negative membrane potential (Wyse & Komor 1984).
Recent molecular work, utilizing cloning and functional complementation in yeast, has revealed numerous transporters in plants that have an affinity for amino acids. Based on the knowledge that amino acid uptake from the soil is dependent on transporters, and to aid in the understanding of plant amino acid uptake, it is critical that the proteins involved are identified and characterised. In this context, *Arabidopsis* has, thus far, been the preferred model plant to investigate the molecular mechanisms for amino acid uptake *in planta*, mainly because its genome has been sequenced and is easily manipulated.

Amino acid transporters in *Arabidopsis*

Amino acid transporters in the *Arabidopsis* genome belong to two families, ATF and APC. The ATF (the amino acid transporter family), also called the AAAP family (amino acid/auxin permease) is the largest family, consisting of 46 members in *Arabidopsis* (Rentsch et al. 2007). The ATFs can be divided into the AAP- (amino acid permease), LHT- (lysine histidine transporter), GAT- (γ-aminobutyric acid transporters), AUX- (auxine resistant), ProT-(proline transporters) and ANT- (aromatic and neutral amino acid transporters) sub-families. The APC family is smaller than the ATF and consists of nine CATs (cationic amino acid transporter) and the LATs (L-type amino acid transporter), although members of the latter have not yet been characterised (Rentsch et al. 2007).

The amino acid permease (AAP) family was discovered after the identification of the *Arabidopsis* AAP1 using yeast complementation (Frommer et al. 1993, Hsu et al. 1993). Since then seven additional AAPs have been discovered in *Arabidopsis* (AAP2–AAP8). AAP homologs have also been found in a number of other plant species (reviewed in Williams & Miller 2001). The AAPs are all similar in size (51-56 kDa) and are believed to have 10-12 membrane spanning regions (Frommer et al., Hsu et al., Kwart et al. 1993). When examined in heterelogous expression systems AAPs generally display an affinity for neutral and acidic amino acids; the exceptions are AAP3 and AAP5, which transport all classes of amino acids, but have the greatest affinity to the cationic amino acids (Fischer et al. 1995, Boorer & Fischer 1997, Fischer et al. 2002, Okumoto et al. 2004).

The LHTs were first thought to have a high affinity for Lysine and Histidine, based on the work on LHT1 in yeast undertaken by Chen & Bush (1997). It has been suggested that LHT2 serves a function in *Arabidopsis* flowering; it is 74% similar to LHT1 at the amino acid

level and has been found to transport neutral- and acidic amino acids, although probably not L-Lys and L-Arg (Lee & Tegeder 2004). Recently, data on LHT family transporters suggest that LHT2/4/5/6 have specific roles in *Arabidopsis* reproduction (Foster et al. 2008). ProTs have been found to transport L-Pro, Glycine betaine and GABA (Grallath et al. 2005). AUX1 has been found to transport auxin (Yang et al. 2006) and is thought to share a common ancestry with amino acid permeases due to the structural similarity between tryptophan and auxin. ANT1 has been found to have an intermediate affinity for aromatic and neutral amino acids (Chen et al. 2001).

Transporter function

The majority of studies on amino acid transporters have been carried out in yeast and *Xenopus* oocytes; this, together with expression data and knowledge about the amino acid concentrations found in plant organs, has lead to suggestions about transporter function. Amino acid transporters have been found to be differentially expressed in plant tissues and the level of transporter expression can vary during the life cycle of a plant (reviewed in Fischer et al. 1998, Ortiz-Lopez et al. 2000, Liu & Bush 2006). In addition to this, amino acid transporters have been found to be responsive to environmental factors such as light, salt stress, water stress, sugars and nutrient availability, and promoter region analysis has corroborated these suggestions (reviewed in Liu & Bush 2006). These findings indicate that transporter function does not rely only on biochemical activity but also on expression, which is influenced by environmental cues and plant development. Suggestions about transporter function are quite speculative and the most common include phloem and xylem loading or unloading, seed loading and sometimes root amino acid uptake (reviewed in Liu & Bush 2006). So far, only a few amino acid transporters have been ascribed a specific function in plants as a result of data derived from *in planta* experiments. Something that complicates the issue of assigning a function to a specific transporter is the fact that most transporters are expressed in more than one location, thus (hypothetically) carrying out more than one function.

Objectives

Plants have access to a number of nitrogenous compounds in soils; of these compounds, ammonium, nitrate and amino acids have been shown to be taken up by plants and a number of transporters facilitating the uptake of nitrate and ammonium have been identified and characterised. In contrast, when this PhD-project was started (in December 2002), many plant amino acid transporters had been discovered, but at that time none had been found to be active in root amino acid uptake. There were, however, examples of amino acid transporter mutants that had been shown to have a reduced capacity for the uptake of L-Lys (Bright et al. 1983, Kumpaisal et al. 1989, Heremans et al. 1997) and L-Pro (Verbruggen et al. 1996). The point of mutation for these transporter mutants could, however, not be mapped accurately in the *Arabidopsis* genome and was, therefore, still to be discovered.

The aim of the studies underlying this thesis was to unravel the molecular background, i.e. the transporters involved in *Arabidopsis* root amino acid uptake. It was imperative that the transporter(s) that facilitate root amino acid uptake were identified in order to assist a thorough and detailed investigation of plant amino acid uptake. Once successful in the task of identifying these transporters, the work of characterising the corresponding transporter mutants with respect to specificity and affinity could follow. Finally, the importance of amino acid uptake for plants and the implications of having altered amino acid transporter expression were investigated.

The final goal was an increased knowledge of plant amino acid uptake and, if possible, to expand the findings into an ecological context.

Methodological reflections

Arabidopsis as a model plant

The experimental setup and the techniques used to answer any specific question are very important. The plant species examined in this thesis was *Arabidopsis thaliana*, perhaps the most widely used model species in plant science. It has many advantages as a model plant, such as being relatively small, easy to grow, it has a short generation time, it can be easily transformed, its genome is sequenced (The *Arabidopsis* Genome Initiative, 2000) and, perhaps the most important factor for my work and this thesis, that publicly available knockout insertion lines exist. Mutagenesis is an effective strategy when trying to establish gene function in *Arabidopsis*; in my project, two different strategies were employed: forward and reverse genetics (for review see Ostergaard &

Yanofsky 2004). In brief, forward genetics describes the approach of screening for a certain phenotype in a randomly mutagenised population; once an interesting phenotype has been found, the point of mutation is investigated. Random mutagenesis can be achieved by the use of chemicals; for example, the seeds derived from ethyl methane sulfonate (EMS) treated plants (Redei & Koncz 1992, Weigel & Glazebrook 2002) used in paper I, or radiation. The downside of random mutagenesis is that it requires efficient screening techniques and time-consuming genome mapping to find the affected gene (Lukowitz et al. 2000, Lister & Dean 1993). Reverse genetics is the approach of ascribing a phenotype when the point of mutation is already known. In reverse genetics, insertions of transferred DNA or transposon elements can be the cause of mutation, often called T-DNA insertion lines, like the SALK- (Alonso et al. 2003) and/or gabi-kat lines used in papers I, II, III and IV. In the case of T-DNA line reverse genetics, the point of mutation is easily detected by PCR/sequencing technology, so the subsequent work focuses on characterising the phenotype of a specific knockout.

Phenotyping wild type and mutant plants

When an interesting mutant phenotype has been found – in the case of this research, one showing an altered capacity to absorb amino acids via roots and indicating an alteration in genes coding for amino acid transporters – the work of characterising the mutants must follow. Experimental design is a complex task; factors like accuracy, reliability, relevance and workload come into play. In this thesis the majority of the plant growth phases and the experiments were carried out in virtually axenic (short term depletion/uptake studies), or axenic conditions, such as on agar or in liquid culture. The reason for this was to avoid the possibility of microbial assimilation and breakdown of the supplied amino acids, since any breakdown could seriously affect the results. In this thesis, axenic systems were utilized both in the growth experiments and to cultivate plants that were going to be used in them. Since data on *Arabidopsis* growth on agar/liquid culture were scarce, it was necessary to develop protocols for these systems. One critical aspect of axenic systems, which has implications for work with agar media, is the limited space/volume, which restricts the amount of nutrients in the agar to that which is added before casting. This has special implications for growth experiments on agar since amino acids have to be supplied at concentrations that cannot be regarded as ecologically relevant.

Figure 3. Depletion setup using pipetting trays on a shaking-table (papers II and IV).

Amino acid uptake studies

After or during the cultivation phase, wild type and mutant *Arabidopsis* plants were used to investigate uptake characteristics of the respective plant line, i.e. in depletion and labelling experiments. The basics of depletion experiments are that plant roots are submerged in a solution with a known concentration of substrate(s). After a defined period of time, a sample is taken from the solution and analysed for substrate content. The difference between starting content and the sample content is used to calculate the plant uptake rate. Depletion experiments are more difficult to carry out than labelling studies since several aspects have to be considered: the substrate concentration has to be relevant, the amount of substrate in the uptake solution has to be adjusted so that a plant can take up a significant proportion of it to facilitate comparisons, in this case using HPLC analysis of amino acids. The advantage of using the depletion method is that it is possible to assess the uptake of several substrates simultaneously, whilst needing to perform only one analysis (in the case of liquid chromatography of amino acids) per sample. In this context, it is also important to highlight the difference between the depletion- and labelling studies in this thesis, namely that, due to inhibition effects, the uptake of amino acids from a mixture can be different to the uptake when single amino acids are supplied (as shown by Borstlap et al. (1986) among others). It can be considered more ecologically relevant to investigate uptake using a solution containing multiple amino acids since such an experimental setup better resembles actual soil conditions. Another difference between labelling and depletion is that depletion experiments measure net uptake, while labelling measures gross uptake. This can in turn affect

the recorded uptake since plants may efflux amino acids during an uptake experiment, i.e. efflux of amino acids from the plant root is measured indirectly when using the depletion setup.

Results and discussion

Identification of transporters involved in root amino acid transport

Historically, scientists trying to identify proteins involved in amino acid transport have employed various strategies. One strategy is to express genes encoding putative plant amino acid transporters in mutant yeast lines that lack transport capacity for one or more amino acids. The idea is that the inserted (plant) transporter will rescue the yeast mutant on amino acid-containing media. After a transporter has been identified, more precise characterisations of it follow, first in yeast or oocytes and then, if possible, in the corresponding plant mutant lines. Another approach when trying to identify transporters involved in the root uptake of amino acids is to screen for mutant seeds on media containing toxic levels of amino acids or amino acid analogues; this was the approach taken by Heremans et al. (1997) and Verbruggen et al. (1996). In our work, when trying to find root active transporters, we took advantage of the knowledge that plants can be sensitive to L-amino acids and in some cases to the D-isomers (Erikson et al. 2004 & 2005). The screening for D-amino acids would assume that the transporters active in root amino acid uptake do not discriminate completely against D-amino acids and that the loss of a root active transporter would result in better survival as compared to wild type on a substrate with toxic levels of D-amino acids. Thus, screening *Arabidopsis* T-DNA insertion lines on toxic levels of D-amino acids would enable us to find mutants defective in the uptake of L-amino acids, or, however unlikely, mutants that have gained the capacity to metabolise D-amino acids. One obvious issue with this strategy was that to achieve effective screening considerably higher D- amino acid concentrations than those found for their L- counterparts in soils had to be used. This could possibly result in the discovery of transporters that do not have any effect on the uptake of amino acids at concentrations that actually occur in the field.

For the first screening experiment (Paper I), a selection of T-DNA lines (Table 1) available at that time, some replicated, and seeds originating from ethyl methane sulfonate (EMS) treated plants were sown onto 3 mM D-Ala. The EMS screening resulted in a few surviving plants, but when the offspring of those plants were re-screened only one survivor proved to be D-Ala resistant. The screening of the T-DNA lines had a similar outcome, also resulting in only one surviving line. Having two

lines that were resistant to D-Ala, one of which was an EMS-mutant that required time-consuming mapping to locate the mutated gene involved, we wanted to test whether the lines were allelic. Since we already knew that the mutation was recessive, the resistant T-DNA and EMS lines were crossed and the offspring of that cross was found to be 100% resistant. Thus, the lines were very likely to be allelic and sequencing confirmed that LHT1 was mutated in the EMS line. The surviving T-DNA line had an insertion in the lysine histidine transporter 1 (LHT1), characterised in yeast by Chen & Bush (1997); in parallel to our work, it was later found to be involved in root amino acid uptake (Hirner et al. 2006). Furthermore, based on mapping data, the *Arabidopsis raz1* mutant, which lacks high affinity transport of L-proline, is also likely to be mutated in LHT1 (Verbruggen et al. 1996).

In the second screening (paper II), we wanted to screen for a root basic amino acid transporter; this was because of the lack of affinity of LHT1 for L-Lys and L-Arg (paper I). Higher concentrations of L-Arg have been found to have a growth retarding effect on plants (Paper IV). Therefore, a screening based on L-Arg could be an effective approach to identifying a root transporter active in the uptake of basic amino acids. By using two separate screening strategies, one utilizing growth retarding effects (1 mM L-Arg + 3 mM nitrate) and one with non-retarding levels (30 µM ^{15}N L-Arg + 3mM nitrate), to look for reduced ^{15}N content in mutant lines, we hypothesised that transporters active both in the high- and low affinity ranges could be identified. In paper II, compared to the screening that resulted in the discovery of LHT1, a more specific selection of T-DNA mutants was used. The 18 knockout lines used in paper II were selected for three different reasons: they were annotated as cationic amino acid transporters; they had a high sequence similarity to LHT1; or they were amino acid transporters with a relatively high root expression (Table 1). As in the D-Ala screening only one line displayed a clearly diverging phenotype. The line found was an AAP5 knockout; in both screenings it exhibited a phenotype that was clearly different to the wild type, suggesting that it was active/of importance for both the high- and low affinity root uptake of L-Arg (Figure 2). Until then, AAP5 had only been characterised in oocytes and yeast (Boorer & Fischer 1997, Fischer et al. 2002), but it is likely to be allelic with the mutants found by Heremans et al. (1997) that had impaired uptake of L-Lys.

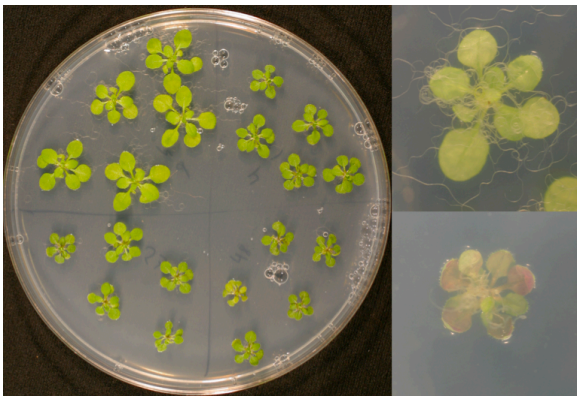

Figure 4. Illustration of the 1 mM L-Arg + 3 mM nitrate screen in paper II. Left picture: *AAP5* mutant plants in the upper left quadrant. Pictures to the right, *AAP5 mutant* (upper) and wild type (lower).

For the screening experiments in papers I & II it is possible that the identified mutants could be exhibiting effects in the upstream transport of amino acids rather than uptake over the root plasma membrane. However, another view on this issue is that the majority of the transporter mutants did not display any phenotype although being expressed in roots. For example AAP3, which along with AAP5 are the only transporters that, thus far, have been found to mediate efficient transport of basic amino acids (Fischer et al. 2002) did not exhibit an identifiable phenotype in paper II; this is an indication that transporters active in the internal transport of amino acids were not singled out in our screenings.

Table 1. List of amino acid transporter families and family members (modified from Rentsch et al. 2007). The use of corresponding mutants in paper 1 and II is indicated.

Family	Locus	Name	Paper I	Paper II
AAP	AT1G10010	AAP8	yes	yes
	AT1G44100	AAP5	yes	yes
	AT1G58360	AAP1		yes
	AT1G77380	AAP3	yes	yes
	AT5G09220	AAP2	yes	
	AT5G23810	AAP7	yes	
	AT5G49630	AAP6	yes	
	AT5G63850	AAP4		
ANT	AT1G80510	-	yes	
	AT2G39130	-		
	AT2G40420	-	yes	
	AT2G41190	-	yes	
	AT2G42005	-		
	AT3G09330	-	yes	
	AT3G09340	-		

	AT3G11900	ANT1	yes	
	AT3G28960	-		
	AT3G30390	-		
	AT3G54830	-		
	AT3G56200	-	yes	
	AT4G38250	-		
	AT5G02170	-	yes	
	AT5G02180	-	yes	
	AT5G15240	-	yes	
	AT5G16740	-	yes	
	AT5G38820	-		
	AT5G65990	-	yes	
AUX	AT1G77690	-		
	AT2G21050	-		
	AT2G38120	AUX1	yes	
	AT5G01240	-		
CAT	AT1G05940	CAT9		
	AT1G17120	CAT8	yes	yes
	AT1G58030	CAT2	yes	
	AT2G34960	CAT5		
	AT3G03720	CAT4		
	AT3G10600	CAT7	yes	yes
	AT4G21120	CAT1	yes	
	AT5G04770	CAT6		
	AT5G36940	CAT3	yes	yes
GAT	AT1G08230	GAT1	yes	
	AT5G41800	-	yes	yes
LAT	AT1G31820	-		
	AT1G31830	-		
	AT3G13620	-		
	AT3G19553	-	yes	
	AT5G05630	-		
LHT	AT1G24400	LHT2	yes	yes
	AT1G25530	-	yes	yes
	AT1G47670	-	yes	yes
	AT1G48640	-	yes	yes
	AT1G61270	-		yes
	AT1G67640	-		yes
	AT1G71680	-	yes	yes
	AT3G01760	-	yes	yes
	AT4G35180	LHT7	yes	
	AT5G40780	LHT1	yes	yes
ProT	AT2G36590	ProT3	yes	
	AT2G39890	ProT1	yes	
	AT3G55740	ProT2	yes	yes

Root active amino acid transporters

So far three transporters (LHT1, AAP1, AAP5) have been found to be involved in root amino acid uptake in *Arabidopsis* (Hirner et al. 2006, Lee et al. 2007, Papers I-II). After identifying the root amino acid defective mutants, the work of characterising them began. As described in the *Methodological reflections* section, *in planta* studies of root amino acid transporters can be quite straightforward, but the experimental setup, the interpretation, relevance and significance of the resulting data is always open to discussion. The *lht1* and *aap5* mutants were subjected to similar characterisations, although the amino acids used in the experiments differed. *Aap1* mutants were acquired later for the work described in paper III. When discovered and first studied in yeast and oocytes, both LHT1 and AAP5 were suggested to carry out other functions than *in planta* root uptake (Chen & Bush 1997, Boorer & Fischer 1997, Fischer et al. 2002) as compared to the function they are suggested to have in roots by Hirner et al. (2006) and Papers I-IV.

Chen & Bush (1997) found that LHT1 had a preference for L-His and L-Lys and that it was primarily expressed in flowers, young leaves and siliques. Expression of LHT1 was also found in older leaves, stems and roots. *In situ* analysis also revealed expression localized to the root surface of young seedlings. It was suggested by Chen & Bush (1997) that LHT1 was involved in nutrient uptake in sink tissues, a suggestion that fits quite well with the work of Hirner et al. (2006) who proposed that, apart from root uptake, LHT1 played a role in mesophyll cell loading. The LHT1 preference for the uptake of cationic amino acids was, however, not confirmed (Hirner et al. 2006).

AAP5, when studied in yeast and oocytes (Boorer & Fischer 1997, Fischer et al. 2002) displays affinity for L-Arg and L-Lys. AAP5 is expressed in mature leaves, stems and flowers (Fischer et al. 2002), but also in roots (Fischer et al. 1998). It has been suggested that the function of AAP5 is xylem to phloem transfer of neutral amino acids, despite it displaying a preference for L-Arg and L-Lys in oocytes (Fischer et al. 2002). This suggestion was based on the fact that the uptake of L-Lys is inhibited by other amino acids and that the concentrations of neutral amino acids are usually much higher than those of basic amino acids.

Examining the regulation of amino acid transport was not within the scope of this thesis, but is an important factor when investigating amino acid uptake and the use of amino acids as a N source. Thus far, there have been few investigations into the regulation of amino acid transport and available data is mainly related to localisation and abundance of expression. There are, however, a few studies that indicate possible

regulation of amino acid uptake by environmental signals. Thornton & Robinson (2005) found that the glycine uptake rate was the same when supplied as the sole N source or in a mixture with nitrate and ammonium, whereas the uptake of ammonium and nitrate decreased when supplied in a mixture with glycine. In contrast, Persson et al. (2006) found that the uptake of L-Ala decreased in *Pinus sylvestris* after pre-treatment with ammonium nitrate, suggesting possible regulation of amino acid uptake by plant N status. Rather than being N inhibited, AAP1 and LHT1 have been shown to be nitrate inducible (Guo 2004, as cited in Liu & Bush 2006) and regulated by light or the photosynthetic production of sugars (Ortiz-Lopez et al. 2000). In a review by Liu and Bush (2006), the promoter regions of 22 plant amino acid transporter genes were analysed with respect to *cis*-elements. The promoter analysis revealed a root-specific motif and possible regulation by N for LHT1 and AAP5. Thus, although some findings are contradictory, amino acid uptake and transporter regulation is likely to be dependent on plant N status.

In all experiments involving the *lht1* and *aap5* transporter mutants it is important to remember that the transporters being studied are expressed in more than one location in the plant. The results, therefore, do not necessarily depend solely on the transporter function in the plasma membrane of root cells. This fact became apparent in the *lht1* growth experiment on fertilised soil, during which yellowing of leaves and stunted growth was observed after approximately 24 days and after 31 days for the *lht1* mutants (Paper I) . Hirner et al. (2006) suggested that this phenomenon is likely to be dependent on LHT1 functioning in the amino acid loading of leaf mesophyll cells. Expression data (www.genevestigator.ethz.ch; Zimmerman et al. 2004) also corroborate this suggestion, showing a clear correlation between raised expression in wild type leaves around the time of bolting, when yellowing of older leaves of the *lht1* mutants was observed (Paper I). As a result, it was decided to characterise the *lht1* mutants within the first 21 days of growth to avoid the lack of leaf mesophyll amino acid loading affecting our results. *Aap5* mutants did not display any distinct phenotype when grown on soil, surprisingly both replicate mutant lines were larger than the wild type, although only one was significantly larger at the final harvest (Paper II). It is difficult to offer a good explanation for this since it is rather unlikely that the lack of a transporter could increase growth and any explanations can only be speculative.

Transporter amino acid transport spectrum

Amino acid uptake characteristics were studied either by measuring the uptake of amino acids from solution (depletion) or by growing wild type and mutant plants on media with µmolar levels of ^{15}N labelled amino

acids. Using the depletion setup, and a 25 µM initial concentration, LHT1 was found to have a general affinity for neutral amino acids and L-His. Significant reductions of up to 100% in L-Ser, D-Ala, Gly, L-Ala, L-Gln and L-His uptake were found (amino acids are listed in ascending order of uptake compared to the wild type). Lack of LHT1 did not, however, have any effect on the uptake of L-Lys and L-Glu in the depletion experiment. The ^{15}N experiment described in paper I corroborated the findings from the depletion experiment, suggesting that LHT1 has an affinity for L-Gln and L-Ala, although the reduction in L-Gln uptake was more pronounced in the labelling experiment. In papers II and III, using depletion and either ^{15}N- or ^{14}C-labeled amino acids, LHT1 was also found to have an affinity for L-Glu, L-Asp, L-Asn and L-Pro; in the case of L-Glu and L-Asp, this supports the results of Hirner et al. (2006) that LHT1 also has an affinity for acidic amino acids. However, the results of studies on the uptake of these anionic amino acids could be dependent on the experimental setup. One possible explanation is that L-Glu and L-Asp uptake is inhibited by other amino acids when supplied as a mixture, but it is also possible that L-Glu and L-Asp are effluxed after uptake, making apparent differences in uptake between the wild type and the *lht1* mutant greater when using isotopes than in depletion studies, since isotope uptake studies do not take efflux into account. In paper II it was surprising to find that the uptake of ^{15}N labelled L-Lys and L-Arg in the *lht1* mutant was significantly reduced, since only a slight insignificant decrease was observed in the depletion experiment. These conflicting findings could be explained if LHT1 transport of L-Lys and L-Arg is inhibited by the presence of other amino acids in the depletion solution. This suggests that LHT1 has a broad affinity for neutral-, and acidic amino acids, and some affinity for L-Lys and L-Arg when supplied individually.

As already suggested, a transporter exhibiting root activity for the uptake of cationic amino acids, or at least for L-Lys and L-Arg, must exist; our efforts to find it resulted in the discovery of AAP5 (Paper II). AAP5 perfectly matched our hypothesis and, in contrast to the broad affinity of LHT1, it is apparently specialised for the uptake of two amino acids, L-Lys and L-Arg. Loss of AAP5 function resulted in a reduction in L-Arg and L-Lys uptake by 87% and 90% respectively, when tested at 10 µM concentrations using a depletion setup. These results were confirmed in the subsequent ^{15}N labelling study (Paper II), although the substrate concentration was higher (30 µM).

Do plants have group-specific carriers for amino acids?

In the two screenings in this thesis (papers I & II), two root active transporters were identified. The affinity spectra of these transporters did not, generally, overlap: LHT1 had an affinity for neutral- and acidic

amino acids whereas AAP5 was found to transport L-Arg and L-Lys. Historically, it has been hypothesised and argued that plants might have distinct amino acid uptake systems for neutral-, acidic- and basic amino acids, i.e. transporters that discriminate on the basis of electrical charge (Soldal & Nissen 1978, Kinraide 1981, Datko & Mudd 1985, Borstlap et al. 1986, Schobert & Komor 1987). The data in papers I-IV suggest that LHT1 has an affinity for all neutral amino acids tested, the basic L-His and probably also for the acidic L-Glu and L-Asp. The finding that LHT1 transports L-His, L-Glu and L-Asp does not support the existence of a specialised transporter for neutral amino acids. However, it has been proposed that both L-His and L-Glu are taken up in their neutral/zwitterionic form (Boorer & Fischer 1997, Fischer et al. 2002). L-Lys and L-Arg, on the other hand, are thought to be transported in their cationic form (Komor et al. 1981, Boorer & Fischer 1997) Thus, it is possible that LHT1 transported L-His, L-Glu and L-Asp in their zwitterionic form even though, due to the pH conditions in the experiments described herein, most of the amino acids in the plant media had a net charge (Papers I-IV). So, even though the electrical charge of the amino acids transported was not investigated in papers I-IV, our findings support the suggestion that plants have distinct uptake systems with respect to an amino acid affinity spectrum and that LHT1 and AAP5 are probably the two most important components of the *Arabidopsis* root amino acid uptake systems.

The biggest overall difference between the *in planta* findings for LHT1 and AAP5 in papers I-IV and previous characterisations in heterelogous expression systems (Chen & Bush 1997, Boorer & Fischer 1997, Fischer et al. 2002) was that LHT1 was not found to have an affinity for L-Lys whereas AAP5 was found to be more or less specific for L-Arg and L-Lys and able to transport these amino acids in a mixture with neutral amino acids. A discussion about the discrepancy between our results relating to the affinity of LHT1 and AAP5 for root amino acid uptake and the results of other authors and the way that these transporters function in heterelogous expression systems, such as yeast and oocytes, is interesting but also problematic. It is questionable whether it is relevant to compare yeast and oocyte derived uptake data to that of plants. Bassham et al. (2000) addressed some of the issues of using yeast to assign a function to a gene product and argued that incorrect localisation of plant transporters when expressed in yeast was a possibility and that the information obtained in yeast needs to be confirmed in the plant. The partly diverging results obtained from heterelogous expression systems and plants also raise questions about the underlying mechanisms. It is likely that the chemical environment in yeast or oocyte cells is different from that which a transporter naturally encounters *in planta*; this could affect the characteristics

recorded for the transporters being studied. Another possible explanation is that some form of transporter regulation is lost in heterelogous expression systems. Thus, the use of heterelogous expressions systems is a good starting point when studying transporters but, as suggested by Bassham et al. (2000) and as indicated by our results, transporter function has to be verified *in planta*.

The kinetics of *Arabidopsis* amino acid uptake

In paper III, the concentration dependency of amino acid uptake was investigated. An experiment was set up where the uptake of L-Gln, L-Arg, L-Ala and L-Asp was tested in a concentration range between 2 and 50 µM. The main purpose was to investigate the effect of substrate concentration on the uptake rates in wild type *Arabidopsis*, the *lht1-*, *aap5-*, *aap1* mutants, the *lht1*aap5* double mutant and the LHT1 over-expressor and, if possible, to calculate kinetic parameters. The uptake of L-Arg, L-Gln and L-Ala became saturated at higher substrate concentrations, allowing for K_m and V_{max} calculations; the uptake of L-Asp followed a more linear pattern in the concentration range, resulting in calculated kinetic constants that do not reflect the recorded uptake characteristics. Although the results found for L-Asp failed to fulfil the prerequisites for Michaelis-Menten kinetics, it is clear that the uptake is carrier-mediated, as indicated by the apparent reduction in uptake in the *lht1* mutant. Also, the kinetic constants for affected mutant lines (except the LHT1 over-expressor) may not truly reflect uptake characteristics of these lines because the residual uptake in these lines was small and follows a linear pattern. Regardless of whether or not the kinetic constants truly reflect uptake characteristics, the uptake of L-Asp was reduced by approximately 70% in the *lht1* mutant. The lack of saturation in L-Asp uptake is likely to be because we simply did not use high enough concentrations; other unknown factors may also have influenced the results. In paper III, *Arabidopsis* was found to have a very high affinity for L-Arg and the rate of uptake in the *aap5* mutant decreased, overall, by at least 68% and had a K_m of 7.6 µM in wild type plants. The K_m for the uptake of L- Gln (41.0 µM) indicates a lower affinity than for L-Arg but is still within the high affinity range. The kinetic constants found for *Arabidopsis* uptake of L-Arg and L-Gln are in the same range as those found in plants or plant cells in several earlier studies (Soldal & Nissen 1978, Wyse & Komor 1984, Borstlap et al. 1986, Kielland 1994, Wallenda & Read 1999, Jämtgård et al. 2008). In comparison, the K_m values derived from measurements in yeast are, in the case of LHT1, similar to the ones in paper III, although using different amino acids (Hirner et al. 2006). Measurements on AAP5 expressed in oocytes resulted in K_m values much higher (140 µM for L-Arg, at pH 5) than found for *Arabidopsis* roots. Furthermore, AAP5 was found to have a K_m for L-Lys of 400 µM (Fischer et al. 2002).

Heremans et al. (1997), who probably studied *in planta Arabidopsis aap5* mutants, suggested that they had identified a low affinity transporter, having a K_m of 159 µM for L-Lys. Thus, when comparing the calculated kinetic constants derived from natural- and artificial expression of transporters, the outcome can be different, i.e. the absolute numbers for affinity vary; however, the relative affinity for different amino acids is quite consistent between experiments. Notably, if only looking at the uptake rates presented in paper III (Table S1), it is clear that the uptake of all amino acids was decreased by approximately 70% in either *lht1* or *aap5* at 2 µM. This substrate concentration is five times lower than that tested before (papers I & II) and further strengthens the view that these transporters have the capacity to carry out uptake at ecologically relevant concentrations.

Saturating versus linear uptake

Studies of amino acid uptake have shown complex kinetic characteristics, indicating single, double or multiple uptake systems with linear or saturating properties or a combination of both (Soldal & Nissen 1978, Borstlap et al. 1986, Schobert & Komor 1987, Wallenda & Read 1999). The observation that uptake, as illustrated in the v vs. s plots (paper III), displays both linear and saturating kinetics for wild type and unaffected knockout lines is interesting and raises questions about the reasons for these opposing uptake characteristics, especially since our results show that, regardless of whether uptake displays linear or saturating properties, it is significantly decreased by mutating the LHT1 or AAP5 transporters. It is possible that uptake of L-Asp saturates at concentrations higher than 50 µM; such concentrations do not occur in field conditions; however, other factors can also influence uptake.

When studying uptake rate concentration dependency, the usual approach is to fit data using Michaelis-Menten type kinetics. But, as pointed out by Reinhold & Kaplan (1984), applying Michaelis-Menten kinetics to uptake data might not be appropriate. It can be argued that the nature of an enzymatic reaction with a substrate and a product is fundamentally different to that of protein-facilitated uptake of a substrate, mainly because substrate transport could result in accumulation which could inhibit further uptake. However, the suggestion that substrate accumulation inhibits further uptake might be correct for unicellular organisms but is not necessarily true for uptake studies on intact roots, since the substrate taken up can be compartmentalized or translocated to neighbouring cells. Thus, the lack of saturation in the uptake of L-Asp and the saturating uptake of L-Gln, L-Arg and L-Ala in paper III could possibly be explained by the concentrations of the corresponding amino acid in root cells.

Uptake of multiple substrates and implications for kinetics

Both the K_m and V_{max} kinetic parameters calculated in paper III varied considerably between amino acids, even if transported by the same protein. Regardless of whether this finding is a matter of mathematical bias or a true observation, it would be valuable to investigate the kinetics of uptake in media containing two or more amino acids. There are a number of reasons why this would be interesting. First, the kinetics of amino acids tend to be investigated one amino acid at a time, whereas plants in the field are exposed to a number of amino acids simultaneously. Secondly, when studying kinetics, compounds are usually referred to as substrates and inhibitors but for amino acid uptake where transporters have transport capacity for more than one substrate it might be wrong to say that one amino acid inhibits the uptake of another, instead it can be argued that they are both taken up. And what do the kinetic parameters found for amino acid uptake in the laboratory tell us about plant uptake in the field? An interesting finding in Paper III, related to this question, was that the wild type had approximately the same K_m for L-Gln and L-Ala whilst the V_{max} values were 2.1 and 15.2, respectively. If tested in a solution containing both amino acids, would the kinetics be the same? As shown in paper I, L-Gln and L-Ala are clearly transported simultaneously by LHT1; in addition, the uptake rates recorded in paper I are also very similar to those recorded in paper III at 10 µM. So, how are uptake kinetics affected by having access to several substrates and should the plant capacity for amino acid uptake be regarded as the sum of uptake rates found for individual amino acids or is the relationship between uptake of individual amino acids and total amino acid uptake more complex? This complex question cannot be answered without more experimental data.

Use of amino acids for plant growth

The studies, utilizing ^{15}N labelled amino acids (papers I, II, IV) do not only allow the determination of the long-term acquisition of amino acids in wild type and mutant plants, they can also provide clues to the amino acid derived N content in plants. When supplied with large amounts of L-Ala and L-Gln (3 mM) in a mix with equal concentrations of nitrate (paper IV), amino acid N constituted between 37 and 47% of plant N. This finding is puzzling and intriguing because of the discrepancy between the large amounts of amino acid N found in the plants and the lack of increased growth when adding amino acids to a background of nitrate. One possible explanation is that the amino acids taken up are not incorporated into plant biomass due to metabolic constraints. However, in the same experiment (paper IV), plant total N (%) was virtually equal in plants given 3 mM amino acid + 3mM nitrate and 30 µM amino acid and 3 mM nitrate, an indication of that amino acid N is used in the same way as N from any other source. Moreover,

Persson et al. (2006) showed that L-Ala and L-Glu derived ^{15}N was quickly transferred to a number of other amino acids in *Pinus sylvestris,* suggesting that plants have the capacity to incorporate absorbed amino acids into proteins; it would be interesting to conduct similar experiments on *Arabidopsis*. It is, however, still possible that amino acids taken up in excess are stored and/or have an adverse effect on plant metabolism. A striking finding by Persson et al. (2006) was that pre-treatment with ammonium nitrate, although decreasing the uptake of L-Ala in absolute amounts, resulted in a large increase in L-Ala derived ^{15}N being transferred into L-Asn and "minor amino acids", whereas the ^{15}N levels being incorporated into L-Gln decreased. This suggests that plant amino acid metabolism could, in some way, be dependent on N status, which in turn could have implications for growth experiments in which plants are either supplied with amino acids as the sole N source or in a mixture with inorganic N. The presence of some amino acids in growth media can also down-regulate the uptake of nitrate (for review see Miller et al. 2007), thereby increasing the relative amounts of amino acid N absorbed by plants. This could, in turn, result in similar growth on nitrate + L-Ala or nitrate + L-Gln as compared to nitrate grown plants, as seen in papers I & IV. Nevertheless, the high levels of amino acid N found in plants supplied with nitrate + amino acids could suggest that the significance of amino acids as a N source is underestimated when N is supplied as amino acids only and that inorganic N is required to some degree for efficient amino acid assimilation.

Studies on amino acids as a source of plant N have produced ambiguous results, suggesting that they promote growth, inhibit growth and are of limited importance. When supplied individually, amino acids are generally poor N sources compared to nitrate (Valle & Virtanen 1965, Bollard 1966, Joy 1969, Bonner et al. 1996,1997, Hirner et al. 2006, papers I & IV). However, there are a few examples where the addition of amino acids to inorganic N sources has had a positive effect on plant growth (Valle & Virtanen 1965, Joy 1969, Papers I & IV). During the course of the work resulting in this thesis, experiments in which *Arabidopsis* was subjected to a number of treatments revealed both capacities and limitations in the utilization of amino acids for growth.

In papers I and IV, glutamine was shown to be the most growth promoting amino acid, although being on average, only about half the size of plants grown on nitrate. Glutamine has also been shown to be a growth supporting amino acid in other species and, sometimes, the only amino acid that supports growth (Turnbull et al. 1995, Bonner et al. 1996, Schmidt et al. 2006). Adding amino acids to nitrate resulted in only a small or non-significant growth increase (Papers I & IV),

corroborating previous findings (Valle & Virtanen 1965, Bollard 1966). An interesting aspect of *Arabidopsis* growth on amino acids is the lack of correlation between the uptake rates found for a specific amino acid and its suitability as a N source (paper IV). There is also evidence that *Arabidopsis* growth on amino acids is limited by transporter function and capacity, as shown in the mutant and over-expressor experiments described in papers I & IV and by Hirner et al. (2006). In the experiments in paper IV, LHT1 over-expressors exhibited much increased growth over a concentration range of L-Gln, L-Glu and L-Asn. Notably, the over-expressor grew equally well on 1.5 mM L-Gln as on 3 mM nitrate, suggesting that uptake not only limit growth but that amino acids are a good N source. A complicating factor regarding the growth experiments in this thesis, in common with all amino acid growth studies, is that the high amino acid concentrations used are sometimes 100 times higher than the ones found in soils. Data in paper IV also suggest that the importance of amino acid uptake for growth is greater at lower amino acid concentrations; showing that the percentage reduction in biomass in the *lht1-5* mutant was greatest at the lowest L-Gln concentration. Thus the agar-based growth experiments in this thesis could be regarded as not ecologically relevant; it is clear that efforts need to be made to try to develop experimental systems supplying plants with µmolar levels of N. Nonetheless, altering the expression of LHT1 has been shown to affect *Arabidopsis* growth on amino acids.

Metabolic constraints of amino acid assimilation

Not only does *Arabidopsis* growth on amino acids seem to be limited by its uptake capacity, also the assimilation of amino acids might be a limiting step for the use of amino acids in plant metabolism and growth. Several studies have also pointed to a metabolic constraint with regard to plant utilization of D-enantiomeric (cf. Erikson et al. 2004; 2005). As a result of genetic engineering, *Arabidopsis* plants were able to metabolize the otherwise toxic D-isomers of some amino acids (Erikson et al. 2004 & 2005). These plants were able to grow on D-Ser and D-Ala as the sole N source and grew better on nitrate + D-Ser than on nitrate only (the latter in comparison to wild type growth on nitrate + L-Gln in paper IV).

In paper IV, *Arabidopsis* growth on a range of amino acids was tested. Of the 15 amino acids used in this experiment, 6 promoted growth when supplied alone and only 3 when supplied together with nitrate. The reason for why some amino acids can sustain growth to some extent whereas others inhibit growth is not well understood. It should however be noted that the results of the growth experiments could be due to the

high amino acid concentrations used, as compared to those found in the field (as discussed in *Methodological reflections)*.

The 6 growth promoting amino acids in paper IV all belong to what Noctor et al. (2002) refer to as the major amino acids, consisting of: L-Gln, L-Glu, Gly, L-Ser, L-Asp, L-Ala and L-Thr. The major amino acids are generally present at high concentrations in plants and are closely linked to carbon metabolism and N assimilation. It is therefore plausible to believe that there is a connection between which amino acids that are central to plant metabolism and those that promote growth. Noctor et al. (2002) also found the concentrations of the remaining amino acids, referred to as minor amino acids, to be co-ordinated, i.e. the relative levels of these amino acids stay relatively constant. This could in turn explain why some amino acids in paper IV inhibit growth, possibly through the inhibition of enzymatic pathways for other amino acids.

Practical applications

One practical application of amino acids as N sources already exists: Öhlund & Näsholm (2001) showed that Scots pine and Norway spruce grew as well on L-Arg and Gly as compared to commercial fertilizer. L-Arg fertilization was then shown to be suitable for the cultivation of conifer seedlings due to the low N losses associated with its use because of the strong retention of L-Arg in soils or growth substrates (Öhlund & Näsholm 2002). The low mobility of L-Arg in soils could, in fact, explain how evolutionary pressure could lead to a transporter having a high affinity for L-Arg. The discovery that AAP5 is active in L-Arg uptake could be used to enhance uptake and possibly to increase plant growth on this new type of fertilizer, in the same way as the LHT1 over-expressor in paper IV that in contrast to the attempts to enhance uptake of mineral N (Fraisier et al. 2000) was successful. Thus, knowledge of amino acid transporters could potentially be used to metabolically engineer plants, creating novel amino acid distribution or growth.

Conclusions and future challenges

Amino acid uptake in plants is an established fact and has been proven for many plant species. Despite its preferred source of N being nitrate, *Arabidopsis* has been found to take up amino acids at ecologically relevant concentrations. The transporters responsible for amino acid uptake in plants were, however, unknown when the work underlying this thesis began.

Since this project started, three amino acid transporters involved in root uptake of amino acids have been discovered; my work contributed to the discovery and characterisation of two of these. LHT1 was the first

transporter to be identified as being active in root amino acid uptake, specifically in the uptake of neutral- and probably also acidic amino acids. The discovery of AAP5 closed the affinity gap left by LHT1; it carries out high affinity transport of L-Lys and L-Arg. Mutating and over expressing LHT1 was shown not only to have an impact on amino acid uptake, but also to influence plant growth on amino acids. An intriguing result in paper IV was that amino acids do not necessarily promote growth, but when the origin of plant N was analysed, amino acid N constituted up to almost 50%. The kinetics of *Arabidopsis* amino acid uptake described here strengthen the view that uptake is "intentional", being carried out by transporters adapted to the amino acid concentrations found in soils. A role in the retrieval of amino acids being effluxed from the roots cannot, however, be dismissed. Almost no uptake occurred in the double mutant of LHT1 and AAP5 for the amino acids tested, suggesting that, at least in the micro-molar range, it is unlikely that any transporter with substantial impact in this affinity range will be discovered. There are, however, transporters involved in root uptake that remain to be identified in other species; that may or may not be similar to LHT1 and AAP5.

The future challenge lies in the work of trying to accurately quantify the significance of amino acid uptake in plants. The results of such work could have a big impact on our view on plant nutrition, as well as having implications for the fertilization and management of plants. In this context, such work could also be valuable in agricultural applications, using plants over-expressing amino acid transporters to promote growth or in some way affect amino acid metabolism in plants.

Acknowledgements

Ja, vad säger man dryga fem år som doktorand?

Först och främst måste jag tacka Torgny, jag skulle vilja beskriva dig som ett jordnära uppslagsverk med en stresstålighet utöver det vanliga och du har tid för alla. Dina idéer är (nästan) alltid bra, du har en sällsynt god förmåga att sålla bra försök från dåliga, detta tycker jag tydligt avspeglas i att undertecknad snart kan lämna universitetet med en känsla av att faktiskt ha åstadkommit något av värde, en känsla jag säkert delar med dina forna doktorander. Du är även för snäll, du kunde gott ha låtit piskan vina över mig någon gång då och då……..

Ulrika, vi kompletterar varandra, jag är slarvig, du är ordentlig. Att vi sedan delar samma positiva livssyn a´la "vad ska det hända för skit idag då?" gör inte saken sämre. Jag räknar också kallt med att vi tar storslam på årets Inefficiency Awards, IGEN!

Tack till medlemmarna i kvävegruppen och mina kontorskumpaner. Ett särskilt tack vill jag ge Maggan, tack vare dig har sannolikt ett antal Henrik-relaterade kemikalieolyckor undvikits!

Tack till er i "hardcore-fika-gänget", ni som i ur och skur trots stor arbetsbelastning alltid finner tid till att fika och diskutera livets väsentligheter såsom trafikregler, växthuseffekten och hjärnslöa program på kanal 3 och 5. Guldstjärna till Kjell som varit en klippa i fotodiskussioner och fototävlingar. Även Erling förtjänar ett hedersomnämnande, trots att du ibland kan bli lite upptrissad så delar jag många av dina åsikter och du behövs verkligen för att hålla ordning på oss osnutna ungdomar i fikarummet!

Tack till min familj som stöttat mig, och sedan länge undrat när jag ska bli klar! Pernilla, tack för att du står ut med mig och att du agerar som talesman när folk undrar vad jag egentligen gör som doktorand (hon brukar säga, -han jobbar med a-r-a-b-i-d-o-p-s-i-s, ett ogräs och någonting med aminosyror), för det är ju det jag gör!

References

Alonso, J. M., A. N. Stepanova, T. J. Leisse, C. J. Kim, H. Chen, P. Shinn, D. K. Stevenson, J. Zimmerman, P. Barajas, R. Cheuk, C. Gadrinab, C. Heller, A. Jeske, E. Koesema, C. C. Meyers, H. Parker, L. Prednis, Y. Ansari, N. Choy, H. Deen, M. Geralt, N. Hazari, E. Hom, M. Karnes, C. Mulholland, R. Ndubaku, I. Schmidt, P. Guzman, L. Aguilar-Henonin, M. Schmid, D. Weigel, D. E. Carter, T. Marchand, E. Risseeuw, D. Brogden, A. Zeko, W. L. Crosby, C. C. Berry, & J. R. Ecker. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301:653-657.

Bardgett, R.D., Streeter, T. & Bol, R. (2003). Soil microbes compete effectively with plants for organic nitrogen inputs to temperate grasslands. *Ecology 84*, 1277-1287.

Bassham, D.C., & Raikhel, N.V. (2000). Plant cells are not just green yeast. *Plant Physiology 122*, 999–1001.

Bollard, E.G. (1966). A comparative study of the ability of organic nitrogenous compounds to serve as sole sources of nitrogen for the growth of plants. *Plant & Soil XXV*, 153-166.

Bonner, C.A. & Jensen, R.A. (1996). Antagonism by L-glutamine of toxicity and growth inhibition caused by other amino acids in suspension cultures of *Nicotiana silvestris*. *Plant Science 113*, 43-58.

Bonner, C.A. & Jensen, R.A. (1997). Recognition of specific patterns of amino acid inhibition of growth in higher plants, uncomplicated by glutamine-reversible 'general amino acid inhibition'. *Plant Science 130,* 133–143.

Boorer, K.J. & Fischer, W.N. (1997). Specificity and stoichiometry of the *Arabidopsis* H+/amino acid transporter AAP5. *Journal of Biological Chemistry 272,* 13040– 13046.

Boorer, K.J., Frommer, W.B., Bush, D.R., Kreman, M., Loo, D.D.F. & Wright, E.M. (1996). Kinetics and specificity of a H+/Amino Acid transporter from *Arabidopsis thaliana*. *Journal of Biological Chemistry 271*, 2213-2220.

Borstlap, A.C., Meenks, J.L.D., van Eck, W.F. & Bicker, J.T.E. (1986). Kinetics and specificity of amino acid uptake by the duckweed *Spirodela polyrhiza* (L.) Schleiden. *Journal of Experimental Botany 37*, 1020-1035.

Bright, S. W. J., Kueh, J. S. H. & Rognes, S. (1983). Lysine transport in two barley mutants with altered uptake of basic amino acids in the root. *Plant Physiology 2*, 821-824.

Bush, D.R. (1993). Proton-coupled sugar and amino acid transporters in plants. *Annual Review of Plant Physiology and Plant Molecular Biology 44*, 513-542.

Bush, D.R. (1999). Amino Acid Transport. In BK Singh, ed, *Plant Amino Acids: Biochemistry and Biotechnology*. Marcel Dekker, New York, 305-318.

Chapin III, F.S., Moilainen, L. & Kielland, K. (1993). Preferential use of organic nitrogen by a non-mycorrhizal arctic sedge. *Nature 361,* 150-153.

Chen, L. & Bush, D.R. (1997). LHT1, a lysine- and histidine-specific amino acid transporter in *Arabidopsis*. *Plant Physiology 115,* 1127-1134.

Chen, L., Ortiz-Lopez, A., Jung, A. & Bush, D.R. (2001). ANT1, an Aromatic and Neutral Amino Acid Transporter in *Arabidopsis*. *Plant Physiology 125,* 1813-1820.

Datko, A. H. & Mudd, S. H. (1985). Uptake of amino acids and other organic compounds by *Lemna paucicostata* Hegelm. *Plant Physiology* 77, 770-778.

Erikson, O., Hertzberg, M. & Näsholm, T. (2004). A conditional marker gene allowing both positive and negative selection in plants. *Nature Biotechnology 22,* 455-458.

Erikson, O., Hertzberg, M. & Näsholm, T. (2005). The dsdA gene from *Escherichia coli* provides a novel selectable marker for plant transformation. *Plant Molecular Biology 57,* 425-433.

Falkengren-Grerup, U., Månsson, K.F. & Olsson, M.O. (2000). Uptake of amino acids by ten grasses and forbs in relation to soil acidity and nitrogen availability. *Environmental and Experimental Botany 44*, 207-219.

Fischer, W.N., Andre, B., Rentsch, D., Krolkiewicz, S., Tegeder, M., Breitkruz, K. & Frommer, W.B. (1998). Amino acid transport in plants. *Trends in Plant Science 3*, 188-195.

Fischer, W. N., Kwart, M., Hummel, S. & Frommer, W. B. (1995). Substrate specificity and expression profile of amino acid transporters (AAPs) in *Arabidopsis*. *The Journal of Biological Chemistry* 270 (27) 16315-16320.

Fischer, W.-N., Loo, D.D.F., Koch, W., Ludewig, U., Boorer, K.J., Tegeder, M., Rentsch, D., Wright, E.M. & Frommer, W.B. (2002). Low and high affinity amino acid H+-cotransporters for cellular import of neutral and charged amino acids. *The Plant Journal 29*, 717-731.

Foster, J., Lee, Y.H. & Tegeder, M. (2008). Distinct expression of members of the LHT amino acid transporter family in flowers

Fraisier, V., Gojon, A., Tillard, P. & Daniel-Vedele F. (2000). Constitutive expression of a putative high-affinity nitrate transporter in Nicotiana plumbaginifolia: evidence for post-transcriptional regulation by a reduced nitrogen source. *Plant Journal 23*: 489–496.

Frommer, W.B., Hummel, S. & Riesmeier, J.W. (1993). Expression cloning in yeast of a cDNA encoding a broad specificity amino acid permease from *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America 90*, 13, 5944-8

Glass, A.D.M., Britto, D.T., Kaiser, B.N., Kinghorn, J.R., Kronzucker, H.J., Kumar, A., Okamoto, M., Rawat, S.R., Siddiqi, M.Y., Unkles, S.E. & Vidmar, J.J. (2002). The regulation of nitrate and ammonium transport systems in plants. J*ournal of Experimental Botany 53*, 855-864.

Grallath, S., Weimar, T., Meyer, A., Gumy, C., Suter-Grotemeyer, M., Neuhaus, J.M. & Rentsch, D. (2005). The AtProT family. Compatible solute transporters with similar substrate specificity but differential expression patterns, *Plant Physiology 137,* 117–126.

Guo, M.G. (2004). Molecular and genomic analysis of nitrogen regulation of amino acid permease 1 (AAP1) in *Arabidopsis*. PhD dissertation, University of Illinois at Urbana-Champaign, URBANA, IL, USA.

Hangs, R.D., Knight, J.D. & Van Rees, C.J. (2003). Nitrogen uptake characteristics for roots of conifer seedlings and common boreal forest competitor species. *Canadian Journal of Forest Research 33*, 156–163.

Harrison, K.A., Bol, R. & Bardgett, R.D. (2008). Do plant species with different growth strategies vary in their ability to compete with soil microbes for chemical forms of nitrogen? *Soil Biology & Biochemsitry 40*, 228-237.

Henry, H.A.L. & Jefferies, R.L. (2002). Free amino acid, ammonium and nitrate concentrations in soil solutions of a grazed coastal marsh in relation to plant growth. *Plant Cell & Environonment 25*: 665-675.

Heremans, B., Borstlap, A.C. & Jacobs, M. (1997). The rlt11 and raec1 mutants of *Arabidopsis thaliana* lack the activity of a basic-amino-acid transporter. *Planta 201*, 219-226.

Hirner, A., Ladwig, F., Stransky, H., Okumoto, S., Keinath, M., Harms, A., Frommer, W.B. & Koch W. (2006). *Arabidopsis* LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. *Plant Cell 18*, 1931–1946.

Hodge, A., Stewart, J., Robinson, D., Griffiths, B.S. & Fitter, A.H. (2000). Competition between roots and soil micro-organisms for nutrients from nitrogen-rich patches of varying complexity. *Journal of Ecology 88*, 150-164.

Hsu, L.C., Chiou, T.J., Chen, L., and Bush, D.R. (1993). Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant. *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* 90, 7441– 7445.

Hutchinson, H.B. & Miller, N.H.J. (1911). The direct assimilation of inorganic and organic forms of nitrogen by higher plants. *Centbl Bakt II 30*, 513-547.

Jones, D.L. & Darrah, P.R. (1993). Influx and efflux of amino acids from *Zea mays* L. roots and their implications for N nutrition and the rhizosphere. *Plant and Soil* 155/156, 87-90.

Jones, D.L. & Darrah, P.R. (1994). Amino-acid influx at the soil-root interface of *Zea mays* L. and its implications in the rhizosphere. *Plant and Soil 163*, 1-12.

Jones, D.L., Healey, J.R., Willett, V.B., Farrar, J.F. & Hodge, A. (2005a). Dissolved organic nitrogen uptake by plants—an important N uptake pathway? *Soil Biology & Biochemistry 37*, 413–423.

Jones, D.L. & Hodge, A. (1999). Biodegradation kinetics and sorption reactions of three differently charged amino acids in soil and their effects on plant organic nitrogen availability. *Soil Biology and Biochemistry* 31, 1331-1342.

Jones, D.L. & Kielland, K. (2002). Soil amino acid turnover dominates the nitrogen flux in permafrost-dominated taiga forest soils. *Soil Biology and Biochemistry 34*, 209-219.

Jones, D.L., Shannon, D., Junvee-Fortune, T. & Farrarc, J.F. (2005b). Plant capture of free amino acids is maximized under high soil amino acid concentrations. *Soil Biology & Biochemistry 37*, 179–181.

Joy, K.W. (1969). Nitrogen metabolism of *Lemna minor*. I. Growth, nitrogen sources and amino acid inhibition. *Plant Physiology 44*, 845-848.

Jämtgård, S., Näsholm, T. & Huss-Danell, K. (2007). Uptake of organic nitrogen by Barley. *Plant & Soil 302*, 221-231.

Kielland, K. (1994). Amino acid absorption by arctic plants: implications for plant nutrition and nitrogen cycling. *Ecology 75*, 2373-2383.

Kielland, K. (1995). Landscape patterns of free amino acids in arctic tundra soils. *Biogeochemistry 31*, 85-98.

Kielland, K., McFarland, J. & Olson K. (2006). Amino acid uptake in deciduous and coniferous taiga ecosystems. *Plant & Soil 288*, 297-307.

Kielland, K., McFarland, J.W., Ruess, R.W. & Olson, K. (2007). Rapid cycling of organic nitrogen in taiga forest ecosystems. *Ecosystems 10*, 360-368.

Kinraide, T. B. (1981). Interamino acid inhibition of transport in higher plants.
Evidence for two transport channels with ascertainable affinities for amino acids.
Plant Physiology 68:1327-1333.

Komor, E., Thom, M. & Marketzki, A. (1981). Mechanism of uptake of L-Arginine by sugar-cane cells. *European Journal of Biochemistry* 116, 527-533.

Kranabetter, J.M., Dawson, C.R. & Dunn, D.E. (2007). Indices of dissolved organic nitrogen ammonium and nitrate across productivity gradients of boreal forests. *Soil Biology & Biochemistry 39*, 3147-3158.

Kumpaisal, R., Hashimoto, T. & Yamada, Y. (1989). Uptake of lysine by wild-type and S-(2-aminoethyl)-L-cysteine-resistant suspension-cultured cells of *Triticum aestivum*. *Plant Cell Physiology 30(8),* 1099-1105.

Kwart, M., Hirner, B., Hummel, S. & Frommer, W.B. (1993). Differential expression of two related amino acid transporters with differing substrate specificity in *Arabidopsis thaliana*. *Plant Journal 4: 6*, 993-1002.

Lee, Y.H., Foster, J., Chen, J., Voll, L.M., Weber, A.P.M. & Tegeder, M. (2007). AAP1 transports uncharged amino acids into roots of *Arabidopsis*. *Plant Journal 50*, 305-319.

Lee, Y.H. & Tegeder, M. (2004). Selective expression of a novel high-affinity transport system for acidic and neutral amino acids in the tapetum cells of *Arabidopsis* flowers. *Plant Journal 40*, 60–74.

Lister, C. & Dean, C. (1993). Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. *Plant Journal 4*, 745–750.

Liu, X. & Bush, D. R. (2006). Expression and transcriptional regulation of amino acid transporters in plants. *Amino Acids* 30, 113-120.

Ludewig, U., Wirén, N. von. & Frommer, W. B. (2002). Uniport of NH_4^+ by the root hair plasma membrane ammonium transporter LeAMT1;1. *The Journal of Biological Chemistry* 277 (16), 13548-13555.

Lukowitz, W., Gillmor, C.S. & Scheible, W.R. (2000). Positional cloning in *Arabidopsis*. Why it feels good to have a genome initiative working for you. *Plant Physiology 123*, 795–805.

McFarland, J.W., Ruess, R.W., Kielland, K. & Doyle A.P. (2002). Cycling dynamics of NH_4^+ and amino acid nitrogen in soils of a deciduous boreal forest ecosystem. *Ecosystems 5*: 775–788.

Miller, A.J., Fan, X., Shen, Q. & Smith, S. (2007). Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *Journal of Experimental Botany 59(1),*111-119.

Min, X., Siddiqi, M.Y., Guy, R.D., Glass, A.D.M. & Kronzucker, H.J. (2000). A comparative kinetic analysis of nitrate and ammonium influx in two early-successional tree species of temperate and boreal forest ecosystems. *Plant, Cell and Environment 23*, 321-328.

Näsholm, T., Ekblad, A., Nordin, A., Giesler, R., Högberg, M.N. & Högberg, P. (1998). Boreal forest plants take up organic nitrogen. *Nature 392*, 914-916.

Näsholm, T., Huss-Danell, K. & Högberg, P. (2000). Uptake of organic nitrogen in the field by four agriculturally important plant species. *Ecology 81*, 1155-1161.

Näsholm, T. & Persson, J. (2001). Plant acquisition of organic nitrogen in boreal forests. *Physiologia Plantarum 111*, 419-426.

Noctor, G., Novitskaya, L., Lea, P. J & Foyer C. H. (2002). Co-ordination of leaf minor amino acid contents in crop species: significance and interpretation. *Journal of Experimental Botany 53*: 939–945.

Nordin, A., Högberg, P. & Näsholm, T. (2001). Soil nitrogen form and plant nitrogen uptake along a boreal forest gradient. *Oecologia 129*, 125-132.

Okumoto, S., Koch, W., Tegeder, M., Fischer, W.N., Biehl, A., Leister, D., Stierhof, Y.D. & Frommer, W.B. (2004). Root phloem-specific expression of the plasma membrane amino acid proton co-transporter AAP3. *Journal of Experimental Botany 55*, 2155–2168.

Østergaard, L. & Yanofsky, M.F. (2004). Establishing gene function by mutagenesis in *Arabidopsis thaliana*. *The Plant Journal* 39, 682–696.

Öhlund, J. (2004). Organic and inorganic nitrogen sources for conifer seedlings. Doctoral diss. Dept. of Forest Genetics and Plant Physiology, SLU. *Acta Universitatis Agriculturae Sueciae. Silvestra vol. 312*.

Öhlund, J. & Näsholm, T. (2001). Growth of conifer seedlings on organic and inorganic nitrogen sources. *Tree Physiology 21*, 1319-1326.

Öhlund, J. & Näsholm, T. (2002). Low nitrogen losses with a new source of nitrogen for cultivation of conifer seedlings. *Environmental Science and Technology* 36 (22), 4854-4859.

Ortiz-Lopez, A., Chang, H.-C. & Bush, D.R. (2000). Amino acid transporters in plants. *Biochimica et Biophysica Acta 1465*, 275-280.

Owen, A.G. & Jones, D.L. (2001). Competition for amino acids between wheat roots and the rhizosphere microorganisms and the role of amino acids in plant N acquisition. *Soil Biology and Biochemistry 33*, 651-657.

Paungfoo-Lonhienne, C., Lonhienne,T. G. A., Rentsch, D., Robinson, N., Christie, M., Webb, R.I., Gamage, H. K., Carroll, B. J., Schenk, P. M. & Schmidt, S. (2008). Plants can use protein as a nitrogen source without assistance from other organisms. *Proceedings of the National Academy of Sciences of the United States of America*

105:11, 4524-4529.
Persson, J. (2003). Organic nitrogen uptake by boreal forest plants. Doctoral diss. Dept. of Forest Genetics and Plant Physiology, SLU. *Acta Universitatis Agriculturae Sueciae. Silvestra vol. 265.*
Persson, J., Gardeström, P. & Näsholm, T. (2006). Uptake, metabolism and distribution of organic and inorganic nitrogen sources by *Pinus sylvestris*. *Journal of Experimental Botany* 57 (11), 2651-2659.
Persson, J. & Näsholm, T. (2001). Amino acid uptake: a widespread ability among boreal forest plants. *Ecology Letters* (4) 434-438.
Raab, T.K., Lipson, D.A. & Monson, R.K. (1996). Non-mycorrhizal uptake of amino acids by roots of the alpine sedge *Kobresia myosuroides*: implications for the alpine nitrogen cycle. *Oecologia 108*, 488–494.
Raab, T.K., Lipson, D.A. & Monson, R.K. (1999). Soil Amino Acid Utilization among Species of the Cyperaceae: Plant and Soil Processes. *Ecology, Vol. 80*, No. 7, 2408-2419.
Redei, G.P. & Koncz, C. (1992). Classical mutagenesis. In Methods in *Arabidopsis* Research (Koncz, C., Chua, N.-H. and J. Schell, eds). *Singapore: World Scientific*, 16–82.
Reinhold, L. & Kaplan, A. (1984). Membrane transport of sugars and amino acids. *Annual review of Plant Physiology* 35, 45-83.
Rentsch, D., Schmidt, S. & Tegeder, M. (2007). Transporters for uptake and allocation of organic nitrogen compounds in plants. *FEBS Letters 581,* 2281-2289.
Salonen, M. L. & Simola, L. K. (1977). Dipeptides and Amino Acids as Nitrogen Sources for the Callus of *Atropa belladonna. Physiologia Plantarum 41, 55–58.*
Schimel, J. & Bennett, J. (2004). Nitrogen mineralization: challenges of a changing paradigm. *Ecology* 85 (3), 591-602.
Schimel, J.P. & Chapin III, F.S. (1996). Tundra plant uptake of amino acid and NH4+ nitrogen *in situ*: Plants compete well for amino acid N. *Ecology 77*, 2142-2147.
Schmidt, S., Handley, L. L. & Sangtiean, T. (2006). Effects of nitrogen source and ectomycorrhizal associations on growth and $\delta\ ^{15}N$ of two subtropical *Eucalyptus* species from contrasting ecosystems. *Functional Plant Biology* 33, 367-379.
Schobert, C., Köckenberger, W. & Komor, E. (1988). Uptake of amino acids by plants from the soil: A comparative study with castor bean seedlings grown under natural and axenic soil conditions. *Plant and Soil 109*, 181-188.
Schobert, C. & Komor, E. (1987). Amino acid uptake by *Ricinus communis* roots: characterization and physiological significance. *Plant, Cell and Environment 10*, 493-500.
Sokolovski, S.G., Meharg, A.A. & Maathuis, F.J.M. (2002). *Calluna vulgaris* root cells show increased capacity for amino acid uptake

when colonized with the mycorrhizal fungus *Hymenoscyphus ericae*. *New Phytologist* 155, 525-530.
Soldal, T. & Nissen, P. (1978). Multiphasic uptake of amino acids by barley roots. *Physiologia Plantarum 43*, 181-188.
Streeter, T.C., Bol, R. & Bardgett, R.D. (2000). Amino acids as a nitrogen source in temperate upland grasslands: the use of dual labelled (13C, 15N) glycine to test for direct uptake by dominant grasses. *Rapid Communications in Mass Spectrometry 14*, 1351-1355.
Tamm, C.O. (1991). Nitrogen in terrestrial ecosystems. *Springer Verlag. Berlin.*
The *Arabidopsis* Genome Initiative (AGI). (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature 408*, 796–815.
Thornton, B. & Robinson, D. (2005). Uptake and assimilation of nitrogen from solutions containing multiple N sources. *Plant, Cell and Environment 28* , 813–821.
Turnbull, M.H., Goodall, R. & Stewart, G.R. (1995). The impact of mycorrhizal colonisation upon nitrogen source utilization and metabolism in seedlings of *Eucalyptus grandis* Hill ex Maiden and Eucalyptis maculata Hook. *Plant, Cell and Environment 18*, 1386-1394.
Vinolas, L. C., Healey, J. R. & Jones D.L. (2001). Kinetics of soil microbial uptake of free amino acids. *Biology and Fertility of Soils 33, 67-74.*
Virtanen, A.I. & Linkola, H. (1946). Organic nitrogen compounds as nitrogen nutrition for higher plants. Nature 157, 515.
Valle E, Virtanen AI. (1965). On the injurious and growth promoting effects in peas and barley of various amino acids given together with nitrate. *Acta Agralica Fennica 107*, 308-319.
Verbruggen, N., Borstlap, A. C., Jacobs, M., Montagu, M. Van. & Messens, E. (1996). The *raz1* mutant of *Arabidopsis thaliana* lacks the activity of a high-affinity amino acid transporter. *Planta* 200, 247-253.
Wallenda, T. & Read, D. J. (1999). Kinetics of amino acid uptake by ectomycorrhizal roots. *Plant, Cell and Environment 22*, 179–187.
Weigel, D. and Glazebrook, J. (2002). *Arabidopsis*: A Laboratory Manual. *Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.*
Weigelt, A., Bol, R. & Bardgett, D. (2005). Preferential uptake of soil nitrogen by grassland plant species. *Oecologia 142*, 627-635
Williams, L.E. & Miller, A.J. (2001). Transporters responsible for the uptake and partitioning of nitrogenous solutes. *Annual Review of Plant Physiology and Plant Molecular Biology 52*, 659-688.

Wright, D.E. (1962). Amino acid uptake by plant roots. *Archives of Biochemsiry & Biophysics 97, 174-180*.

Wyse, R.E. & Komor, E. (1984). Mechanism of amino acid uptake by sugar cane suspension cells. *Plant Physiology 29, 865–870*.

Xu, X.L., Ouyang, H., Cao, G.M., Pei, Z.Y. & Zhou, C.P. (2004). Uptake of organic nitrogen by eight dominant plant species in *Kobresia* meadows. *Nutrient Cycling in Agroecosystems 69*, 5–10.

Yang, Y., Hammes, U.Z., Taylor, C.G., Schachtman, D.P., & Nielsen, E. (2006). High-affinity auxin transport by the AUX1 influx carrier protein. *Current Biology 16*, 1123–1127.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L, & Gruissem, W. (2004). GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. *Plant Physiology*. 136, 2621–2632.