New Phytologist

Research

Transporters in Arabidopsis roots mediating uptake of amino acids at naturally occurring concentrations

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Received: *19 January 2011*
Accepted: *16 February 2011*

New Phytologist (2011) **191**: 459–467
doi: 10.1111/j.1469-8137.2011.03699.x

Key words: amino acid permease 1 (AAP1), amino acid permease 5 (AAP5), kinetics, lysine histidine transporter 1 (LHT1), mutants.

Summary

• Recent studies of Arabidopsis have identified several transporters as being important for amino acid uptake.

• We used Arabidopsis plants with altered expression of lysine histidine transporter 1 (LHT1), amino acid permease 1 (AAP1) and amino acid permease 5 (AAP5) with the aim of disentangling the roles of each transporter in the uptake of different amino acids at naturally occurring concentrations (2–50 μM).

• *LHT1* mutants displayed reduced uptake rates of L-Gln, L-Ala, L-Glu and L-Asp but not of L-Arg or L-Lys, while *AAP5* mutants were affected in the uptake of L-Arg and L-Lys only. Double mutants (*lht1aap5*) exhibited reduced uptake of all tested amino acids. In the concentration range tested, *AAP1* mutants did not display altered uptake rates for any of the studied amino acids. Expression analysis of amino acid transporter genes with important root functions revealed no major differences in the individual mutants other than for genes targeted for mutation.

• We conclude that LHT1 and AAP5, but not AAP1, are crucial for amino acid uptake at concentrations typically found in soils. LHT1 and AAP5 displayed complementary affinity spectra, and no redundancy with respect to gene expression was found between the two transporters, suggesting these two transporters have separate roles in amino acid uptake.

Introduction

Organic nitrogen (N) compounds, in particular amino acids, may function as N sources for plants in various ecosystems, and the capacity to absorb amino acids is present in both mycorrhizal and nonmycorrhizal plants (Näsholm *et al.*, 2009). Plant uptake of amino acids was first described in the early 20th century (Hutchinson & Miller, 1911; Brigham, 1917) but has lately attracted renewed interest, as demonstrated by the range of more recent studies published in this field (for recent reviews, see Lipson & Näsholm, 2001; Näsholm & Persson, 2001; Schimel & Bennett, 2004; Rentsch *et al.*, 2007; Näsholm *et al.*, 2009). The actual benefit to plants absorbing such compounds is, however, still uncertain. Moreover, in contrast to the extensive information available on the molecular biology and physiology of plant inorganic N nutrition, our knowledge of the mechanisms underpinning plant organic N nutrition is still very limited.

Plant uptake of amino acids is energized by the proton gradient over the plasma membrane and facilitated by transport proteins (cf. Liu & Bush, 2006; Rentsch *et al.*, 2007; Näsholm *et al.*, 2009). 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; Näsholm *et al.*, 2009). Early studies 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). The kinetics of root amino acid uptake has been investigated over widely varying amino acid concentration ranges. Because of this, available information about the kinetics of plant amino acid uptake is associated with a high degree of variability both

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within and between species, and the concentrations used have ranged from the low μM range (Soldal & Nissen, 1978; Schobert & Komor, 1987; Jämtgård *et al.*, 2008) to several mM (Soldal & Nissen, 1978; Borstlap *et al.*, 1986; Schobert & Komor, 1987).

Arabidopsis lysine histidine transporter 1 (LHT1), originally identified by Chen & Bush (1997), was the first transporter shown to be involved in amino acid uptake (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007). LHT1 displays high affinity for neutral amino acids, L-His (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007) and acidic amino acids (Hirner *et al.*, 2006). The second transporter identified as having a role in amino acid uptake was Arabidopsis amino acid permease 1 (AAP1; Lee *et al.*, 2007), which was shown to mediate uptake of several neutral amino acids as well as L-Glu and L-His but, similar to LHT1, activity for L-Arg and L-Lys was not detected. As neither AAP1 nor LHT1 displayed activity for L-Arg and L-Lys, Svennerstam *et al.* (2008) searched for a transporter mediating uptake of basic amino acids and found Arabidopsis amino acid permease 5 (AAP5) to be crucial for this function, thereby providing evidence for a third transporter mediating amino acid uptake.

In the work of Hirner *et al.* (2006), Lee *et al.* (2007) and Svennerstam *et al.* (2007, 2008), several studies of LHT1, AAP1 and AAP5 were performed *in planta*, in yeast and in Xenopus oocytes, thereby providing invaluable information about the biochemical and physiological properties of these transporters and of amino acid uptake in general. However, the relative importance of each of these three transporters for uptake of different amino acids at field-relevant concentrations is presently unclear. Moreover, the potential effects of mutations in single genes involved in amino acid uptake on expression of other candidate genes in this process are not known. Our aim in this study was therefore to disentangle the roles of individual amino acid transporters in uptake of neutral, acidic and basic amino acids at concentrations spanning the range typical of soils in agricultural and in temperate and boreal forest ecosystems. Concentrations of amino acids in soil solution in these soils generally have been found to be below 50 μM, with the exception of single measurements (Kielland, 1994; Raab *et al.*, 1996, 1999; Henry & Jefferies, 2002; Jones *et al.*, 2002, 2005; Yu *et al.*, 2002; Öhlund, 2004; Jämtgård *et al.*, 2008, 2010). We compared amino acid uptake rates in Arabidopsis wild-type plants with uptake rates in plants lacking functional expression of *LHT1*, *AAP1* or *AAP5* as well as a double mutant (*lht1aap5*) and a mutant in which *LHT1* is overexpressed. To reveal potential interactions between amino acid transporters, we studied the effects of the mutations on gene expression of the targeted amino acid transporters (LHT1, AAP1 and AAP5) as well as expression of genes encoding amino acid permease 2 (AAP2) and amino acid permease 3 (AAP3), two transporters with potential roles in xylem/phloem transport of amino acids (Hirner *et al.*, 1998; Okumoto *et al.*, 2004).

Materials and Methods

Plant material and growth conditions

Wild-type *Arabidopsis thaliana* L. Heynh. (Columbia (Col-0)), the amino acid transporter mutants *lht1-5* (Svennerstam *et al.*, 2007), *aap5-1* (Svennerstam *et al.*, 2008), *aap1-3* (see description in the next section) and *lht1-5aap5-1* (double mutant; Svennerstam *et al.*, 2008) and an *LHT1* overexpressor (*35SLHT1-2*; Forsum *et al.*, 2008) were grown on sterile vertical agar plates containing half-strength N-free Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), 3 mM NO_3^-, 1% (w/v) agar and 0.5% (w/v) sucrose, buffered to pH 5.8 using 7.7 mM MES. Seeds were surface-sterilized (Forsum *et al.*, 2008), sown onto plates and incubated in a cold room for 48 h (to optimize germination). The plates were then transferred to a climate chamber. All plant lines were grown for 18 d at 22°C with an 8 : 16 h light : dark regime (photosynthetic photon flux density 200 μmol m^{-2} s^{-1}).

Arabidopsis mutants lacking functional *AAP1* expression were originally characterized by Lee *et al.* (2007). We obtained a T-DNA mutant line from the GABI-Kat knockout collection (GABI-135G05; Rosso *et al.*, 2003), and named the mutant *aap1-3* to avoid confusion with the mutant lines used in Lee *et al.* (2007). Confirmation of T-DNA insertion in the *AAP1* gene of *aap1-3* was performed by PCR using an *AAP1*-specific primer and a primer specific for the T-DNA insert (data not shown). According to sequencing data from GABI-Kat, the insert was located in the first intron of *AAP1* (Fig. 1a). RT-PCR reactions to confirm repression of *AAP1* were performed using *AAP1*-specific primers spanning the insertion site (Fig. 1b). The constitutively expressed Arabidopsis actin gene (*ACT2*) was used as a control for equal

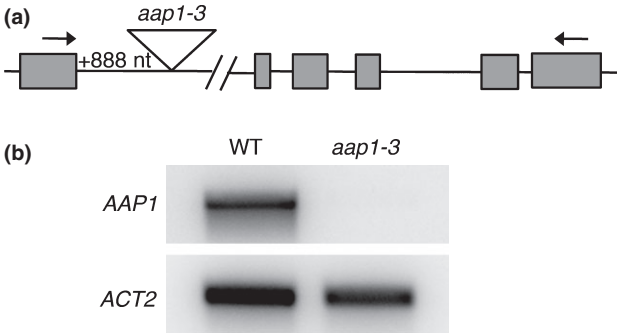

Fig. 1 Characterization of an Arabidopsis amino acid permease 1 (*AAP1*) T-DNA mutant line. (a) Graphic depiction of *AAP1* and the estimated location of the T-DNA insert in *aap1-3* (marked with a triangle; *c.* 888 nt from ATG). Exons are depicted as filled boxes. The positions of the primers used for RT-PCR in (b) are marked with arrows. (b) RT-PCR analysis of *AAP1* expression using gene-specific primers spanning the targeted T-DNA insertion site, showing that the expression of *AAP1* is repressed in *aap1-3* plants (WT, wild type). Trace amounts of transcript could be detected if the image was overexposed (data not shown). Specific primers for the Arabidopsis actin gene *ACT2* were used as controls (An *et al.*, 1996).

loading of RNA in each reaction (An *et al.*, 1996). Similarly to Lee *et al.* (2007), we found that *aap1-3* plants were resistant to 10 mM L-Phe on agar media (data not shown).

Measurements of amino acid uptake

The uptake experiment was carried out using 2.5 kBq ml^{-1} of L-[U-^{14}C]Gln (7.4 TBq mol^{-1}), L-[U-^{14}C]Asp (8.14 TBq mol^{-1}), L-[U-^{14}C]Ala (6.475 TBq mol^{-1}), L-[U-^{14}C]Arg (11.47 TBq mol^{-1}), L-[U-^{14}C]Glu (9.99 TBq mol^{-1}) or L-[U-^{14}C]Lys (9.25 TBq mol^{-1}) at the concentrations 2, 5, 10, 25 and 50 μM.

Uptake solutions were buffered with 2.85 mM MES to pH 5.8 and contained 0.5 mM $CaCl_2$ to preserve membrane integrity (Epstein, 1961). Immediately before the uptake experiment, plants were removed from the agar plates and their roots were washed in 0.5 mM $CaCl_2$ and gently blotted on tissue paper. Roots of the intact plants were then submerged in 1 ml of a solution of the desired amino acid at the desired concentration for 60 min. In the same experiment, five plants were used for each plant line, amino acid and concentration, with each individual plant representing one replicate. Roots were washed three times in 0.5 mM $CaCl_2$ and the plants were divided into roots and shoots, dried at 60°C and weighed. Roots and shoots were rehydrated separately in 200 μl of distilled water overnight. Plant tissues were digested by adding 1 ml of Soluene 350 (Perkin Elmer, Boston, MA, USA) in capped vials at 50°C overnight. After the addition of 6 ml of scintillation cocktail (Hionic Fluor; Perkin Elmer), the samples were assayed for ^{14}C in a Beckman LS6500 scintillation counter (Beckman Coulter, Brea, CA, USA). Amino acid uptake was calculated from the sum of ^{14}C in shoots and roots and was expressed per unit root dry mass. A control experiment, performed as described in the beginning of this section, established that amino acid uptake rates were constant during 90 min at amino acid concentrations of 2 and 50 μM (data not shown).

Data analysis

The maximum uptake rate (V_{max}) and half saturation constant (K_m) parameters were determined using both Hanes–Woolf plots and Michaelis–Menten nonlinear regression (PRISM 5; Graphpad Software, La Jolla, CA, USA) using all replicate samples (Table 1). The two methods gave similar results (Table 1), and therefore we only discuss the results for the Hanes–Woolf plots.

RT-PCR

Roots were collected from plants growing under the same conditions as the plants used in the uptake experiment. Roots were harvested from three biological replicates (three pooled plants per replicate), briefly rinsed in water, gently blotted dry on tissue paper and frozen in liquid N_2. RNA was prepared using the EZNA Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA) and the samples were DNaseI-treated using DNA-*free* (Ambion Inc., Austin, TX, USA). One biological replicate from *aap1-3* did not yield any RNA, and therefore the corresponding average is only based on two biological replicates. First-strand cDNA synthesis was performed using the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) and amplification of the target genes was performed using Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Gene-specific primers were used for each gene: (*LHT1*: 5′-AGTCATCGTTGCTTACATCGTCGT and 5′-TGGCGATAGGACCATCAAGAAAAGA; *AAP1*: 5′-TCTTACTCATTTTCTCGTTCATTAC and 5′-ACAATTTGGCTCAATAAACAGTCC; *AAP2*: 5′-ATAACCACCGTCACCACCAC and 5′-CAAGAGCTAGACCAATGGCAG; *AAP3*: 5′-TGCCGTCACTTATTTCACTTCTT and 5′-TTGAACTCGAAACCTGCTCTG; *AAP5*: 5′-TTGGGACAGTGACACTGAGTG and 5′-AACAATGCCAATAACAGATCCC). The linear range for each primer pair was determined. For each biological sample, three technical replicates of PCR amplification were performed. The gel images were visualized and the intensity of each band was analysed using the GelDoc System and QUANTITYONE software from Bio-Rad Laboratories (http://www.bio-rad.com). The Arabidopsis *ACT2* gene (An *et al.*, 1996) was used as an external standard. The wild-type gene expression relative to *ACT2* was set to equal 1.0.

Table 1 Calculated kinetic parameters for Arabidopsis wild type and plants overexpressing the amino acid transporter lysine histidine transporter 1 (*35SLHT1*)

Amino acid	Genotype	K_m (μM)		V_{max} (μmol g^{-1} root DW h^{-1})	
		NLR	H–W	NLR	H–W
L-Gln	Wild type	44.8 ± 21.8	41.0 ± 11.8	**2.3** ± 0.64	**2.1** ± 0.39
	35SLHT1	26.1 ± 6.5	36.7 ± 7.3	**6.7** ± 0.8	**7.5** ± 0.95
L-Arg	Wild type	7.4 ± 0.9	7.6 ± 1.2	**4.0** ± 0.16	**4.0** ± 0.15
	35SLHT1	7.8 ± 1.1	7.5 ± 1.5	**4.7** ± 0.2	**4.6** ± 0.23
L-Lys	Wild type	27.7 ± 7.3	26.1 ± 5.6	7.7 ± 0.98	7.2 ± 0.85
	35SLHT1	34.4 ± 6.9	25.1 ± 4.1	9.1 ± 0.96	7.8 ± 0.68

Comparison between the wild type and *35SLHT1* for K_m (half saturation constant) and V_{max} (maximum uptake rate) was performed using a two-sample *t*-test. Significant differences between the wild type and *35SLHT1* for each amino acid are indicated by bold characters ($P < 0.01$).
Kinetic parameters were calculated using nonlinear regression (NLR) or Hanes–Woolf plots (H–W) and are given as mean values ± SEM ($n = 5$).

Results

Uptake of amino acids at field-relevant concentrations

To investigate the amino acid uptake characteristics of Arabidopsis at naturally occurring concentrations, wild-type

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Arabidopsis plants, amino acid transporter T-DNA mutants for *LHT1*, *AAP1* and *AAP5*, a double mutant (*lht1aap5*) and an *LHT1* overexpressor were subjected to 2–50 μM of a neutral (L-Gln or L-Ala), acidic (L-Asp, L-Glu) or basic (L-Arg, L-Lys) amino acid.

Uptake rates vs substrate concentration plots are shown in Fig. 2. The uptake of L-Gln in the wild type, *AAP5* mutants and *AAP1* mutants saturated to varying degrees at higher substrate concentrations, whereas the uptake of L-Ala, L-Asp and L-Glu followed a linear pattern within the concentration range tested. Similarly, uptake of L-Arg and L-Lys in the wild-type, *LHT1* mutants and *AAP1* mutants was also saturated at higher substrate concentrations. In T-DNA knockout mutants with an altered amino acid uptake phenotype, the remaining uptake displayed linear kinetics within the concentration range tested.

In wild-type plants, the highest uptake rates were found for L-Ala, ranging from 0.56 μmol g^{-1} root DW h^{-1} at 2 μM amino acid to 8.11 μmol g^{-1} DW h^{-1} at 50 μM amino acid. Uptake of L-Lys was 0.56 μmol g^{-1} root DW h^{-1} at 2 μM and 5.01 μmol g^{-1} root DW h^{-1} at 50 μM. Uptake of L-Arg was intermediate at 0.67 to 3.44 μmol g^{-1} DW h^{-1}, followed by L-Gln and L-Asp at 0.1 to 1.23 and 0.07 to 1.76 μmol g^{-1} root DW h^{-1}, respectively. L-Glu was taken up at the lowest rates 0.02 μmol g^{-1} root DW h^{-1} at 2 μM and 0.48 μmol g^{-1} root DW h^{-1} at 50 μM.

As root uptake of the amino acids tested in this study displayed both saturating and linear kinetics, we also wanted to investigate how overexpression of an amino acid transporter affected amino acid uptake kinetics. Plants overexpressing *AtLHT1*, with increased uptake of neutral and acidic amino acids, have been characterized previously (Hirner *et al.*, 2006; Forsum *et al.*, 2008), and are excellent model plants with which to investigate whether uptake rates can be improved by increasing the expression of a gene encoding a transporter. The strong effect of overexpressing *LHT1* is thus principally interesting as a contrast to the knock-out mutant of *LHT1*. Therefore, we subjected plants overexpressing *LHT1* to the same experimental set-up as for the amino acid transporter deficient mutants (Fig. 3). No difference in uptake kinetics was seen for L-Arg and L-Lys. Overexpression of *LHT1* resulted in increased uptake and saturating kinetics for L-Gln and increased but linear kinetics for L-Ala, L-Asp and L-Glu, in comparison to the wild-type plants (Fig. 3).

To further illustrate the impact of the individual amino acid transporter mutations on amino acid uptake, we

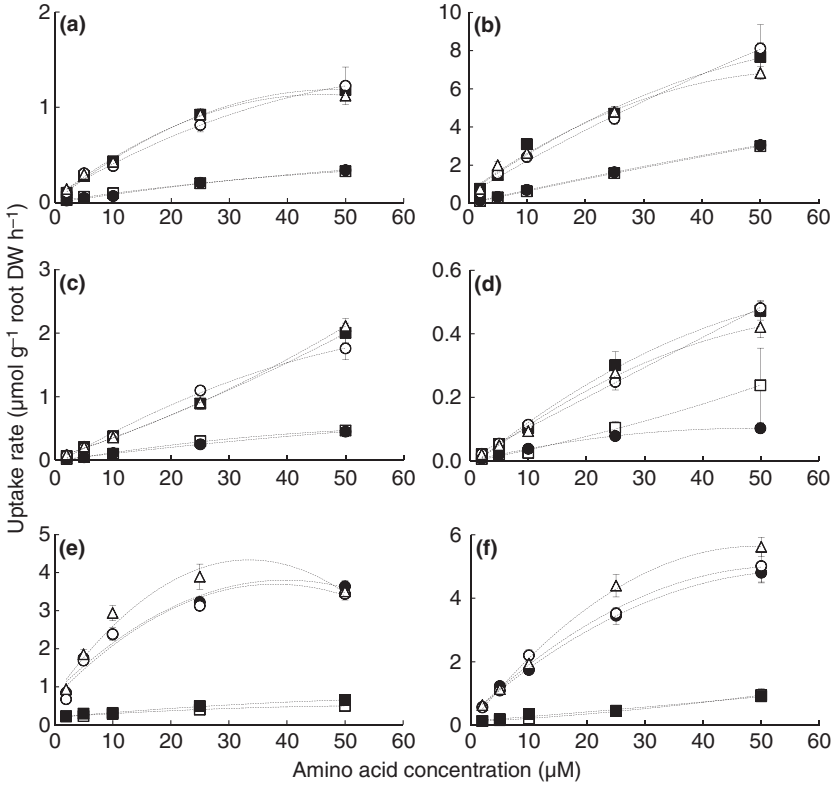


Fig. 2 Amino acid uptake in Arabidopsis wild type and mutants with altered amino acid transporter expression. Uptake of ^{14}C-labelled L-Gln (a), L-Ala (b), L-Asp (c), L-Glu (d), L-Arg (e) and L-Lys (f) was assessed by immersing roots of intact, axenically grown plants in 2, 5, 10, 25 and 50 μM concentrations of the respective amino acid. Uptake for the wild type (open circles), *lht1-5* (closed circles; *lht*, lysine histidine transporter), *lht1aap5* (open squares; *aap*, amino acid permease), *aap5-1* (closed squares) and *aap1-3* (open triangles) is shown. Amino acid uptake was calculated from the sum of ^{14}C in the shoots and roots, expressed per unit root dry mass. Each data point represents the mean of five individual replicate plants ± SEM. The curves show the polynomial regression fitted to the data.

calculated the uptake rates as compared with the wild type (Fig. 4). In the *LHT1* mutant, uptake of L-Gln, L-Ala, L-Glu and L-Asp was greatly reduced by on average 61–85% over the entire concentration range, while the uptake of L-Arg and L-Lys was unaffected. Uptake of L-Arg and L-Lys was strongly affected in the *AAP5* mutants, being reduced by on average 68–88%. *AAP1* mutants did not display any major differences in the uptake of any of the amino acids tested. However, uptake of L-Arg at 2, 10 and 25 μM, and of L-Lys at 25 μM was slightly increased, being 123–140% of wild-type values. Uptake of all amino acids tested was greatly decreased in the double mutants as compared with the wild type and was similar to the uptake rates found for *lht1-5* (L-Gln, L-Ala, L-Glu and L-Asp) and *aap5-1* (L-Arg and L-Lys). The *LHT1* overexpressor showed strongly increased uptake of L-Gln, L-Ala, L-Glu and L-Asp, with uptake rates of between 219 and 456% of wild-type uptake.

Kinetics of amino acid uptake

The primary goal of the current study was to characterize amino acid uptake at concentrations relevant for soils of different ecosystems. Nevertheless, this concentration range allowed for calculations of kinetic parameters of uptake of three of the studied amino acids (Table 1). We calculated K_m and V_{max} only on wild-type plants and the *LHT1* overexpressor plants because of the lack of saturation of uptake rates in the other genotypes. Similarly, kinetic parameters were only calculated for L-Gln, L-Arg and L-Lys because L-Ala, L-Glu and L-Asp uptake did not saturate within the concentration range tested. The calculations of K_m and V_{max} revealed possible differences in affinity for the amino acids tested in Arabidopsis. Uptake of L-Gln in wild-type plants displayed a K_m of 41 μM and a V_{max} of 2.1 μmol g^{-1} root DW h^{-1}. The *LHT1* overexpressor had a similar K_m for L-Gln uptake but V_{max} was approx. 3 times higher than in the wild type. Arabidopsis wild-type plants displayed *c.* 4 and 5 times higher affinity for L-Arg than for L-Lys and L-Gln, respectively. The *LHT1* overexpressor was not significantly different from the wild type with respect to K_m and V_{max} for L-Arg or L-Lys, corroborating a lack of function of this transporter for basic amino acids (cf. Svennerstam *et al.*, 2008).

Expression of amino acid transporter genes in roots of the transporter mutants

We examined whether there were any major alterations in gene expression that can affect amino acid uptake in the

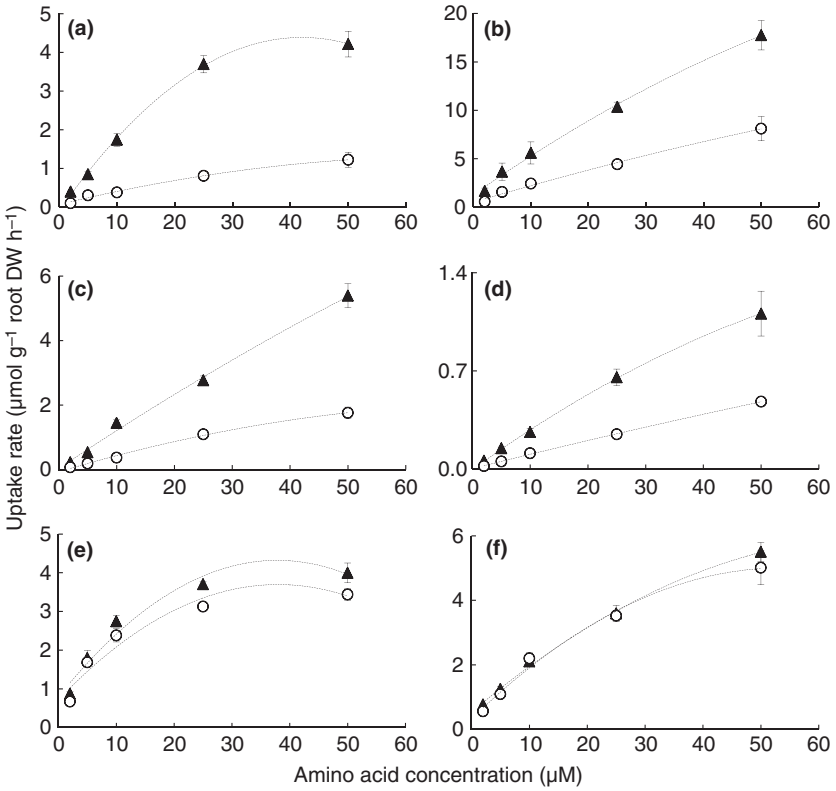
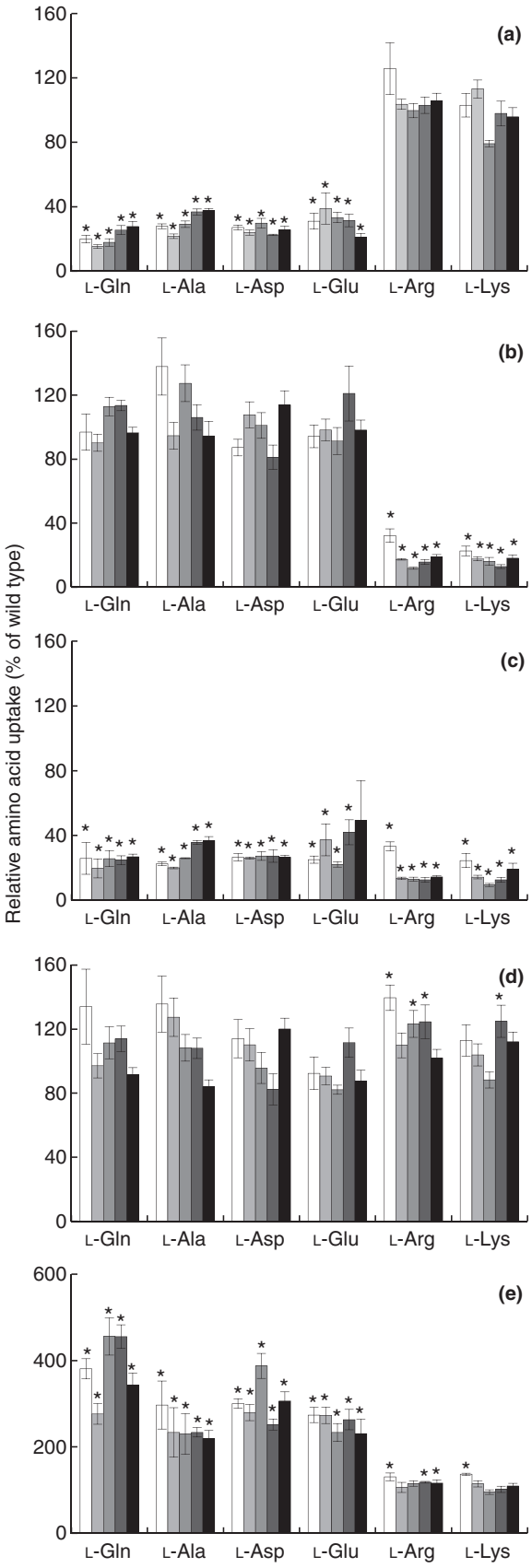


Fig. 3 Amino acid uptake in Arabidopsis wild type and plants overexpressing the amino acid transporter lysine histidine transporter 1 (*35SLHT1*). Uptake of ^{14}C-labelled L-Gln (a), L-Ala (b), L-Asp (c), L-Glu (d), L-Arg (e) and L-Lys (f) was assessed by immersing roots of intact, axenically grown plants in 2, 5, 10, 25 and 50 μM concentrations of the respective amino acid. Uptake for wild type (open circles) and *35SLHT1* (closed triangles) is shown. Amino acid uptake was calculated from the sum of ^{14}C in shoots and roots, expressed per unit root dry mass. Each data point represents the mean of five individual plants ± SEM. The curves show the polynomial regression fitted to the data.

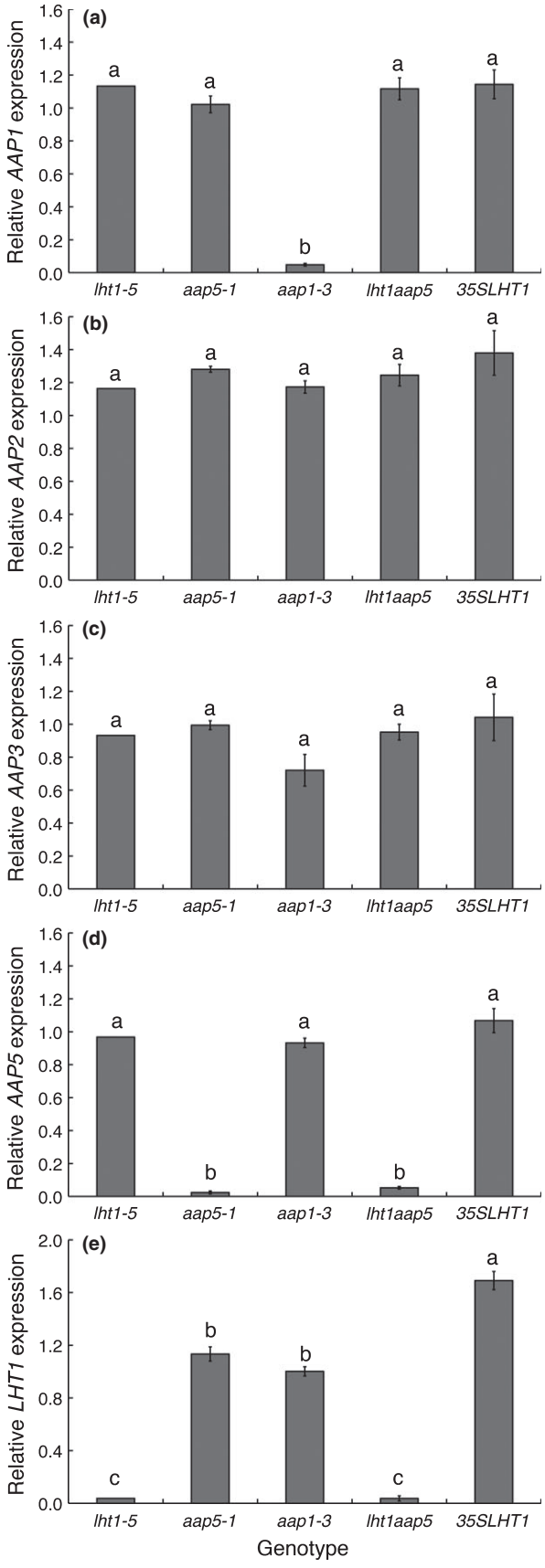


mutants (other than in the genes targeted for mutation). The relative gene expression of the amino acid transporters known to be involved in amino acid uptake, LHT1, AAP1 and AAP5 (Hirner *et al.*, 2006; Lee *et al.*, 2007; Svennerstam *et al.*, 2007, 2008), was analysed in each genotype. In addition, the expression of the genes encoding AAP2 and AAP3 was analysed, as these transporters have been shown to have important functions in roots (Hirner *et al.*, 1998; Okumoto *et al.*, 2004) (Fig. 5). Transcripts corresponding to the genes targeted for mutation were not detected or were only present in trace amounts in the four T-DNA mutants. In addition, the expression of *LHT1* in the overexpressor was 1.7 times as high as in the wild type. No major differences in gene expression of the other amino acid transporters were detected: their root expression levels of amino acid transporter genes ranged from 80 to 140% of wild-type values.

Discussion

The present study shows that the activity of two amino acid transporters, LHT1 and AAP5, accounts for the majority of amino acid uptake in Arabidopsis at concentrations relevant for soil solution in cultivated and natural ecosystems. Moreover, LHT1 and AAP5 were found to be largely complementary to each other with respect to affinity spectra, so that LHT1 accounts for uptake of neutral and acidic amino acids while AAP5 accounts for uptake of basic amino acids. By contrast, the current study does not support a function of AAP1 in amino acid uptake at naturally occurring concentrations. The individual amino acid transporter mutations did not induce any changes in gene expression of two other transporters with potential function in amino acid uptake, which suggests that no redundancy between the studied transporters exists. These findings have major implications for our understanding of the physiology of plant organic N nutrition and suggest that nonmycorrhizal plants may rely on LHT1 and AAP5 to acquire N in the form of amino acids from soil.

Fig. 4 Relative amino acid uptake in Arabidopsis plants with altered amino acid transporter expression. Uptake rates of each amino acid and concentration were compared between the wild type (100%) and single mutant lines (a) *lht1-5*, (b) *aap5-1*, (c) *lht1aap5*, (d) *aap1-3* and (e) *35SLHT1* (*aap*, amino acid permease; *lht*, lysine histidine transporter). Uptake of ^{14}C-labelled L-Gln, L-Ala, L-Asp, L-Glu, L-Arg and L-Lys was assessed by immersing roots of intact, axenically grown plants in 2 μM (white bars), 5 μM (light grey bars), 10 μM (medium grey bars), 25 μM (dark grey bars) and 50 μM (black bars) of the respective amino acid. Comparison of the wild type and single mutant lines of each amino acid at each concentration was performed using one-way ANOVA followed by Dunnett's comparison with a control. No data were transformed except L-Ala 2–10 μM ($\log_{10}$). Bars represent mean values ± SEM; $n = 5$. Data significantly different from wild-type data for the corresponding amino acid and concentration are indicated with an asterisk (*, $P < 0.05$).



Arabidopsis plants lacking functional expression of *LHT1* or overexpressing *LHT1* were affected in L-Gln, L-Ala and L-Asp uptake (Figs 2, 3), suggesting LHT1 to be crucial for L-Gln uptake at low concentrations. Further, the magnitude of the increase in the uptake rate for L-Gln (*c.* 400%) was similar to the magnitude of the increase in the growth response (*c.* 300%) of plants overexpressing *LHT1* when grown on 0.5 mM L-Gln as the single N source (Forsum *et al.*, 2008). Hence, a strong relationship between root uptake capacity and growth of Arabidopsis on L-Gln appears to exist.

L-Ala uptake was also significantly affected by altered *LHT1* expression, as seen both in *LHT1* mutants and in the *LHT1* overexpressor (Figs 2, 3). These findings corroborate the suggestions by Hirner *et al.* (2006) and Svennerstam *et al.* (2007) that LHT1 is probably the most important transporter for root uptake of L-Ala at low concentrations. Furthermore, altering the expression of *LHT1* also had a profound impact on the uptake of L-Asp, which is consistent with Hirner *et al.* (2006), who found that mutants with repressed or increased expression of *LHT1* displayed significant reductions and increases, respectively, in uptake rates of L-Asp. In the current study, the concentration dependence of L-Asp uptake exhibited a clear linear pattern (Fig. 2). This lack of Michaelis–Menten kinetics for root uptake of acidic amino acids was also found in a study by Schobert & Komor (1987) and was interpreted as a sign of a separate uptake mechanism for such compounds. The present study shows, in agreement with the study by Hirner *et al.* (2006), that LHT1 is crucial for uptake of L-Asp as well as L-Gln and L-Ala, in spite of the different patterns of concentration dependence that these three amino acids display. Clearly, the idea that linear and saturated concentration-dependent uptake of amino acids indicates that different transporters are involved in the uptake process (Schobert & Komor, 1987) is not supported by the data presented here.

The strongly reduced uptake of L-Arg in the *AAP5* mutant as compared with the wild type (Figs 2, 3) is similar to earlier findings (Svennerstam *et al.*, 2008) suggesting that the main high-affinity transporter for L-Arg uptake in Arabidopsis roots is AAP5, and extends these findings to concentrations as low as 2 μM. Also, the double (*lht1aap5*) mutant displayed reduced uptake with rates only marginally lower than those in the *AAP5* mutant (Figs 2, 3). Earlier studies

Fig. 5 Relative gene expression of amino acid transporters in roots of Arabidopsis plants with altered root amino acid uptake. Relative gene expression levels for (a) amino acid permease 1 (*AAP1*), (b) *AAP2*, (c) *AAP3*, (d) *AAP5* and (e) lysine histidine transporter 1 (*LHT1*) were analysed using semiquantitative RT-PCR. Gene expression in the wild type was set to 1.0. The Arabidopsis actin gene *ACT2* (An *et al.*, 1996) was used as an external reference. Bars represent mean values ± SEM; $n = 3$ (except for *lht1-5*; $n = 2$). Comparison of gene expression in each mutant line was performed using one-way ANOVA followed by Tukey's test. Data significantly different are indicated with letters ($P < 0.05$).

have suggested that AAP5 may have high affinity for uptake of basic amino acids, in particular L-Lys and L-Arg, but also for neutral and acidic amino acids (Fischer *et al.*, 1995; Boorer & Fischer, 1997). In the present study of intact plants, only uptake rates of L-Arg and L-Lys were affected, which suggests that the function of AAP5 *in planta* may be limited to uptake of basic amino acids.

Earlier studies suggested AAP1 to function in amino acid acquisition, as Arabidopsis mutants lacking AAP1 expression had severely reduced uptake of the neutral amino acids, L-Glu and L-His (but not L-Asp and L-Lys; Lee *et al.*, 2007). Those uptake studies were performed on intact plants with roots supplied either with 10 mM of individual amino acids or with 150 μM of L-Ala or L-Glu. Further, the affinity of AAP1 for L-Ala determined in heterologous expression systems was estimated to 290 μM (yeast; Boorer *et al.*, 1996) and 600 μM (oocytes; Hsu *et al.*, 1993). It seems that AAP1 mediates uptake of amino acids such as L-Glu and L-Ala, but primarily when concentrations of amino acids exceed 100 μM. In the present study, the maximum concentration of individual amino acids was 50 μM; that is, a third of the lowest concentration employed by Lee *et al.* (2007). Under these conditions, we could not detect a significant effect of the *aap1* mutation on uptake rates (Figs 2, 3). Furthermore, the remaining uptake rates of e.g. L-Ala in *LHT1* mutants were only *c.* 20%, underscoring the importance of this transporter at the low end of concentration ranges in soil solutions of various ecosystems.

In the present study, the amino acid concentration range was chosen to represent soil solutions. For some amino acids, this concentration range was too narrow to allow calculation of kinetic properties. In spite of these shortcomings, the application of standard techniques for evaluation of kinetic parameters to our data gave interesting insights into uptake characteristics and effects of loss of individual transporters on these processes. For the amino acids tested, Arabidopsis displayed the highest affinity for L-Arg: wild-type plants had a K_m of 7.6 μM, which is consistent with earlier studies (Soldal & Nissen, 1978; Jämtgård *et al.*, 2008). The affinity for L-Gln was 41 μM, which is in accordance with a previous study by Wallenda & Read (1999), who found K_m for L-Gln to be 19–130 μM in mycorrhizal roots of different forest tree species. The affinity for L-Lys also was within that range, at 26 μM (Table 1). The *LHT1* overexpressor had a threefold higher maximum uptake rate as compared to wild type for L-Gln, while the half saturation constant was only marginally affected (Table 1). This illustrates how alterations in expression of genes encoding amino acid transporters may directly affect amino acid uptake rates but not uptake affinities.

Amino acid transporters are members of a vast gene family with overlapping expression patterns and substrate specificities. In Arabidopsis, at least 63 genes have been annotated as involved in amino acid transport (Wipf *et al.*, 2002; Rentsch *et al.*, 2007). Therefore, it is reasonable to assume that some degree of redundancy exists between amino acid transporters, and that the repression of one amino acid transporter may regulate other members of the gene family to compensate for the expression changes of the targeted gene product. In the present study, no major changes in gene expression other than in the genes targeted could be detected in the mutants (Fig. 5), which suggests that no redundancy exists amongst these genes. However, several amino acid transporters are expressed in Arabidopsis roots (Liu & Bush, 2006; Rentsch *et al.*, 2007) and the exact function of each transporter has not yet been established. Thus, amino acid transporter genes or genes involved in N metabolism, but not analysed in this study, may be regulated in response to the mutations. Similarly, post-transcriptional regulation that can affect root amino acid uptake may occur in these plants.

Concluding remarks

Our results clearly show that Arabidopsis, similarly to barley (*Hordeum vulgare*; Jämtgård *et al.*, 2008), has the capacity to take up amino acids at concentrations as low as 2 μM and throughout the entire concentration range tested. We can also conclude that the recorded amino acid uptake, whether it displayed saturating or linear characteristics, was carrier mediated, as the uptake of all amino acids tested was greatly reduced at all concentrations tested in Arabidopsis lines carrying a mutation in either the *LHT1* or *AAP5* gene. Our results support the suggestion by Svennerstam *et al.* (2008) that any overlap in the affinity spectra of LHT1 and AAP5 is limited. Our findings and previous *in planta* characterizations of LHT1 and AAP5 (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007, 2008; Forsum *et al.*, 2008) suggest these two transporters to be the most important for amino acid uptake in Arabidopsis, as little residual uptake was recorded in the double mutant. Together with the suggestion that L-Glu and L-His predominantly are taken up in their neutral form (Fischer *et al.*, 2002) and the experimentally demonstrated LHT1 affinity for L-His, L-Glu and L-Asp (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007), these findings indicate that it is possible that LHT1 and AAP5 represent the two transport systems that Kinraide (1981) postulated to exist.

Acknowledgements

We would like to thank Ann Sehlstedt and Margareta Zetherström for skilful work in the laboratory and Jun Yu and Kristi Kuljus for valuable advice regarding the statistical analysis. This study was supported by grants from the Kempe Foundation (T.N. and U.G.), the Swedish Research Council FORMAS (K.H.D. and T.N.), the Foundation for Strategic Research (T.N.), the Foundation for Strategic Environmental Research (T.N.), and the Carl Trygger Foundation for Scientific Research (U.G.).

References

An YQ, McDowell JM, Huang SR, McKinney EC, Chambliss S, Meagher RB. 1996. Strong, constitutive expression of the *Arabidopsis ACT2/ACT8* actin subclass in vegetative tissues. *Plant Journal* **10**: 107–121.

Boorer KJ, Fischer W-N. 1997. Specificity and stoichiometry of the *Arabidopsis* H^+/amino acid transporter AAP5. *Journal of Biological Chemistry* **272**: 13040–13046.

Boorer KJ, Frommer WB, Bush DR, Kreman M, Loo DDF, Wright EM. 1996. Kinetics and specificity of a H^+/amino acid transporter from *Arabidopsis haliana*. *Journal of Biological Chemistry* **271**: 2213–2220.

Borstlap AC, Meenks JLD, Vaneck WF, Bicker JTE. 1986. Kinetics and specificity of amino acid uptake by the duckweed *Spirodela polyrhiza* (L.) Schleiden. *Journal of Experimental Botany* **37**: 1020–1035.

Brigham RO. 1917. Assimilation of organic nitrogen by *Zea mays* and the influence of *Bacillus subtilis* on such assimilation. *Soil Science* **3**: 155–195.

Chen L, Bush DR. 1997. LHT1, a lysine- and histidine-specific amino acid transporter in *Arabidopsis*. *Plant Physiology* **115**: 1127–1134.

Datko AH, Mudd SH. 1985. Uptake of amino acids and other organic compounds by *Lemna paucicostata* Hegelm 6746. *Plant Physiology* **77**: 770–778.

Epstein E. 1961. The essential role of calcium in selective cation transport by plant cells. *Plant Physiology* **36**: 437–444.

Fischer WN, Kwart M, Hummel S, Frommer WB. 1995. Substrate specificity and expression profile of amino acid transporters (AAPs) in *Arabidopsis*. *Journal of Biological Chemistry* **270**: 16315–16320.

Fischer W-N, Loo DDF, Koch W, Ludewig U, Boorer KJ, Tegeder M, Rentsch D, Wright EM, Frommer WB. 2002. Low and high affinity amino acid H^+-cotransporters for cellular import of neutral and charged amino acids. *Plant Journal* **29**: 717–731.

Forsum O, Svennerstam H, Ganeteg U, Näsholm T. 2008. Capacities and constraints of amino acid utilization in *Arabidopsis*. *New Phytologist* **179**: 1058–1069.

Henry HAL, Jefferies RL. 2002. Free amino acid, ammonium and nitrate concentrations in soil solutions of a grazed coastal marsh in relation to plant growth. *Plant, Cell & Environment* **25**: 665–675.

Hirner A, Ladwig F, Stransky H, Okumoto S, Keinath M, Harms A, Frommer WB, 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.

Hirner B, Fischer W-N, Rentsch D, Kwart M, Frommer WB. 1998. Developmental control of H^+/amino acid permease gene expression during seed development of *Arabidopsis*. *Plant Journal* **14**: 535–544.

Hsu LC, Chiou TJ, Chen LS, Bush DR. 1993. Cloning a plant amino-acid transporter by functional complementation of a yeast amino acid transport mutant. *Proceedings of the National Academy of Sciences, USA* **90**: 7441–7445.

Hutchinson H, Miller N. 1911. The direct assimilation of inorganic and organic forms of nitrogen by higher plants. *Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankenheit. Zweite Abteilung* **30**: 513–547.

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

Jämtgård S, Näsholm T, Huss-Danell K. 2010. Nitrogen compounds in soil solution of agricultural land. *Soil Biology & Biochemistry* **42**: 2325–2330.

Jones DL, Owen AG, Farrar JF. 2002. Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. *Soil Biology & Biochemistry* **34**: 1893–1902.

Jones DL, Shannon D, Junvee-Fortune T, Farrarc JF. 2005. Plant capture of free amino acids is maximized under high soil amino acid concentrations. *Soil Biology & Biochemistry* **37**: 179–181.

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

Kinraide TB. 1981. Interamino acid inhibition of transport in higher plants – evidence for 2 transport channels with ascertainable affinities for amino acids. *Plant Physiology* **68**: 1327–1333.

Lee YH, Foster J, Chen J, Voll LM, Weber APM, Tegeder M. 2007. AAP1 transports uncharged amino acids into roots of *Arabidopsis*. *Plant Journal* **50**: 305–319.

Lipson D, Näsholm T. 2001. The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. *Oecologia* **128**: 305–316.

Liu X, Bush DR. 2006. Expression and transcriptional regulation of amino acid transporters in plants. *Amino Acids* **30**: 113–120.

Murashige T, Skoog FA. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**: 310–313.

Näsholm T, Kielland K, Ganeteg U. 2009. Uptake of organic nitrogen by plants. *New Phytologist* **182**: 31–48.

Näsholm T, Persson J. 2001. Plant acquisition of organic nitrogen in boreal forests. *Physiologia Plantarum* **111**: 419–426.

Öhlund J. 2004. *Organic and inorganic nitrogen sources for conifer seedlings*. Doctoral dissertation, Department of Forest Genetics and Plant Physiology, SLU, Umeå, Sweden. *Acta Universitatis Agriculturae Sueciae. Silvestra vol. 312*.

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

Raab TK, Lipson DA, Monson RK. 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 TK, Lipson DA, Monson RK. 1999. Soil amino acid utilization among species of the *Cyperaceae*: plant and soil processes. *Ecology* **80**: 2408–2419.

Rentsch D, Schmidt S, Tegeder M. 2007. Transporters for uptake and allocation of organic nitrogen compounds in plants. *FEBS Letters* **581**: 2281–2289.

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B. 2003. An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Molecular Biology* **53**: 247–259.

Schimel JP, Bennett J. 2004. Nitrogen mineralization: challenges of a changing paradigm. *Ecology* **85**: 591–602.

Schobert C, Komor E. 1987. Amino acid uptake by *Ricinus communis* roots – characterization and physiological significance. *Plant, Cell & Environment* **10**: 493–500.

Soldal T, Nissen P. 1978. Multiphasic uptake of amino acids by barley roots. *Physiologia Plantarum* **43**: 181–188.

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.

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* **180**: 620–630.

Wallenda T, Read DJ. 1999. Kinetics of amino acid uptake by ectomycorrhizal roots. *Plant, Cell and Environment* **22**: 179–187.

Wipf D, Ludewig U, Tegeder M, Rentsch D, Koch W, Frommer WB. 2002. Conservation of amino acid transporters in fungi, plants and animals. *TIBS* **27**: 139–147.

Yu Z, Zhang Q, Kraus TEC, Dahlgren RA, Anastasio C, Zasoski RJ. 2002. Contribution of amino compounds to dissolved organic nitrogen in forest soils. *Biogeochemistry* **61**: 173–198.