The Occurrence of Amino Acids in Agricultural Soil and their Uptake by Plants

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Doctoral Thesis
Swedish University of Agricultural Sciences
Umeå 2010

Acta Universitatis agriculturae Sueciae
2010:27

Cover: The art of root dipping
(photo: Sandra Jämtgård)

ISSN 1652-6880
ISBN 978-91-576-7504-0

Print: Arkitektkopia AB, Umeå 2010

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Abstract

The ability of plants to take up amino acids is widespread among plants, but the ecological and physiological implications of this ability are not fully understood. Therefore, in the investigations this thesis is based upon, key aspects of the uptake of amino acids by agricultural plants were explored in field studies (to ensure ecological relevance) and laboratory analyses (to ensure precision). Small tension lysimeters were used to collect soil solution from several agricultural soils with minimal disturbance. Concentrations of free amino acids were found to be low (0-12.7 μM). However, they may be continuously replenished from bound amino acid pools and were found to be sufficiently high (generally) for uptake by hydroponically grown barley, Hordeum vulgare L., and Arabidopsis, Arabidopsis thaliana L. Hence, the effective minimum concentrations for uptake by these species do not seem to exceed most of the field-measured concentrations. The uptake affinity in both barley and Arabidopsis was found to be comparable to reported values for nitrate at corresponding concentrations and for uptake of amino acids by soil micro-organisms. The amino acid transporters lysine histidine transporter 1 (LHT1) and amino acid permease 5 (AAP5) were found to be largely responsible for amino acid uptake in Arabidopsis at these concentrations. These transporters have complementary affinities for amino acids with differing properties; LHT1 transporting acidic and neutral amino acids, and AAP5 basic amino acids. Furthermore, the gene expression of LHT1 and AAP5 clearly increased after roots were exposed to amino acids, even in the presence of inorganic nitrogen, resulting in up to 15-fold increases in the rate of amino acid uptake. The induced amino acid uptake rates were up to 10-fold higher than nitrate uptake rates in Arabidopsis.

According to standard textbooks, nitrate and ammonium are the major nitrogen sources for plants. However, the results of these studies indicate that plants have the capacity to take up amino acids at field concentrations in presence of nitrate and ammonium. This capacity requires gene expression, synthesis and regulation of amino acid transporters, and the ability of plants to sense and respond to amino acid concentrations at ambient concentrations. There is, therefore, little doubt that plants can take up amino acids in their natural environment. Thus, it is time to reconsider traditional views of the nitrogen compounds used by agricultural plants.*Keywords:* Arabidopsis thaliana, barley, Bound amino acids, Free amino acids, Induction, Inorganic nitrogen, Lysimeter, Nitrogen, Amino acid transporter

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To F and I

Först piggade han upp sig med en arg och krigisk dans.
Sen högg han sina tänder djupt i morrans kalla svans.

Tove Jansson, Vem ska trösta knyttet

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 Jämtgård S, Näsholm T and Huss-Danell K. Nitrogen compounds in soil solutions of agricultural land. (submitted)

II Jämtgård S, Näsholm T and Huss-Danell K (2008). Characteristics of amino acid uptake in barley. *Plant and Soil* 302, 221-231.

III Svennerstam H, Jämtgård S, Huss-Danell K, Näsholm T and Ganeteg U. Transporters in Arabidopsis roots mediating uptake of amino acids at field relevant concentrations. (manuscript)

IV Jämtgård S, Holmlund M, Cambui Aguetoni C, Inselsbacher E, Huss-Danell K and Näsholm T. Induction of amino acid uptake in Arabidopsis and barley. (manuscript)

The contribution of Sandra Jämtgård to the papers included in this thesis was as follows:

I Planned the work jointly with the co-authors, analysed the data, wrote the paper.

II Planned the work jointly with the co-authors, performed the experiments and analysed the data, wrote the paper.

III Planned the work jointly with the co-authors, performed uptake experiments, analysed data, took part in writing the paper.

IV Planned the work jointly with Holmlund, Huss-Danell and Näsholm, performed and analysed uptake experiments, wrote the paper.

Abbreviations

AAP1	Amino acid permease 1
AAP5	Amino acid permease 5
AMT	Ammonium transporter family
BAA	Bound amino acid(s)
cHATS	Constitutive high affinity transporter systems
C_{min}	Minimum concentration for uptake
DON	Dissolved organic nitrogen
FAA	Free amino acid(s)
HATS	High affinity transporter system(s)
IN	Inorganic nitrogen
iHATS	Inducible high affinity transporter system(s)
K_m	Uptake affinity
LATS	Low affinity transporter system(s)
LHT1	Lysine histidine transporter 1
N	Nitrogen
NRT	Nitrate transporter family
ON	Organic nitrogen
V_{max}	Maximum uptake rate

1 Introduction

1.1 Why nitrogen and why amino acids?

This thesis focuses on the role of nitrogen generally, and amino acids specifically, in plant nutrition. This is important from both plant productivity and global warming perspectives, for the following reasons. Nitrogen is the fourth most abundant compound in plants. It is a component of proteins, nucleic acids, chlorophyll, diverse secondary compounds and many cellular structures. It also plays essential roles in all plant growth and development processes, including transport, cell division and catalysis of biochemical reactions. Plant availability of N is therefore tightly coupled to plant productivity in both natural and agricultural ecosystems. Indeed, high applications of N fertilizer together with the development of high-yielding crop varieties were major drivers of the enormous increase in crop production during the "green revolution" in the 1950's and 1960's. Agricultural production today is heavily dependent on inputs of inorganic N (IN) fertilizer to maintain global primary production and food production, which also dramatically affect the N cycle and associated processes (Fig. 1) (Vitousek *et al.*, 1997; Galloway *et al.*, 2008). With increasing carbon (C) dioxide concentrations in the atmosphere knowledge about the connections between the C and N cycles is becoming increasingly important. Theoretically, global warming could be reduced by globally enhancing the primary production of photosynthesis, but that would require enormous N inputs (Gruber & Galloway, 2008). Therefore, there are profound reasons for improving our knowledge of plant nutrition, and if possible tailoring inputs to optimize desired outputs. An important aspect to consider in this context is the relative importance of different N sources for crop plants and other photosynthetic organisms. Roots have the ability to take up N in both organic and inorganic forms. In particular, the

ability to take up organic N (ON) in the form of amino acids is known to be widespread among plants (Näsholm *et al.*, 2009). However, although this ability was first investigated at the beginning of the last century (e.g. Hutchinson & Miller, 1911), the quantitative importance of amino acids in the plant N budget is still uncertain.

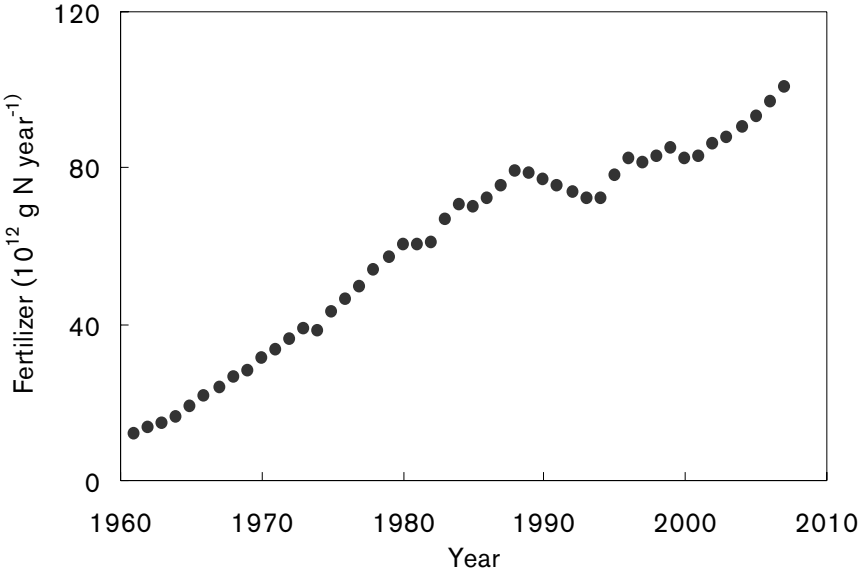

Figure 1. World consumption of N-containing fertilizers between 1961-2007. Nitrogen-containing fertilizers include ammonium nitrate, ammonium phosphate, ammonium sulphate, urea, calcium ammonium nitrate, ammonia direct application, and combinations including NP, NK and NPK. (Plotted data from International Fertiliser Industry Association, www.fertilizer.org).

To investigate the importance of root uptake of amino acids in the plant N budget the influence of many factors needs to be evaluated. Firstly, amino acids have to be available in the root environment for uptake to be potentially possible. Concentrations of free amino acids (FAA) in soil solution are therefore important factors to determine. Ideally, the FAA production rates and diffusion rates should also be estimated, since the concentration of FAA available for plant uptake in the rhizosphere is dependent upon these processes (Leadley *et al.*, 1997). In addition, for amino acids to be significant contributors to plant N nutrition, roots have to be capable of taking them up from solutions with field-relevant concentrations. Thus, root uptake capacity has to be quantified in terms of both uptake affinity and maximum potential uptake rates, to define circumstances in

which plant amino acid uptake may be important. Root uptake capacity is affected by diverse features of plants, notably the amounts, types and activities of amino acid transporters in cell membranes in contact with soil solutions. Thus, knowledge of the expression and regulation of these transporters is also needed.

1.2 Nitrogen in soil

Nitrogen is present in soils in a huge number of chemical forms, including complex forms such as proteins, and simple forms like inorganic ions such as ammonium and nitrate (Fig. 2). Soil N also occurs in both solid forms, absorbed to surfaces of soil mineral particles, and as solutes in the soil solution, but plant-available nutrients are usually present in the soil solution. Both ON and IN are present as solutes in the soil solution, and varying fractions of these N compounds' pools are considered to be available for uptake by plants. Briefly, the thermodynamically "downhill" phase of the N cycle, in which N compounds are degraded, starts with the breakdown of organic matter and proceeds, via protein and peptide depolymerisation, through the liberation of amino acids and (if these substances are not taken up by roots or microbes) further degradation to ammonium, which is often subsequently nitrified to nitrate (Fig. 2). All of these compounds and/or intermediates are present in the soil.

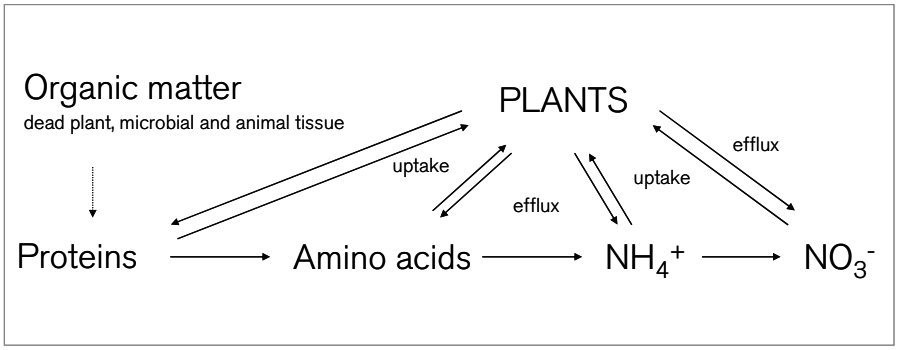

Figure 2. Simplified view of chemical forms of N found in soil, relationships between them and their plant availability. IN is represented by ammonium and nitrate. Arrows pointing in opposite directions indicate uptake and efflux.

1.2.1 Amino acids in soil

Studies of plant uptake of ON have mostly focused on the uptake of amino acids, which have been shown to be present in soils around the world (Sowden *et al.*, 1977; Kielland, 1994; Raab *et al.*, 1999; Nordin *et al.*, 2001;

Schmidt & Stewart, 1999). As shown in Figure 3, amino acids in soils and/or soil solution can be divided into three pools: (i) those dissolved in the soil solution, which are referred to as free amino acids (FAA) and are considered to be directly available to plants; (ii) exchangeable amino acids bound to charged surfaces on clay particles and soil organic matter; and (iii) bound amino acids (BAA) – the largest fraction of amino acids, mostly proteinaceous amino acids in proteins and peptides (Schulten & Schnitzen, 1997). Most of the BAA fraction is only indirectly available to plants, and can be regarded as a reservoir from which FAA is replenished.

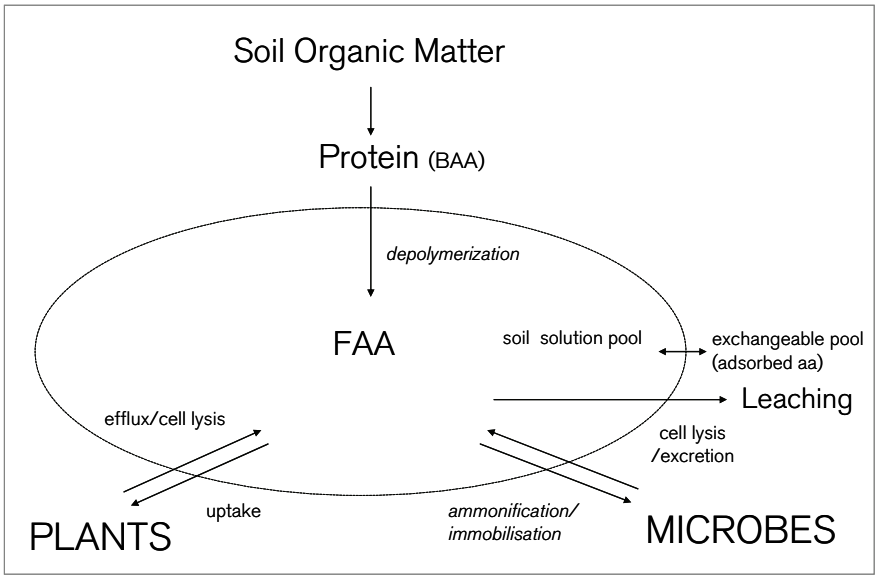

Figure 3. Processes affecting concentrations of free amino acids, FAA, in soil solution

For FAA to serve as N sources for plants, they must be available in concentrations at which uptake is possible. Many studies of FAA concentrations in soil have been carried out in natural ecosystems, fewer have investigated their concentrations in agricultural soils (but see Jones *et al.*, 2005). The relatively few studies of FAA concentrations in soil solution have found concentrations ranging from 0 to 158 μM (Raab *et al.*, 1999; Jones *et al.*, 2005). Ascertaining concentrations of FAA is not straightforward since they are affected by diverse factors, both abiotic and biotic. Abiotic factors like pH affect the charge of amino acids, and hence the rate of their movement in the soil solution through diffusion. Amino acids are grouped into acidic, basic and neutral amino acids according to the charge of the side

chain at pH 7. Basic amino acids, like arginine, are usually positively charged at the pH of most soil solutions (around neutral). The positive charge promotes adsorption to negatively charged surfaces of soil particles (clay) and soil organic matter (cf. Lipson & Näsholm, 2001), which in turn retards their diffusion in the soil solution and movement to the root surface. The size of an amino acid molecule also affects its diffusion rate, and the presence of Ca-carbonates or alkaline salts has been found to severely inhibit the movement of FAA (cf. Lipson & Näsholm, 2001). Soil amino acids can also bind to quinones and reducing sugars (Stevenson, 1994). All these factors affect the FAA concentration in the rhizosphere, and hence may reduce the effective concentration of FAA in the soil solution. Accordingly, the supply rate rather than root uptake kinetics has been found to be the most limiting parameter for the uptake of ammonium and glycine by arctic sedge (*Eriophorum vaginatum* L.) by Leadley *et al.* (1997). The supply rate (of any substance) to the root surface is a function of the diffusion rate and production rate. In the case of FAA, the production rate depends mainly on depolymerisation.

FAA are produced through depolymerisation of proteins by extracellular enzymes. Plant litter is the main form of ON inputs in most soils (Figs. 1, 2) (Stevenson, 1982) and root turnover may contribute the greatest inputs of FAA and BAA (proteins and peptides) in soil (Jones *et al.*, 2005a). Other sources include dead bacteria, fungal and animal tissues, excretions from microbes and animals, and effluxes or leakage from roots. Organic forms of N predominate in soil N, and approximately 40 % of total soil N is generally present in the form of proteins and peptides (Schulten & Schnitzer, 1997). Protein might therefore be the largest and most reliable source of FAA (Schulten & Schnitzer, 1997). Therefore, depolymerisation of protein N to amino acid N has been suggested to be the rate-limiting step in the overall N cycle of soils (Schimel & Bennett, 2004; Rennenberg, 2009). This hypothesis is supported by findings of increases in FAA concentrations along a successional gradient in forest soils (Werdin-Pfister *et al.*, 2009) that appeared to be related to increases in the rate of depolymerisation rather than reductions in FAA degradation (Kielland *et al.*, 2007).

The degradation of FAA in soils devoid of plants has been found to be very rapid, with measured half-lives usually less than three hours and ranging from less than one to 20 hours (Jones, 1999; Lipson *et al.*, 2001; Owen & Jones, 2001; Jones & Kielland, 2002; Jones *et al.*, 2005). Furthermore, in quantitative assessments of plant FAA consumption, knowledge of their fluxes in addition to their concentrations may be required. As soon as FAA are released into the soil solution several processes cause their disappearance,

and thus reduce the concentration of FAA in the soil solution, including: (*i*) mineralization (ammonification, nitrification) to IN, (*ii*) binding in microbial biomass (immobilization), (*iii*) uptake by plants, (*iv*) losses by leaching, and (*v*) adsorption to charged surfaces (Fig 2) (Stevenson, 1982; Yu *et al.*, 2002).

1.3 Plant uptake of amino acids

Traditionally, plant physiologists have regarded the main route of ON uptake by plants as involving ON capture by mycorrhizae, followed by transfer of some of the acquired N to associated plants (Read, 1991). Accordingly, various mycorrhizal plants have been found to be able to access peptides (Bajwa and Read, 1985; Abuzinadah & Read, 1989), proteins (Abuzinadah & Read, 1986; Finlay *et al.*, 1992), and even chitin (Kerley & Read, 1995) as N sources. However, some of these compounds have also been recently shown to be taken up by non-mycorrhizal plants (Fig. 1) (Chalot & Brun, 1998; Komarova *et al.*, 2008; Paungfoo-Lohienne *et al.*, 2008).

As mentioned above, in most studies on ON uptake in plants amino acids have been used as test substances. The uptake of amino acids has been studied under various circumstances with various plant species. Furthermore, plant uptake of amino acids has been detected in both laboratory studies and the field, in ecosystems as diverse as arctic tundra (Kielland, 1994; Schimel & Chapin, 1996), boreal forests (Näsholm *et al.*, 1998), alpine ecosystems (Raab *et al.*, 1996, 1999) and both sub-Antarctic and tropical ecosystems (Schmidt & Stewart, 1999). Among agricultural plants both mycorrhizal grassland species (Näsholm *et al.*, 2000; Weigelt *et al.*, 2005) and non-mycorrhizal winter wheat (Näsholm *et al.*, 2001) have been shown to take up double-labelled glycine in the field, and several plant species, including N_2-fixing legumes, have been shown to take up organic N in laboratory studies (Virtanen & Linkola, 1946; Soldal & Nissen, 1978; Schobert & Komor, 1987; Jones & Darrah, 1994; Reeve *et al.*, 2008; Ge *et al.*, 2009).

1.3.1 Characteristics of amino acid uptake by intact roots

Plant uptake of amino acids has been found to be concentration dependent. For amino acids to play a role in plant nutrition, uptake has to be possible at field concentrations. The minimum concentration (C_{min}), uptake affinity (K_m) and maximum uptake rate (V_{max}) are important parameters for evaluating whether root uptake may occur at field concentrations, and if so its potential importance. C_{min} is defined as the lowest concentration at which net uptake of a compound can occur, and the concept has been shown to be applicable

to phosphate uptake in plants (Lambers *et al.*, 1998). Hence, determining C_{min} is essential for ascertaining if amino acid uptake is possible under prevailing concentrations (if there is a C_{min}, and it is higher than field concentrations, then plant uptake is likely to be non-existent). In addition, the uptake of amino acids has been found to be transporter-mediated (Bush, 1993), hence knowledge of uptake kinetic parameters (K_m and V_{max}) may be valuable for assessing the functional status of the system, e.g. its level of expression/induction and potential fluxes through it.

Despite the findings that FAA generally occur in low μM concentrations, considerably higher concentrations have been used in most uptake studies, both when characterising root uptake kinetics and when investigating plant uptake of amino acids under field conditions. In most laboratory studies amino acid concentrations in the range 100-8000 μM have been used (Wright, 1962; Raab *et al.*, 1999; Schmidt & Stewart, 1999; Owen & Jones, 2001). However, in a few studies uptake has been detected from solutions with ecologically relevant amino acid concentrations (0.1-10 μM), indicating that uptake might not be limited by a C_{min} (Soldal & Nissen, 1978). Some of these studies (Soldal & Nissen, 1978; Jones & Darrah, 1994; Kielland, 1994) were performed on excised roots, which have been found to display different uptake kinetics compared to intact plants (Falkengren-Grerup *et al.*, 2000).

Amino acid uptake rates have been investigated over a wide range of concentrations; from 0.1 μM to 10 mM. Therefore, published rates of plant amino acid uptake vary widely both within and between species, from 0.32 to 100 μmol amino acid (g root DM)$^{-1}$ h^{-1} (Schobert & Komor, 1987; Kielland, 1994; Raab *et al.*, 1999; Falkengren-Grerup *et al.*, 2000; Persson & Näsholm, 2001a; Persson & Näsholm, 2001b; Persson & Näsholm, 2002).

Amino acid uptake rates by plants have been shown to increase with increases in the external amino acid concentration, and the concentration dependency of amino acid uptake in plants is often described by Michaelis-Menten kinetic equations, which are commonly used to model enzyme kinetics, based on correlations between enzyme activity and substrate concentration. There are similarities between enzyme kinetics and membrane transporter kinetics, which have made Michaelis-Menten kinetics useful for characterizing root uptake of nutrients. In the latter context, they are used to define the affinity of the uptake system, expressed as K_m, which is the concentration at which the uptake rate is half the maximum value (V_{max}). K_m is therefore a useful quantitative measure for comparing different plant species' capacities to take up an amino acid, or root uptake and microbial uptake of the same substance at a given concentration. K_m is also used to

assign transporters to an uptake affinity range. Further, K_m can be used as a qualitative measure to compare uptake affinities for different compounds, e. g. amino acids and IN. V_{max} is also a valuable measure since not only the affinity but also the potential uptake rate is informative (*inter alia*) for assessing competition and preferences. The approximation of Michaelis-Menten kinetics to transporter activities is based on the assumption that there will be a concentration at which uptake is saturated. This is derived from the definition of V_{max}, as the maximum velocity at which the modelled reaction possibly can occur, when the binding sites in all transporter proteins are occupied.

A wide range of K_m values have been determined for plant root uptake of amino acids, from 1.6 μM (barley) to 12 900 μM (*Arabidopsis thaliana*) (Soldal & Nissen, 1978; Schobert & Komor, 1987; Jones & Darrah, 1994; Kielland, 1994; Frommer *et al.*, 1995; Chalot & Brun, 1998; Breitkreuz *et al.*, 1999; Wallenda & Read, 1999). In comparison, reported K_m values for mycorrhizal fungi and heterotrophic microbes range from 1.6 to 233 μM, and from 0.5 to 180 μM, respectively (Lipson & Näsholm, 2001). The correlation between amino acid uptake rate and external concentration may display a multiphasic relationship – e.g. in barley (Soldal & Nissen, 1978) – which can be plotted as a series of saturation kinetics curves for consecutive (or overlapping) concentration intervals, each with specific K_m and V_{max} values. This indicates that some of the large range in K_m values may be due to differences in kinetic parameters of different components of the uptake system, i.e. the presence of two or more distinct transport systems with varying uptake affinities (see section *1.4.1 IN transporters in roots* for further details).

Despite the wide variation in affinity constants there is evidence that roots have high-affinity uptake systems for amino acids, capable of activity within ranges of FAA concentrations found in soil and with K_m values comparable to those of microbial uptake systems (Lipson & Näsholm, 2001). This suggests that plants are potential competitors with soil microbes for FAA. However, in order to investigate the importance of amino acids in the N-nutrition of agricultural plants, K_m values need to be determined within the range of ecologically relevant concentrations.

1.4 Inorganic N and ON transporters in roots

Traditionally, the IN forms nitrate and ammonium are considered to be the main N sources for plants. Hence, the importance of amino acids as N

sources for plants is often assessed by comparing their uptake with IN uptake.

1.4.1 Inorganic N transporters in roots

Nitrate transporters in roots can be divided into three classes. At low external concentrations (<0.50 mM) nitrate is taken up by high affinity transporter systems (HATS). The HATS can be further divided into constitutively expressed systems (cHATS), which are present prior to exposure of nitrate in situations where nitrate becomes available after a period in which none was accessible and nitrate induced systems (iHATS) which are only expressed in the presence of low concentrations of nitrate (for review see Williams & Miller, 2001; Glass *et al.*, 2002; Glass, 2009). At higher concentrations (0.50 mM to 50 mM) low-affinity transporter systems (LATS) are largely responsible for nitrate uptake. Depending on species LATS can be both constitutive and inducible, leading to four nitrate transporter systems in Arabidopsis (Tsay *et al.*, 2007; Glass, 2009).The transporter systems responsible for ammonium uptake, like nitrate uptake systems, are divided into HATS and LATS (Williams & Miller, 2001), HATS being chiefly responsible for ammonium uptake at concentrations up to 200 μM (Williams & Miller, 2001). Several transporters belonging to the nitrate transporter family (NRT) and others belonging to the ammonium transporter family (AMT) have been identified in Arabidopsis (Tsay *et al.*, 2007; Williams & Miller, 2001; Glass *et al.*, 2002).

1.4.2 Amino acid transporters in roots

The confirmation that amino acid uptake in plants is an active process has been important in physiological studies of amino acid uptake in plants. When I started my PhD studies amino acid transporters had been identified, but none had been localized to roots and shown to be specifically involved in root uptake. Physiological studies had led to the hypothesis that plants have two separate transport systems, one for neutral/acidic amino acids and one for basic amino acids (Kinraide, 1981; Datko & Mudd, 1985; Borstlap *et al.*, 1986; Schobert & Komor, 1987). Today, plant uptake of amino acids is thought to be energized by the proton gradient across the plasma membrane and facilitated by transport proteins (cf. Liu & Bush, 2006; Rentsch *et al.*, 2007). These transporters may function in the acquisition of amino acids from the soil solution as well as in the recapture of amino acids leaking from roots (Jones *et al.*, 2005). Three amino acid transporters have been identified as components of the amino acid uptake system in the model plant Arabidopsis: lysine histidine transporter 1, LHT1 (Hirner *et al.*, 2006;

Svennerstam *et al.*, 2007), amino acid permease 1, AAP1 (Lee *et al.*, 2007) and amino acid permease 5, AAP5 (Svennerstam *et al.*, 2008). LHT1 displays high affinity for neutral amino acids, histidine (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007) and acidic amino acids (Hirner *et al.*, 2006), AAP1 (Lee *et al.*, 2007) has been shown to mediate uptake of several neutral amino acids, glutamic acid and histidine, AAP5 displays activity for arginine and lysine, neither of which are taken up by LHT1 or AAP1 (Svennerstam *et al.*, 2008).

The mechanisms regulating of the activity of each of these transporters are still relatively unknown. Their relative importance for uptake of different amino acids at field-relevant concentrations is also currently unclear. In this context it should be noted that it is not just the ability of plants to take up FAA that needs to be elucidated, but also their importance as plant N sources. For instance, a factor that might regulate the activity of these transporters is the presence of IN, but interactions between uptake of amino acids and IN has not been thoroughly studied. If IN inhibits or abolishes the uptake of amino acids, the ecological relevance of amino acid transporters in IN-rich environments could be questioned. Alternatively, amino acid uptake might be less inhibited by the presence of IN than vice versa (Thornton & Robinson, 2005). Indeed, the presence of amino acids (e. g. glutamine) has been found to inhibit uptake of nitrate and ammonium (Rawat *et al.*, 1999; Vidmar et at., 2000; Aslam *et al.*, 2001; Thornton, 2004). Amino acid transporter systems might also share similarities with IN transporter systems in addition to being concentration-dependent.

The LHT1 and AAP5 amino acid transporters might be HATS for amino acid uptake (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007, 2008). If so, it would be interesting to determine whether they were constitutive or inducible transporter systems, and the ecological implications of their status. However, despite the similarities between IN and amino acid transporter systems the inducibility of the latter has rarely been explored (Hirner *et al.*, 2006; Liu & Bush, 2006).

2 Objectives

The overall aims of the studies this thesis is based upon were to elucidate key aspects of amino acid uptake by agricultural plants and assess its importance. To do this, a series of both field and laboratory studies were designed and performed in order to combine the relevance of the former and precision of the latter. The studies and results are described in detail in the four papers (Papers I-IV) appended to the thesis. Briefly, however, the amino acids that occur in agricultural soils were identified and quantified (Papers I and II). Based on this information, uptake experiments were carried out to assess whether plants can take up the identified amino acids at field-relevant concentrations (Papers II and III). Further, to acquire more information about the mechanisms involved, attempts were made to characterise the transporters involved in the uptake of amino acids at ecologically relevant concentrations (Paper III). In addition, given the simultaneous presence of IN and amino acids in soil, and the fact that IN is considered to be the main N source for plants, a further field-relevant issue investigated whether IN influences uptake of amino acids and/or vice versa (Paper IV). Finally, since uptake of amino acids shares similarities with IN uptake, the inducibility of amino acid uptake systems was investigated (Paper IV).

The studies described in the four papers are schematically presented in Figure 4.

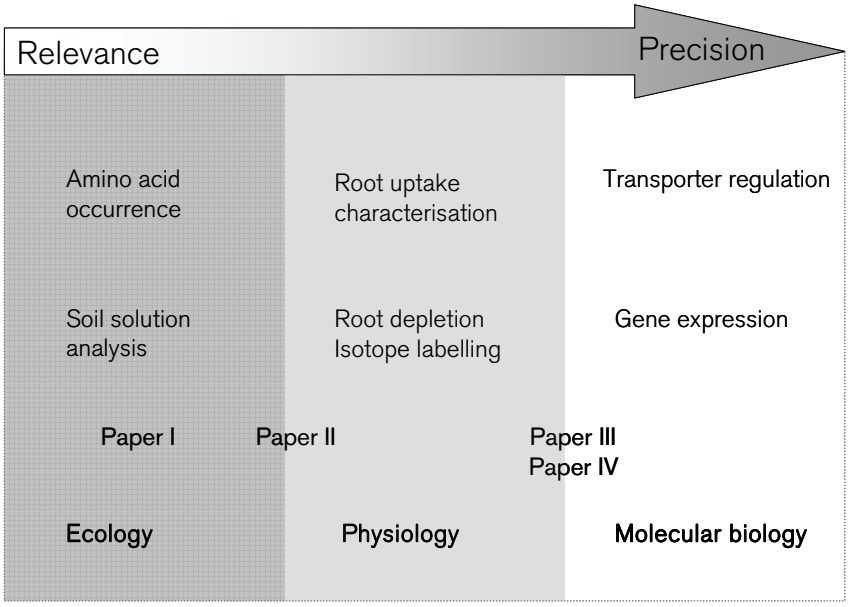

Figure 4. Schematic view of the relevance and precision extended by the included studies of this thesis.

3 Materials and methods

3.1 Soil solution sampling

In order for a substance to be taken up by an organism, it needs to be in the vicinity of the organism. Thus, for amino acids to be taken up by roots, they must be present in the soil solution. However, most investigations on the occurrence of amino acids in soil have examined their presence in soil extracts (Schulten & Schnitzen, 1997, Kielland *et al.*, 2007). This complicates comparisons of relevant published data, since amino acid concentrations in soil extracts are usually expressed in relation to soil dry mass (g N g^{-1}), rather than as (molar) concentrations in soil solution *per se*.

Due to the rapid turnover of FAA in soil (Jones, 1999; Lipson *et al.*, 2001; Owen & Jones, 2001; Jones & Kielland, 2002; Jones *et al.*, 2005; Jones *et al.*, 2009) it is important to ensure that the technique used for sampling them causes minimal disturbance, and hence minimal changes to the concentrations of the measured compounds during sampling and processing. Soil extraction methods are convenient for acquiring samples for analyzing FAA contents in soil without tedious preparation in the field or laborious adjustments to meet exacting instrument requirements. However, there are inevitable time lags between sampling and soil extractions, which given the rapidity of FAA turnover in soils could cause chemical alterations prior to analysis. They also inevitably involve disruption of the *in situ* soil structure since the soil is sampled, roots are removed from samples and soils are sieved or only small amounts of soil are used. Therefore, the measured amino acid concentrations do not necessarily show the amounts that are directly available to plant roots. Further, there are risks of substantially over- or under-estimating amino acid concentrations in soil solutions when using water extractions or centrifugation, because of the difficulties involved in

sampling the solution without destroying soil aggregates, fine roots and fungal hyphae. Excluding roots always entails a risk of cell lysis or exudative burst (Jones *et al.*, 2005a). This may be particularly important when measuring BAA in soil HCl extracts rather than soil solution. The risk of lysing living microbial biomass is reduced when the aqueous phase of the soil rather than the soil *per se* is hydrolysed (Roberts & Jones, 2008).

In order to obtain representative samples to analyse soil solution concentrations of amino acids, ideally both disturbance of the soil-plant system and the risks of over- or under-estimating soluble N pools due to the production or decomposition of N compounds during sampling and handling of soil samples should be minimised. Recent studies suggest that sampling of soil solutions using small tension lysimeters (Fig. 5) may fulfil these requirements (Andersson, 2003; Andersson & Berggren, 2005; Robert & Jones, 2008). The use of small tension lysimeters (P2.30-1 Rhizon Soil Moisture Samplers, Eijkelkamp, Giesbeek, The Netherlands) reduces the risk of degradation of N compounds before analysis (Andersson, 2003) since the likelihood of microbial decomposition of organic compounds is minimized when the solution is sampled by percolation through the small pores (0.1 μm diameter) of the lysimeter (Andersson, 2003). When using this method a lysimeter (2.5 mm diameter and 10 cm long) is carefully inserted into the soil to be sampled and soil solution is collected by suction (Fig. 5). Some disturbance of the soil can be expected when the lysimeter is inserted into the soil and when solution is removed, but it will be very minor in comparison with extractions. The solution can be sampled in the presence of plant roots, and with minimal risk of altering chemical composition, enabling robust assessment of *in situ* levels of organic and inorganic N compounds.

Figure 5. Lysimeter installed in the field for sampling soil solution (Papers I, II). Soil has been excavated to show the position of the lysimeter. Photo: Ines Barth and Kerstin Huss-Danell.

However, lysimeter sampling is limited by its dependence on quite high soil moisture contents, and it gives bulk concentrations, probably biased towards the concentrations in the largest water-filled soil pores, since pores tend to empty in order of size, starting with the largest soil pores, in accordance with associated differences in water potential. Measured concentrations may not therefore reflect true amplitudes of concentration, i.e. the maximum concentrations will be diluted. In addition, despite sampling at (relatively) micro-scale the proximity of the analytes to roots is unknown. Further, the variations of concentrations that are probably present in the soil cannot be elucidated. Turnover of soil organic matter most likely leads to some microsites having considerably higher FAA concentrations than those indicated by results from available methods. The high FAA concentrations found in, for example, plant root cells (1-10 mM; Jones & Darrah, 1994) support this hypothesis. Further studies of the usefulness of lysimeters and other methods for assessing the heterogeneity of amino acid concentrations available to plant roots in the micro-scale of the rhizosphere would therefore be of great value.

However, an important advantage of small tension lysimeters is the possibility they provide to investigate temporal and spatial variations in the occurrence and concentrations of compounds. This might give a wider understanding of the environment that roots encounter. Because of the advantages of sampling soil solution from intact soil and the possibility of repeated measurements, they were used to investigate the concentrations of FAA, nitrate, ammonium (Papers I and II) and BAA (Paper I) available to plants in soil solution of various kinds of agricultural land.

3.2 Amino acid uptake

3.2.1 Solution depletion

To study amino acid uptake in barley and Arabidopsis at ecologically relevant concentrations, and lower, plants were grown in hydroponic culture and depletion of amino acids in a known volume of medium was monitored over time, as illustrated in Figures 6 and 7 (Papers II, IV). Solution depletion is convenient for such studies, since the concentration and composition of the amino acid solution can be easily controlled, the composition of the medium can be kept homogeneous around the roots by gently bubbling air through it, and it is not altered by amino acids (or other substances) physically or chemically binding to soil particles. Hence, it is a good method for investigating the factors involved in regulating amino acid

uptake, e.g. concentration dependencies (Paper II) and the compounds plants prefer to take up, when more than one is available (Paper IV). Unlike the use of isotopically-labelled amino acids this method gives indications of the net uptake of amino acids, i.e. uptake minus efflux. In the perspective of a whole plant N budget this is advantageous, but it might result in underestimations of uptake rates.

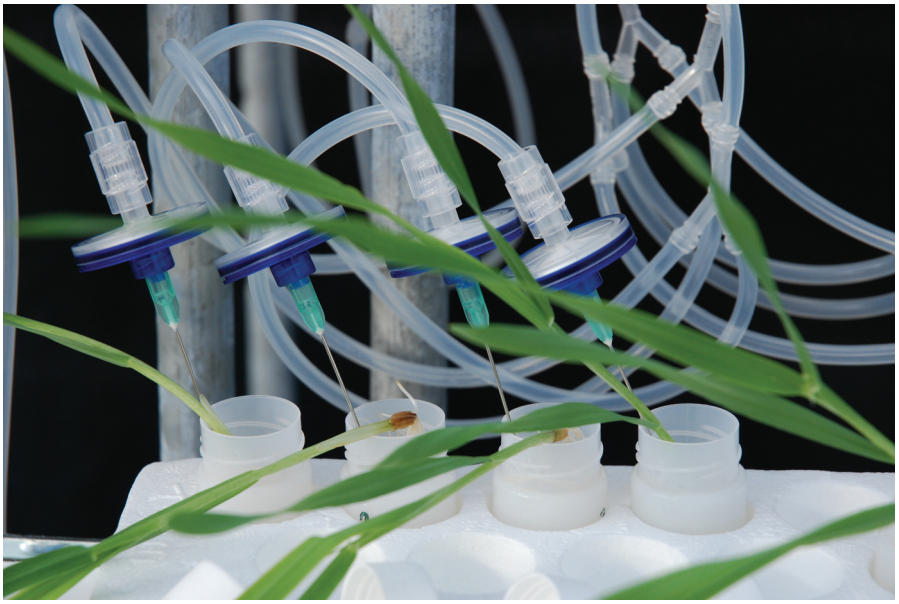

Figure 6. Barley plants in a solution uptake study (Papers II, IV). Tubes for aeration of solution and sterile filters attached to needles are seen in the background.

The relevance and applicability of the results from solution depletion studies to natural conditions can, of course, be questioned because of the exclusion of physical and chemical binding of amino acid to soil particles, heterogeneity of the nutrient concentration and microbial competition (Jones et al., 2005a). Uptake rates measured in solution should not therefore be extrapolated to soil because of these factors, and plant-related factors, e.g. changes in root architecture, lack of mycorrhiza and poor root hair development (Jones et al., 2005a). Other disadvantages of solution depletion studies are the often unrealistic temperatures, unrealistic nutrient enrichments and homogeneous concentrations used (Hodge et al., 2000). Complementary field studies should ideally be conducted, of course, to verify the ecological relevance of any findings, but are difficult to perform. However, solution depletion studies in which the disappearance of amino acids from a solution of known concentration is monitored in short-term

laboratory experiments are valuable for quantifying uptake, and for assessing differences between uptake systems in relation to concentration, e.g. between amino acid and IN transporters (Paper IV).

Figure 7. Arabidopsis plants in a solution uptake study (Paper IV). Sterile filters on needles for aeration of solution are seen from above.

3.2.2 Isotopically-labelled amino acids

When measuring amino acid uptake in plants it is essential to check that the amino acid is taken up in intact form and not as breakdown products of microbial activities. For this purpose, dual (^{13}C and ^{15}N)-labelled amino acids can be used. The relationship between excess ^{13}C and excess ^{15}N in plants at the end of uptake experiments can then be used to calculate the amount of intact amino acid they took up (Näsholm *et al.*, 1998; Näsholm *et al.*, 2000). Dual-labelled amino acids are useful in field studies and have been used to study the uptake of intact amino acids in agricultural plants in field (Näsholm *et al.* 2000; Näsholm *et al.*, 2001). This method was used to evaluate measured uptake by solution depletion (Paper II).

A methodological limitation to keep in mind when using ^{13}C,^{15}N-labelled amino acids is the high natural abundance of ^{13}C (Näsholm & Persson, 2001; Nordin *et al.*, 2001; Miller & Bowman, 2003; Persson *et al.*, 2003). This can make detection of excess ^{13}C in plant material impossible when using low concentrations of ^{13}C because of the dilution from naturally occurring ^{13}C.

Another restriction is that dual-labelled amino acids can only be used to indicate uptake of intact amino acids in short-term studies since C may be lost through respiration from the plant, resulting in alterations of the $^{13}C/^{15}N$ ratio. Because of the risk of such changes in ratio and the importance of verifying uptake of amino acids, long-term studies of root uptake of intact amino acids are currently only possible under axenic conditions in the laboratory.

^{14}C-labelled amino acids were used in the study described in Paper III to measure amino acid uptake during 1 h. As for the use of dual-labelled amino acids, this is a useful method for measuring gross uptake during a short time, provided that the amino acid remains intact until taken up and risks of respiration losses prior to analysis are avoided. Since the natural abundance of ^{14}C is very much lower than that of ^{13}C it is a practical method for measuring uptake from low concentration solutions (Paper III).

3.3 Amino acid uptake mechanisms

In the studies reported in Papers III and IV, Arabidopsis (a member of the Brassicaceae family) was used because abundant genetic information, huge arrays of mutants and advanced molecular tools are available for analyzing this species (more so than for any other plant species, e.g. barley). Therefore, a selection of the available tools, and mutants, were used to explore the molecular background of amino acid uptake and its putative role in plant N nutrition.

In attempts to identify transporters involved in the high affinity uptake of amino acids (Paper III), mutants lacking expression of the genes *LHT1*, *AAP1* or *AAP5* and plants overexpressing *LHT1* – according to previous findings (Hirner *et al.*, 2006; Lee *et al.*, 2007; Svennerstam *et al.*, 2007, 2008) – were used. Regulation of the transporters these genes encode was studied, with and without exposure to amino acids, in the presence of IN (Paper IV). The amino acids used in these experiments were glycine and arginine, since they had been shown to be taken up by separate transporter systems at field-relevant concentrations (Paper III). To assess uptake preferences the plants were exposed to these amino acids, nitrate and ammonium at two, equimolar concentrations that were thought to represent high and low affinity concentrations (50 and 500 μM, respectively).

4 Results and discussion

4.1 Occurrence of amino acids in soil solution

In assessments of the relevance of plant amino acid uptake under ecological conditions it is essential to have information on FAA concentrations in soil. Therefore, FAA concentrations were measured in soil solutions from five kinds of agricultural (or formerly agricultural) soils: under organically grown (fertilized) iceberg lettuce, organically grown (unfertilized) ley, old grassland and thinned birch forest on old pasture (Paper I) and fertilized barley (Paper II). In all cases they were found to be low (0.1-12.7 μM). FAA concentrations in agricultural soils are generally thought to be lower than in boreal or alpine ecosystems, and they have been found to increase along a boreal forest succesional gradient (Werdin-Pfister et al., 2009). However, the use of different methods (see Material and Methods) by different authors complicates comparisons, as does the predominance of studies in natural ecosystems of northern latitudes. Nevertheless, the measured concentrations of FAA in soil solutions (Papers I, II) were clearly in the lower end of previous findings, which range from 0-158 μM (Raab *et al.* 1999; Jones *et al.*, 2005).

In contrast to the low FAA concentrations, the concentrations of BAA were high, up to 50 times higher (10-75 μM, sum of hydrolysed BAA) indicating that BAA is a potential replenishment pool of FAA (Paper I). BAA was the dominant N pool measured (Paper I), and present at higher concentrations than nitrate and ammonium, except in fertilized soil. The nitrate concentration was approximately 2 mM in soil solution under both lettuce (Paper I) and barley (Paper II), just after fertilization. The concentrations then decreased very rapidly (due to plant uptake, immobilization, leaching etc.) to levels even lower than those of FAA in

soils under barley in October. The ammonium level was quite constant throughout the seasons in both of these arable soils. Concentrations declined in the order BAA>>ammonium>nitrate and FAA in the unfertilized soils (old grassland, birch forest and organically grown ley), but although concentrations of FAA were the lowest there was no significant difference between them and either the ammonium or nitrate concentrations (Paper I). These results, which are consistent with findings reported by Young & Aldag (1982), show that the reserves of FAA were higher than those of ammonium and nitrate. The size of the replenishment pool might therefore be of importance since the FAA production rate, together with the diffusion rate, is thought to be a major determinant of FAA availability in the rhizosphere (Leadley *et al.*, 1997).

The size of each N-pool was surprisingly similar in all of the soils analyzed in the study reported in Paper I, despite major differences in vegetation types and previous histories. Hence (*inter alia*), in contrast to findings by Werdin-Pfister at al. (2009), growth of birch forest on the old pasture does not seem to have significantly influenced the concentration of FAA in the soil, raising questions about the reasons for the differences in results. One likely factor is that the successional gradient between vegetation types was probably larger in the study by Werdin-Pfisterer *et al.* (2009). Differences in FAA concentrations related to the boreal forest succession stage have been attributed to rates of protein depolymerisation increasing more rapidly than rates of amino acid breakdown as succession proceeds (Kielland *et al.*, 2007). Depolymerisation of protein N is considered to limit not only plant availability of FAA, but also the overall rate of the N cycle of soils (Schimel & Bennett, 2004, Rennenberg, 2009). From our measurements (Paper I) it is not possible to draw any conclusions regarding flux rates in soil. Hypothetically, depolymerisation could limit FAA availability (Jan *et al.*, 2009) in the studied soils, but there are many other influential factors, for example plant uptake.

In three of the soil types considered in Paper I, BAA dominated over IN. The main FAAs in all soils examined, including the soil under barley (Paper II), were serine, glycine and alanine. However, the relative proportions of FAA differed between the two geographical locations of the sites (Timrå and Ängersjö) in Paper I). Assuming that BAA acts as a replenishment pool for FAA, differences in proportions between the two locations indicate that processes involved in BAA depolymerisation and/or consumption of FAA could alter the FAA profile. However, relative proportions of BAA in the four soil types described in Paper I were very similar. This could be due to the BAA at the sites having similar origins, the amino acid composition of

proteins from different organisms being very similar, or the decomposition of organic matter or uptake by organisms (fungi, bacteria, plants) being in some way selective. BAA and FAA have been hypothesised to be of mainly microbial origin (Sowden *et al.*, 1977; Stevenson *et al.*, 1982; Schulten & Schnitzen, 1997), thus the composition of BAA could reflect the proportions of amino acids in recalcitrant microbial cell structures. In contrast, cytoplasmic proteins have been hypothesized to be readily accessible for degradation, leading to decreased abundance of the amino acids they contain during breakdown. Subsequently, N from resistant structures such as cell walls from bacteria (peptidoglycan), fungi and plants might accumulate (Rovira *et al.*, 2008). According to proteomic studies the amount of microbial (bacterial and fungal) protein might increase with increasing decomposition of plant debris (Schulze, 2005). Another way to identify the origin of some amino acids is to analyse the isomers of alanine, aspartic acid and glutamic acid, since the D-isomers of these amino acids are found predominantly in cell walls of bacteria (Davies, 1977).

Analysis of soil solution concentrations of FAA provides a snapshot of the concentrations, but no indications of their production and consumption rates (fluxes). To evaluate uptake ability by plants, it is essential to supply them in ecologically relevant concentrations, but to estimate the quantitative importance of FAA, knowledge of their fluxes is also important, since (for instance) a low concentration might be due to high consumption rates and/or low production rates. Some relevant information, on both the ability of plants to take up FAA and fluxes, can be obtained by using isotopically labelled substances. Despite the problems and uncertainties, and the low concentrations of FAA detected in soil solutions in agricultural lands (Papers II, III), the results presented in Papers II and III strongly indicate that plants have the capacity to take them up. In addition, there seems to be a large potential replenishment pool of BAA.

4.2 Characterisation of amino acid uptake at field-relevant concentrations

In most studies of plant uptake of amino acids have been done at concentrations considerably higher concentrations than those measured in the field. Concentrations of FAA in studied soils (Papers I, II) were in the lower μM range. However, the studies described in Papers II and III show that barley and Arabidopsis, respectively, have the capacity to take up amino acids at these concentrations. This indicates that the capacity of these plants to take up amino acids is not restricted by a C_{min}, since depletion of the

uptake solution continued even when concentrations were very low (Paper II). These results are in line with previous studies of excised roots (Soldal & Nissen, 1978; Kielland, 1994).

4.2.1 Amino acid uptake kinetics

From data presented in Papers II and III conclusions can be made that uptake of amino acids is possible at ecologically relevant concentrations and, hence, uptake kinetics can be used to evaluate the importance of amino acid uptake systems in plants under such conditions. Uptake of amino acids showed concentration-dependency, increasing with increased concentrations of single amino acids (Paper III), five amino acids simultaneously present (Paper II) and single amino acids in the presence of IN (Paper IV). To evaluate uptake capacities of barley and Arabidopsis for the tested amino acids at ecologically relevant concentrations, the concentration dependencies reported in Papers II and III were described by Michaelis-Menten kinetics. The results could be compared with published data on both uptake by soil micro-organisms (Paper II) and the kinetic parameters of several amino acid transporters involved in amino acid uptake at these concentrations (Paper III). Uptake capacities of any uptake system depend on both K_m and V_{max}, which govern potential influxes. Uptake affinities of each of the five tested amino acids in barley varied between 19.6 and 33.2 μM (for arginine and alanine, respectively; Paper II). In wild type Arabidopsis plants the affinity for arginine was found to be within the same range as in barley (7.6 μM), which is lower than most, but not all, published values (Lipson & Näsholm, 2001). These results, in agreement with Soldal and Nissen (1978), show that barley and Arabidopsis have uptake affinities for amino acids within the lower μM range and indicate that they do not have C_{min} uptake values that are higher than concentrations measured in the field.

Transporters shown to be involved in amino acid uptake in plants seem to have broad specificity (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007, 2008, Lee *et al.*, 2007), indicating that uptake rates by a specific transporter may depend on the total concentration of amino acids that it is potentially capable of transporting, rather than the concentration of individual amino acids. In soil solution amino acids occur in mixtures, which could explain the similar concentration dependencies shown in Paper II for the tested amino acids. Further, the uptake kinetics reported in Paper II may be more strongly related to the sum of concentrations and uptake rates of the four amino acids (serine, glutamic acid, glycine and alanine) probably transported by LHT1 (Paper III) rather than to any individual amino acid. However, it is not possible to draw definite conclusions regarding this matter since there

have been no *in planta* comparisons of single and multiple amino acid uptakes by a certain transporter within the same species. Uptake rates for single amino acids are presented in Paper III and for simultaneous uptake of five amino acids in Paper II, but in different plant species.

Besides the similarities there are also differences between enzyme and transporter kinetics. Notably, transportation might not be controlled solely by substrate availability on the outside of the plasma membrane, but also by various factors inside the cell. For instance, amino acid transport over the plasma membrane could result in the accumulation of amino acids in the cytosol, which in turn might inhibit further uptake. Therefore, the applicability of Michaelis-Menten kinetics to amino acid uptake has been questioned, since only changes in external concentrations of the amino acids are generally considered (Reinhold & Kaplan, 1984). Furthermore, in contrast to unicellular organisms, amino acids may not necessarily be accumulated in the cytosol of the cells that take them up when studying uptake in intact roots since the amino acids could be compartmentalized, translocated to other cells or metabolized. Despite all these differences, Michaelis-Menten kinetics is useful for transporter characterisation.

Published values of K_m in plants show large variations (Soldal & Nissen, 1978; Schobert & Komor, 1987; Jones & Darrah, 1994; Kielland, 1994; Frommer *et al.*, 1995; Chalot & Brun, 1998; Breitkreuz *et al.*, 1999; Wallenda & Read, 1999). However, the results outlined above indicate that amino acid uptake systems in plants have affinities within concentration ranges of soil solutions in the field, and the kinetic parameters of the uptake appear to reflect those of the corresponding amino acid transporters (as discussed in further detail below).

4.2.2 Transporters involved in amino acid uptake at field-relevant concentrations

To date, three amino acid transporters involved in amino acid uptake in plants have been identified: LHT1 (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007), AAP1 (Lee *et al.*, 2007) and AAP5 (Svennerstam *et al.*, 2008). The results presented in Paper III support the hypothesis that there are two separate transporter systems for amino acids, accounting for most of the uptake at field-relevant concentrations ≤50 μM (Kinraide, 1981; Datko & Mudd, 1985; Borstlap *et al.*, 1986; Schobert & Komor, 1987); one for neutral and acidic amino acids (LHT1) and one for basic amino aids (AAP5).

However, uptake of amino acids has been found to be multiphasic over large concentration ranges (Soldal & Nissen, 1978). This, together with the variations in K_m (see previous section), indicates that transporter systems with

different affinities are probably involved in the uptake of amino acids at different concentrations, analogously to the uptake of IN at low and high concentrations predominantly by HATS and LATS, respectively (Williams & Miller, 2001; Tsay *et al.*, 1997; Glass, 2009). Plants lacking expression of *AAP1* did not show any significant differences in uptake in comparison with wild type plants at concentrations ≤50 μM (Paper III). This finding, together with results of studies by Lee *et al.* (2007), indicate that AAP1 is a potential candidate for uptake at higher concentrations (LATS), above 50-150 μM. If plants have separate systems for uptake of amino acids at high and low concentrations LHT1 and AAP5 are probably HATS (Paper III), while AAP1 is probably a LATS (Lee *et al.*, 2007).

Measured uptake affinities for these potentially high affinity transporters (Paper III) were found to be comparable to those of nitrate iHATS in Arabidopsis (Touraine & Glass, 1997), which is interesting given the similarity of the concentrations of FAA and nitrate found in unfertilized agricultural soils (Paper I). In addition to being IN concentration-dependent, IN uptake has been found to be IN-induced (Glass, 2009), and influenced by the presence of amino acids (Miller & Cramer, 2005), but corresponding interactions for uptake of amino acids had not been as thoroughly studied, therefore, these aspects of amino acid uptake were also examined, as discussed below.

4.2.3 Amino acids induce increases in uptake capacities, even in the presence of IN

What similarities are there between amino acid and IN transporter systems? Uptake of amino acids has been found to be dependent on concentration, as is uptake of IN. In accordance with uptake of IN, results presented in Paper IV clearly show that amino acid uptake is also inducible; uptake of glycine and arginine by both barley and Arabidopsis increased with the duration of exposure to these amino acids in the presence of IN. Uptake rates of glycine, arginine increased up to 15-fold following exposure to them. Exposure to the amino acids for 6-24 h also increased expression of *LHT1* and *AAP5* genes, indicating that this effect is related to increased synthesis of transporters. The results suggest that both of these systems might be inducible HATS (iHATS) rather than constitutive HATS (cHATS), raising questions about some of the conclusions (and implications) of previous studies, in which uptake rates have usually been measured in plants cultivated on ammonium nitrate as the sole N source. The novel findings that induction can dramatically increase amino acid uptake rates suggest that previous studies of amino acid uptake, including those described in Papers II

and III, could have characterised non-induced uptake rates (which may be substantially lower than post-induction rates, analogously to iHATS nitrate uptake patterns). The induction systems may help to conserve valuable resources by tailoring N uptake capacity to current N availability, allowing amino acids to be taken up by weakly expressed constitutive systems when they are present at low concentrations, and at greatly increased rates by induced systems when present at high concentrations.

There are also indications that amino acid induction influences plants' preferences for N compounds (Paper IV; Thornton & Robinson, 2005). The observed induction of amino acid uptake indicates that root uptake capacities are closely related to the recent soil solution composition, since the capacity for amino acid uptake increased under inductive conditions for 24 h, and did not reach a steady state or decline during this time. Further studies of this process are needed to assess the effects of amino acid induction on the preferential uptake of N compounds from the soil, notably interactions between the uptake of amino acids and IN, which might have important ecological implications. Results of such studies, together with those described above, may significantly enhance our understanding of the role and significance of amino acid uptake in plants (Jones *et al.*, 2005).

4.2.4 Uptake of amino acids in the presence of IN

Early studies of amino acid uptake attempted to assess the importance of amino acid uptake by comparing it with IN uptake, but usually in the absence of IN (Chapin *et al.*, 1993; Schimel & Chapin, 1996; Raab *et al.*, 1999). The effects of increasing internal cytosolic concentrations of amino acids on IN uptake have also been investigated (Rawat *et al.*, 1999; Vidmar *et al.*, 2000; Thornton, 2004). However, plants' preferences for taking up amino acids, relative to other N sources, have rarely been addressed (Thornton & Robinson, 2005). This is an important issue, because in soil solutions IN and amino acids are usually present simultaneously, at highly variable but often similar concentrations (Paper I). Uptake of both nitrate and ammonium seems to be inhibited by increased cytosolic concentrations of amino acids, especially glutamine (Rawat *et al.*, 1999; Vidmar *et al.*, 2000). Thus, high internal concentrations of amino acids are known to affect IN uptake. A further aim of the studies this thesis is based upon was to determine whether IN may similarly affect amino acid uptake. More specifically, my colleagues and I explored the effects of exposure to mixtures of amino acids and IN on the amino acid uptake rates of root systems cultivated in amino acid-free media. In Arabidopsis roots that had not previously been exposed to amino acids nitrate was taken up at the highest

rates from solutions containing 50 μM or 500 μM of glycine, arginine, nitrate and ammonium (0 h, Paper IV). These measured uptake rates of nitrate and simultaneous uptake rates of ammonium were similar to published uptake rates for each compound supplied alone (Touraine & Glass, 1997; Rawat *et al.*, 1999). After amino acid exposure (at 50 μM) for 24 h, uptake of glycine and arginine increased, to similar rates to those of nitrate and at 500 μM the uptake rate for each amino acid was 6-10 times higher than that of nitrate (Paper IV). This effect was partly due to a reduction in the nitrate uptake rate, but mainly to a dramatic increase in amino acid uptake. These results imply that measurements of the uptake of single IN sources might be overestimates (Thornton & Robinson, 2005).

5 Conclusions

According to standard textbooks, nitrate and ammonium are considered to be the major N sources for plants. Is that consistent with current knowledge, and should plants also be regarded as consumers of FAA? The ability to take up amino acids is widespread among plant species, but the ecological and physiological implications of this ability are not fully understood. The results presented in this thesis indicate that FAA concentrations in agricultural soils are low, but might be continuously replenished (Papers I, II). Furthermore, uptake systems in barley and Arabidopsis do not seem to be constrained by a C_{min} higher than these concentrations (Papers I, II, III). The uptake affinity in both barley and Arabidopsis was found to be comparable to reported values for nitrate at corresponding concentrations, and for uptake of amino acids by soil micro-organisms. Thus, uptake of amino acids by these species could occur in the field, from soil solutions with observed concentrations. At these concentrations two transporter systems, LHT1 and AAP5, were identified as likely mediators of most amino acid uptake in Arabidopsis (Paper III). These two transporters were found to have complementary, non-overlapping affinities for different amino acids, LHT1 transporting acidic and neutral amino acids and AAP5 basic amino acids, while the importance of other transporters, such as AAP1, appeared to be low. The expression of *LHT1* and *AAP5* was clearly shown to increase following exposure of roots of Arabidopsis to amino acids, despite the simultaneous presence of IN (Paper IV). In addition, increased uptake of amino acids related to induction was shown in both barley and Arabidopsis and resulted in up to 15-fold increases in uptake rates. It is therefore highly likely that amino acids are taken up simultaneously with IN in the field. The finding that the presence of amino acids induces increases in uptake rates complicates attempts to define plants' preferences for N sources, since uptake preferences were found (*inter alia*) to depend on the duration of

amino acid exposure. However, induced uptake rates of amino acids in Arabidopsis were up to 10 times higher than nitrate uptake rates. Thus, the recent history of proportions and concentrations of these N sources encountered by roots in the soil may be important determinants of plant uptake preferences for N sources. The results show, that plants possess organic nitrogen uptake systems that have many features similar to those used for inorganic nitrogen uptake.

The results of the work underlying this thesis show that plants have the capacity to take up amino acids at field concentrations. This capacity requires gene expression, synthesis and regulation of amino acid transporters, and the ability of plants to sense and respond to changes in external amino acid concentrations. One can expect, therefore, that plants can take up amino acids in their natural environment. Thus, it is time to reconsider traditional views of the nitrogen compounds used by agricultural plants.

6 Future perspectives

Insights gained into various aspects of plant amino acid uptake are presented above. A future challenge is to understand how this ability can be exploited. To make full use of this ability further knowledge on the regulation of uptake, induction, metabolism and growth on different amino acids as well as other ON compounds is needed. Due to the wide distribution of this ability among plant species it would be valuable to include species with different growth strategies in studies of these phenomena. It is also important to study the regulation of amino acid uptake in the presence of IN, since the metabolism and regulation of organic and inorganic nutrient sources are probably interconnected. Another great challenge is to elucidate the influence of soil properties on uptake capacities and preferences of ON and IN, to ensure that any conclusions have ecological relevance. However, the greatest challenge might be to develop methods capable of providing a clear understanding of the fluxes and short-term, micro-scale variations of ON and IN concentrations that roots encounter in intact root-soil systems.

Knowledge gained through further research of nitrogen inputs in agricultural systems is important for the challenge to develop ways to maintain rates of food production, without causing eutrophication of surrounding environments via N leaching, and possibly for optimising fertilization by tailoring it to match plant uptake capacities. Further improvements could also be potentially gained by identifying, breeding or engineering plants with increased N uptake capacities, which may need less surplus N additions.

7 Sammanfattning

Förekomst av aminosyror i jordbruksmark och hur de tas upp av växter

För att få så stor skörd som möjligt i dagens jordbruk och för att få kraftiga, gröna pelargonior i köksfönstret är det framför allt kvävet i den gödsel som tillförs som ger denna effekt.

Enligt många läroböcker anses nitrat och ammonium vara de två kväveföreningar som växterna tar upp i rötterna och använder för sin tillväxt. Hur stämmer den uppfattningen överrens med den kunskap som vi har idag? Finns det andra kväveformer som växter kan använda? Ny forskning visar att växters förmåga att ta upp aminosyror är vida spridd bland olika växtarter. Trots detta är den ekologiska och den fysiologiska betydelsen av denna förmåga dåligt känd. Denna avhandling fokuserar på de förutsättningar som finns för aminosyraupptag i jordbruksväxter, genom att kombinera relevansen i fältförsök med precisionen i lab-experiment.

Med hjälp av små lysimetrar samlades markvätska från olika typer av jordbruksmark med minimal störning av marken. Markvätskans innehåll av olika kväveföreningar analyserades. Koncentrationen av fria aminosyror var låg, men sannolikt sker det en kontinuerlig tillförsel av aminosyror från nerbrytningsprocesser i marken. De aminosyrakoncentrationer som uppmätts i markvätskan användes sedan i upptagsförsök. Trots de låga koncentrationerna kunde växthusodlade plantor av både korn och backtrav ta upp aminosyrorna. Upptaget begränsades inte av någon lägsta upptagbar koncentration. Hos både korn och backtrav var upptagsaffiniteten för aminosyror jämförbar med litteraturuppgifter för motsvarande koncentrationer av nitrat och jämförbar med upptag av aminosyror hos markmikroorganismer.

Det studerade aminosyraupptaget skedde till största delen med hjälp av två så kallade aminosyratransportörer i rötterna hos backtrav, nämligen LHT1 (lysine histidine transporter 1) och AAP5 (amino acid permease 5). Tack vare olika egenskaper hos de två transportörerna kompletterar de varandra i upptag av aminosyror: LHT1 transporterar sura och neutrala aminosyror och AAP5 basiska aminosyror. Betydelsen av andra aminosyratransportörer såsom AAP1 var liten i de utförda experimenten.

Genuttrycket av *LHT1* och *AAP5* visade en tydlig ökning efter att rötterna exponerats för aminosyror, och detta skedde trots samtidig närvaro av nitrat och ammonium i lösningen. Då rötterna hade exponerats för aminosyror innan mätningarna började blev aminosyraupptaget upp till 15 gånger så högt som upptaget utan föregående exponering, det skedde således en induktion av aminosyraupptag, både hos korn och hos backtrav. När växterna hade tillgång till såväl aminosyror som nitrat och ammonium i lösningen och då rötterna exponerades för aminosyror innan mätningarna började blev upptaget av aminosyror upp till 10 gånger så högt som upptaget av nitrat. Dessa molekylära och fysiologiska effekter av att exponera rötterna för aminosyror indikerar att växter har system för aminosyraupptag samt att systemen för aminosyraupptag och systemen för upptag av oorganiskt kväve har liknande egenskaper.

Resultaten från de arbeten som denna avhandling är baserad på indikerar att växter har kapacitet att ta upp aminosyror vid koncentrationer som förekommer i markvätska. Denna kapacitet kräver en serie av händelser i växten. Det krävs genuttryck, syntes och reglering av aminosyratransportörer men också att växten kan uppfatta att det finns en viss koncentration av aminosyror i rotmiljön och därvid reagera med denna serie av händelser. De studier som denna avhandling beskriver ger stöd för påståendet att växter, i sin naturliga miljö, tar upp aminosyror. Det finns således anledning att ompröva uppfattningen att jordbruksväxter endast nyttjar nitrat och ammonium.

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Acknowledgements

This has certainly not been a one woman show. This thesis and the projects behind it is a joint effort of several persons to whom I would like to express my deepest gratitude.

First and foremost I would like to extend my gratitude to the directors and producers:

Kerstin and **Torgny**, my super-supervisors! Thank you for introducing me to the exciting research of plant-soil interactions! This journey has, thanks to you, been a creative adventure. I have really appreciated to work with you, true nerds of plants! You complement each other perfectly. It's been fun.

I would like to give my humble thanks to the **Opponent** and the **Examining Committee** for taking your time to read my thesis.

My co-authors, thank you for great collaboration

Henrik S, you sure are talkative but entertaining during tedious experiment preparations.

Camila, lab work is so easy in your company.

Mattias H, for excellent molecular analyses.

Ulrika G, for your straight forward and warm attitude and for expanding my molecular knowledge.

Erich I, for enormously efficient nitrate analysis.

I have gotten so much invaluable support during these years from the technical staff at NJV and UPSC. Thank you **Ann-Sofi**, **Margareta**, **Siv, Janne K, Marja-Leena** and **Lasse** for help whenever needed with introducing me to HPLC, general lab behaviour, method development, experiments, massive autoclaving and so much more, always in a very nice way. **Britt- Inger**, for all administrative support. Thank you, **Ingemar** and **Stefan**, for technical support with my computer.

I would like to thank, **Jun Yu**, **Kristi Kuljus** and **Bo Ranneby** for efficient statistical advices and rewarding statistical discussions.

Ines B, thank you for your commitment during an unusually dry and crappy season.

Former and present members of the N-group (**Åsa**, **Oskar**, **Henrik**, **Lena**, **Camila**, **Mattias**, **Ulrika**, **Erich**, **Linda G**, **Jonas**, **Takahide**, **Annika**, **Margareta**, **Ann**, et al.). Thanks for great collaborations, rewarding discussions, great professional and personal support.

My thanks to all you fantastic people I met during the way, present and former employees at NJV and UPSC for all work related and personal discussions. My special appreciation to **Ioana**, **Steve**, **Olivier**, **Jonathan**, **Ilka**, **Ann-Sofi**, **Cissi**, **Georg**, **Anja**, **Osei,** all PhD colleagues and Post-Docs, which are too many to mention but you know who your are.

During time water passes under the bridge meanwhile important people pass over it. I have met some really good friends during these years. Words are limiting but:

Sara AG, for bringing joy to running, for the discussions, and for your nerdiness and thoughtfulness.

Aurora, for your spirit and for making me laugh out loud.

Janne (and your wonderful **Jenny**), thank you for the music…all conversations, chemistry input and computer support.

Junko, you are so much fun.

Annika J, **Sara VP**, **Anna K**, my sisters in arms, thank you for the occasional reality check.

Karin and **Henrik**, I still think the common swift is the coolest bird ever. You two are rare birds.

All friends on the outside,

The best ex-neighbours, **Hanna** and **Pelle**, for spontaneous visits and relaxing atmosphere.

Ellinor and **Patrik**, for your enormous hospitality.

Ann-Sophie and **Roger**, my long distance science supporters.

Charlotta, who is always there.

Mia and **Tobbe**, for being sweet and enthusiastic.

Berika and **Lina**, for ever lasting friendship.

Daniel and **Helen**, I miss beer in Majorna.

Helle! Min Helle! "Det är inte så noga…"

Annika, **Hasse**, **Mimmi** and **Julle**. You are the best in-laws one could ever wish for.

Mamma och **Pappa**, **Evelina** och **Petter**. Tack för allt! Utan er så vore jag inte jag! Tack för att ni bryr er fastän ni inte alltid förstår!

All you need is love…

Frasse, I love you around the world and back again.

Ingrid, oändligt är vårt stora äventyr!

The language was corrected by John Blackwell at Sees-editing Ltd, UK.
My position as a PhD student was funded by FORMAS, grant to KHD.

Det mesta blir väl inte som man tänkt sig och ingen annan kan man skylla på. Och baken har man bakåt fast man vänt sig, å knappast blir de nå bättre.

Allan Edwall, Förhoppning