



Root uptake of cationic amino acids by *Arabidopsis* depends on functional expression of amino acid permease 5

Henrik Svennerstam¹, Ulrika Ganeteg¹ and Torgny Näsholm^{1,2}

¹Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre, Swedish University of Agricultural Sciences, SE-901 83 Umeå; ²Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, SE-901 83 Umeå

Summary

Author for correspondence:

Torgny Näsholm

Tel: +46 90 786 6302

Fax: +46 90 786 5901

Email: torgny.nasholm@genfys.slu.se

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• Specific transporters mediate uptake of amino acids by plant roots. Earlier studies have indicated that the lysine histidine transporter 1 and amino acid permease 1 participate in this process, but although plant roots have been shown to absorb cationic amino acids with high affinity, neither of these transporters seems to mediate transport of L-arginine (L-Arg) or L-lysine (L-Lys).

• Here, a collection of T-DNA knockout mutants were screened for alterations in *Arabidopsis* root uptake rates of L-Arg and it was found that only the *AAP5* mutant displayed clear phenotypic divergence on high concentrations of L-Arg. A second screen using low concentrations of ¹⁵N-labelled L-Arg in the growth media also identified *AAP5* as being involved in L-Arg acquisition.

• Momentaneous root uptake of basic amino acids was strongly affected in *AAP5* mutant lines, but their uptake of other types of amino acids was only marginally affected. Comparisons of the root uptake characteristics of *AAP5* and *LHT1* mutants corroborated the hypothesis that the two transporters have distinct affinity spectra *in planta*.

• Root uptake of all tested amino acids, except L-aspartic acid (L-Asp), was significantly affected in double *AAP5***LHT1* mutants, suggesting that these two transporters account for a major proportion of roots' uptake of amino acids at low concentrations.

Key words: amino acid, amino acid permease 5, double mutant, lysine histidine transporter 1, nitrogen uptake.

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Introduction

Plants have been shown to utilize amino acids as sources of nitrogen (N) in various ecosystems (Lipson & Näsholm, 2001; Schimel & Bennett, 2004). For nonmycorrhizal plants, specific amino acid transporters, some of which have not yet been identified, mediate root amino acid acquisition (Williams & Miller, 2001; Rentsch *et al.*, 2007). A large number of proteins may facilitate amino acid transport in plants; in the *Arabidopsis* (*Arabidopsis thaliana*) genome at least 53 genes have been annotated as amino acid transporters or putative amino acid transporters (Wipf *et al.*, 2002;

Lalonde *et al.*, 2004). Amino acid transporters play essential roles in plants, notably in xylem and phloem loading, transport of amino acids from one plant organ or compartment to another, and uptake of amino acids from the external environment. The necessity of amino acid transport is mirrored not only by the great number of plant amino acid transporters but also by the wide variation in spatial and temporal expression patterns of single plant amino acid transporters, indicating that a specific transporter may have several different functions *in planta* (Liu & Bush, 2006). Furthermore, most amino acid transporters display broad affinity for amino acids when characterized in yeast and

oocyte expression systems, suggesting functional redundancy, at least at the level of amino acid specificity (Fischer *et al.*, 1998, 2002). Clearly, much remains to be discovered about amino acid transporters and associated processes. In order to improve our understanding of plant amino acid acquisition, it is essential to identify the transporters that are involved in amino acid uptake by roots.

Recent work has identified the lysine histidine transporter 1 (LHT1) (Chen & Bush, 1997; Hirner *et al.*, 2006; Svennerstam *et al.*, 2007) and the amino acid permease 1 (AAP1) (Lee *et al.*, 2007) as active participants in root amino acid uptake processes in Arabidopsis. The first characterization of LHT1, published by Chen & Bush (1997), suggested that in addition to having high affinity for the basic amino acids L-lysine (L-Lys) and L-histidine (L-His) (but not L-arginine (L-Arg)) it also mediated transport of various neutral amino acids. By contrast, Hirner *et al.* (2006) studied LHT1 *in planta* and in yeast expression systems, and concluded that this transporter has low affinity for basic amino acids, but it has broad affinity for, and efficiently mediates the transport of, most neutral amino acids. Further, using promoter-GUS and northern blot, Hirner *et al.* (2006) obtained evidence that LHT1 is expressed in several tissues, as well as indications that it may play roles in both apoplastic transport of amino acids in leaves and their uptake by roots. The cited study also indicated that RNAi lines with decreased expression of LHT1 display reduced root uptake rates for a number of amino acids, including L-glutamine (L-Gln), L-glutamic acid (L-Glu) and L-asparagine (L-Asn). Svennerstam *et al.* (2007), using both reverse and forward genetic approaches, identified LHT1 as crucial for the root uptake of several amino acids and, in accordance with Hirner *et al.* (2006), found it to be less significant for the uptake of L-Lys. Thus, taken together, these studies suggest that lack of LHT1 expression reduces root uptake rates of several amino acids, including L-Gln, L-serine (L-Ser), L-alanine (L-Ala), glycine (Gly), L-aspartic acid (L-Asp), L-Glu and L-His, but not either L-Lys or L-Arg.

In planta studies of AAP1 (Lee *et al.*, 2007) have suggested it to be a low-affinity amino acid transporter, mediating the transport of several neutral amino acids, L-Glu and L-His, while its affinities for L-Lys and L-Arg are low. When studied in heterologous expression systems, AAP1 displayed highest affinity for L-Ala, with a $K_{0.5}$ of 0.29 mM in yeast and a $K_{0.5}$ of 0.6 mM in oocytes (cf. Lee *et al.*, 2007). These findings suggest that AAP1 may preferably mediate root uptake of amino acids in the low-affinity range.

The weak affinities of both AAP1 and LHT1 for cationic amino acids such as L-Lys and L-Arg, the limited effects of mutations in either *AAP1* or *LHT1* on rates of root uptake of these amino acids and the rapid rates at which these amino acids are absorbed by plant roots all suggest the existence of additional root amino acid transporters in Arabidopsis. Our aim in the present study was therefore to identify transporter(s) that are important for root uptake of cationic amino

acids. In earlier attempts to isolate mutants in which the uptake of basic amino acids is affected, the toxic L-Lys analogue S-2-aminoethyl-L-cysteine was used (Bright *et al.*, 1983; Kumpaisal *et al.*, 1989; Heremans *et al.*, 1997). The mutant lines isolated with this screening technique have been thoroughly characterized and shown to be severely impaired with respect to root uptake rates of L-Lys, but the mutated genes have not been identified. It has also been suggested that uptake of basic amino acids may be mediated by several different transporters, some with high affinity and others with low affinity (Heremans *et al.*, 1997).

We used two strategies to screen a number of T-DNA lines that were deemed likely to include relevant mutants based on information regarding levels of root expression, affinity for basic amino acids and/or sequence similarity to LHT1. The first approach was to grow all the selected mutant lines on media containing growth-inhibiting concentrations of L-Arg. In this case, our hypothesis was that any mutant with reduced capacity to absorb L-Arg through its roots would be less affected than other mutant lines and wild-type plants. The second strategy was to grow the various lines on a medium containing a low concentration of ^{15}N -labelled L-Arg and to screen for mutants displaying significant reductions in ^{15}N contents. In the first strategy, a relatively high concentration of L-Arg was used, enabling the identification of a low-affinity transporter, while in the second strategy a relatively low amino acid concentration was applied to enable identification of a high-affinity transporter. Both screening strategies, however, resulted in the identification of the *AAP5* as being important for L-Arg uptake. Following this identification, we studied *AAP5* mutants, *LHT1* mutants and double mutants of *AAP5* and *LHT1* to evaluate how loss of either or both of these transporters affects short- and long-term root amino acid uptake.

Materials and methods

Plant material and mutant screening

Arabidopsis (*Arabidopsis thaliana* (L.) Heynh. genes encoding or putatively encoding proteins annotated as cationic amino acid transporters, having high sequence similarity to *LHT1*, or relatively high expression in roots (www.geneinvestigator.ethz.ch; Zimmermann *et al.*, 2004), were targeted in this study. Twenty-three T-DNA lines from the SALK institute (Scholl *et al.*, 2000; Nottingham Arabidopsis Stock Centre, Nottingham, UK) or Gabi-Kat (Rosso *et al.*, 2003) with insertions in 18 individual genes were obtained (Supporting Information, Table S1). All mutant lines were analysed by PCR, using gene-specific primers (not shown) and a T-DNA-specific primer (SALK, 5'-GAGAAACGCCGGAGATGGAAT; Gabi-Kat, 5'-CCCATTTGGACGTGAATGTAGACAC), to confirm that the T-DNA insertions in the targeted genes were homozygous (data not shown).

Seeds were sown on agar media containing either 1 mM L-Arg or 30 μM ^{15}N L-Arg. Thus, the screening utilized retarding and nonretarding concentrations of L-Arg (Forsum *et al.*, 2008), enabling the identification of transporters with activities in both the low- and high-affinity concentration ranges. Mutant screens were performed on sterile agar plates containing N-free, half-strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), with 0.65% w/v agar (plant agar, Duchefa Biochemie, Haarlem, the Netherlands), 0.5% w/v sucrose, and N supplied as either 3 mM nitrate and 1 mM L-Arg, or 3 mM nitrate and 30 μM ^{15}N ($\geq 96\%$ ^{15}N) L-Arg. The media was buffered to pH 5.8 with 3.6 mM MES (2N-morpholinoethanesulfonic acid). For the screen on nitrate and 1 mM L-Arg, plants were grown for 17 d, harvested, dried and weighed ($n = 4$). Plants grown on nitrate and 30 μM ^{15}N L-Arg were grown for 19 d, after which five plants per genotype were harvested and their shoots and roots separated. Roots were rinsed and cleaned thoroughly three times in 0.5 mM CaCl_2 to remove ^{15}N from their surfaces. The harvested shoots and roots were dried at 60°C overnight, weighed and homogenized (all five shoots or roots together). Finally, samples were analysed using a Europa Scientific isotope ratio mass spectrometer to determine total N and ^{15}N contents. In the screening using ^{15}N -labelled L-Arg, samples from wild-type plants, *AAP5* and *PROT2* mutants were replicated ($n = 4$), but only one sample was analysed from each of the other lines. Both screens resulted in the isolation of an *AAP5* (At1g44100) mutant (SALK_041999, *aap5-1*), following which an additional *AAP5* T-DNA mutant was obtained (SALK_099586, *aap5-2*). In addition, we constructed a double mutant of *LHT1* and *AAP5* by crossing *lht1-5* (Svennerstam *et al.*, 2007) with *aap5-1* (*lht1*aap5*). Individual seeds from the cross were selfed and T2 seeds were screened on 3 mM D-Ala (Svennerstam *et al.*, 2007) to isolate plants containing the *LHT1* mutation. D-Ala resistant plants were subsequently screened for the *AAP5* mutation using PCR (see later). Wild-type Arabidopsis (ecotype Columbia-0) was used as a control in all experiments described.

Analysis of T-DNA knockout lines

The presence of a T-DNA insert in *aap5-1*, *aap5-2* and *lht1*aap5* plants was verified by PCR using specific primers for each gene together with a T-DNA left border primer (*aap5-1*, 5'-TTGTTGTCATCACGAAGAACG; *aap5-2*, 5'-AACAAATGCCAATAACAGATCCC; *lht1-5*, 5'-ATTTCA-GACCAACCACAACCTCTTCG; T-DNA, 5'-GAGAAACG-CCGGAGATGGAAT). The PCR products from *aap5-1* and *aap5-2* were sequenced to confirm that *aap5-1* and *aap5-2* originated from unique transformation events. The homozygosity of the plants was verified using the following gene-specific primers for each side of the T-DNA insertion site: Fw, 5'-AAAGATTCGAAAGAACGGCTC and Rv, 5'-TTGTTGTCATCACGAAGAACG for *aap5-1*; Fw, 5'-TTGGGACAGTGACTGAGTG and Rv, 5'-AACAA-

TGCCAATAACAGATCCC for *aap5-2*; Fw, 5'-ATTTTCAG-ACCAACCACAACCTCTTCG and Rv, 5'-TGGCGATAGG-ACCATCAAGAAAAGA for *lht1-5*. Total RNA was prepared from leaves using an EZNA plant RNA kit (Omega Bio-Tek, Norcross, GA, USA). First-strand cDNA synthesis was performed using a First-strand Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA) as recommended by the vendor. RT-PCR reactions to confirm repression of the targeted genes were performed using gene-specific primers spanning the T-DNA insert (Fw, 5'-TTGGGACAGTGACTGAGTG and Rv, 5'-AACAAATGCCAATAACAGATCCC for both *aap5-1* and *aap5-2*; Fw, 5'-AGTCATCGTTGCTTACATCGTCGT and Rv, 5'-TGGCGATAGGACCATCAAGAAAAGA for *lht1-5*). Primers amplifying the constitutively expressed Arabidopsis actin gene *ACT2* were used as controls (Fw, 5'-CCAATCGTGTGTGACAATGGTACCG and Rv, 5'-GGTTGTACGACCACTGGCGTACAAG; An *et al.*, 1996).

Plant L-Arg concentration

Wild-type and *aap5-1* plants were grown under sterile conditions as described for the selection on media containing 1 mM L-Arg and 3 mM nitrate. After 17 d of growth, three replicates, each consisting of three plants, of each plant type were harvested for amino acid analysis. Plants were cleaned of agar, homogenized, extracted in 0.01 M HCl and their L-Arg concentrations were measured using the UPLC-AccQTag method (UPLC Amino Acid Analysis System Solution, application note 720001683EN; www.waters.com).

Amino acid uptake assay

Wild-type, *aap5-1*, *aap5-2*, *lht1-5* and *lht1*aap5* plants were grown under sterile conditions in a climate chamber with an 8 : 16 h light : dark (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and 23 : 18°C (day : night) regime, on agar containing 5 g l^{-1} sucrose, 7 g l^{-1} plant agar (Duchefa Biochemie), N-free half-strength MS and 3 mM nitrate. The agar solution was buffered to pH 5.8 using MES. Plants were grown on vertical plates to avoid roots penetrating the agar surface, facilitating harvesting and cleaning of the roots. After 18 d (19 d for the CCCP-treated plants, see later), 15 plants, divided into five biological replicates of each line, were selected for the uptake experiment. The roots of the three intact plants in each replicate were rinsed in 0.5 mM CaCl_2 , blotted with paper tissue, placed in 2 ml of uptake solution and incubated in the climate chamber on a shaking table. The uptake solution contained 0.5 mM CaCl_2 and the following compounds, each at a concentration of 10 μM and with pH adjusted to 5.8: L-Gln, L-Glu, L-Asn, L-Asp, Gly, L-Ala, L-Ser, L-Arg, L-Val, L-Lys, L-His and L-Pro. Samples of the uptake solution were taken after 1, 2 and 4 h, the concentrations of the amino acids in these samples were measured using the UPLC-AccQTag method, and the data acquired from the 4 h samples were used to calculate the roots' amino acid uptake

rates ($\mu\text{mol g}^{-1}$ DW root h^{-1}) from the decline in concentration of each amino acid in the solution. After the uptake period, the roots were dried and weighed. The passive amino acid uptake of wild-type plants was measured in a separate experiment by submerging root samples in 100 μM CCCP (carbonyl cyanide 3-chlorophenylhydrazone) for 1 h before the uptake period, in an otherwise identical uptake experiment.

^{15}N amino acid uptake experiment

Wild-type, *aap5-1*, *aap5-2*, *lht1-5* and *lht1*aap5* plants were grown on plates containing 0.5% sucrose, 0.65% agar, half-strength N-free MS buffered to pH 5.8 with MES and N supplied as 3 mM NO_3^- and 0.03 mM of L- ^{15}N -Gln, L- ^{15}N -Glu, L- ^{15}N -Ala, L- ^{15}N -Arg or L- ^{15}N -Lys (all > 96% ^{15}N). Amino acid solutions were sterile-filtered and added separately to the autoclaved solutions. Eight replicate plates, each with three seeds, were prepared for each genotype and treatment. The agar plates were incubated in a cold room for 1 d and then transferred to a climate chamber with a 16 : 8 h light : dark (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and 23 : 18°C day : night regime. After 20 d, plants from five plates per treatment and genotype were harvested. Roots were rinsed and cleaned thoroughly three times in 0.5 mM CaCl_2 to remove ^{15}N -labelled amino acid from their surfaces, then the plants were dried at 60°C overnight, weighed and homogenized. Finally, samples were analysed for total N and ^{15}N contents (CPSIL analytical services, USA, www.npcer.nau.edu/isotopelab).

Growth on soil

To determine growth rates of the plants in soil, wild-type, *aap5-1*, *aap5-2*, *lht1-5* and *lht1*aap5* seeds were sown on soil : perlite (3 : 1) and grown in a climate chamber under a 16 : 8 h, 23 : 18°C day : night regime with 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the light periods. After 23 and 34 d, the above-ground biomass of six replicate plants was harvested, dried overnight at 60°C and weighed. The final biomass was recorded after plants had completely senesced and as described.

Growth on L-Gln

Wild-type, *aap5-1*, *aap5-2*, *lht1-5* and *lht1*aap5* plants were grown on plates containing 0.5% sucrose, 0.65% agar and N-free half-strength MS agar media amended with either 0.5 mM L-Gln or 3 mM nitrate. Since L-Gln may be degraded during autoclaving, a L-Gln solution was sterile-filtered and added to the agar mix after autoclaving. Seven replicate plates, each with six seeds, were prepared for each treatment and genotype. After sowing, plates were incubated in a cold room for 2 d and then transferred to a climate chamber under a 16 : 8 h, 23 : 18°C day : night regime with 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during the light periods. After 21 d, plants were harvested, dried overnight at 60°C and weighed.

Results

Earlier studies of Arabidopsis growth responses to various amino acids in the root medium have shown that L-Arg, at a concentration of 0.75 mM, supplied in combination with 3 mM nitrate, strongly hampers biomass production (Forsum *et al.*, 2008). We therefore envisaged that screening on high concentrations of L-Arg could be a suitable approach to search for mutants lacking amino acid uptake mechanisms, especially the uptake of cationic amino acids in the low-affinity range. Therefore, using medium containing 1 mM L-Arg and 3 mM nitrate we screened 23 selected T-DNA lines for growth alterations compared with wild-type plants. Out of the 23 T-DNA lines tested, only one (with an insertion in the gene encoding AAP5) displayed a clearly diverging phenotype compared with wild-type plants (Fig. 1). *AAP5* mutant (*aap5-1*) plants were substantially larger ($44 \pm 8.2\%$, average \pm SE, more biomass) than wild-type plants after growth on 1 mM L-Arg + 3 mM nitrate for 19 d. Furthermore, analysis of the L-Arg content of plants exposed to this treatment revealed high concentrations of L-Arg in wild-type plants ($7.8 \mu\text{mol g}^{-1}$ FW), while the L-Arg content of *aap5-1* plants was only $0.4 \mu\text{mol g}^{-1}$ FW, again suggesting that uptake of L-Arg was reduced by this mutation.

In the second screen, we exposed the selected T-DNA lines to a combination of 3 mM nitrate and 0.03 mM ^{15}N L-Arg (U- ^{15}N L-Arg). Our rationale was that it would be possible to assess variations between the wild-type and mutant lines' capacity to take up L-Arg in the high-affinity range at this relatively low concentration of L-Arg. The *AAP5* mutant line also showed by far the clearest phenotypic divergence in this screen, having only 22 ± 1.1 and $27 \pm 0.9\%$ (average \pm SE) of wild-type ^{15}N contents in its shoots and roots, respectively (Fig. 2a,b), after 19 d of growth.

Following the identification of AAP5 as crucial for L-Arg uptake, a second *AAP5* mutant (*aap5-2*) and a double *LHT1* and *AAP5* (*lht1*aap5*) mutant were obtained (see the Materials and Methods section). The locations of the two T-DNA insertions in the *AAP5* mutants were determined by sequencing (Fig. 3a). RT-PCR analysis of the *AAP5* mutants showed that transcripts spanning the T-DNA inserts were absent in both lines (Fig. 3b). The two *AAP5* mutant lines, a previously characterized *LHT1* mutant (*lht1-5*; Svennerstam *et al.*, 2007) and a double mutant were subjected to several experiments addressing the role of AAP5 in root uptake of amino acids in Arabidopsis and the relationship between the AAP5 and LHT1 transporters in root amino acid acquisition.

Amino acid uptake

Root uptake rates of 12 amino acids were assessed to obtain an overview of the effects of the *AAP5* mutation on plant amino acid uptake characteristics (Fig. 4). Uptake of two cationic amino acids was severely affected by loss of AAP5, since it reduced the uptake of L-Arg and L-Lys by 87 ± 4.1

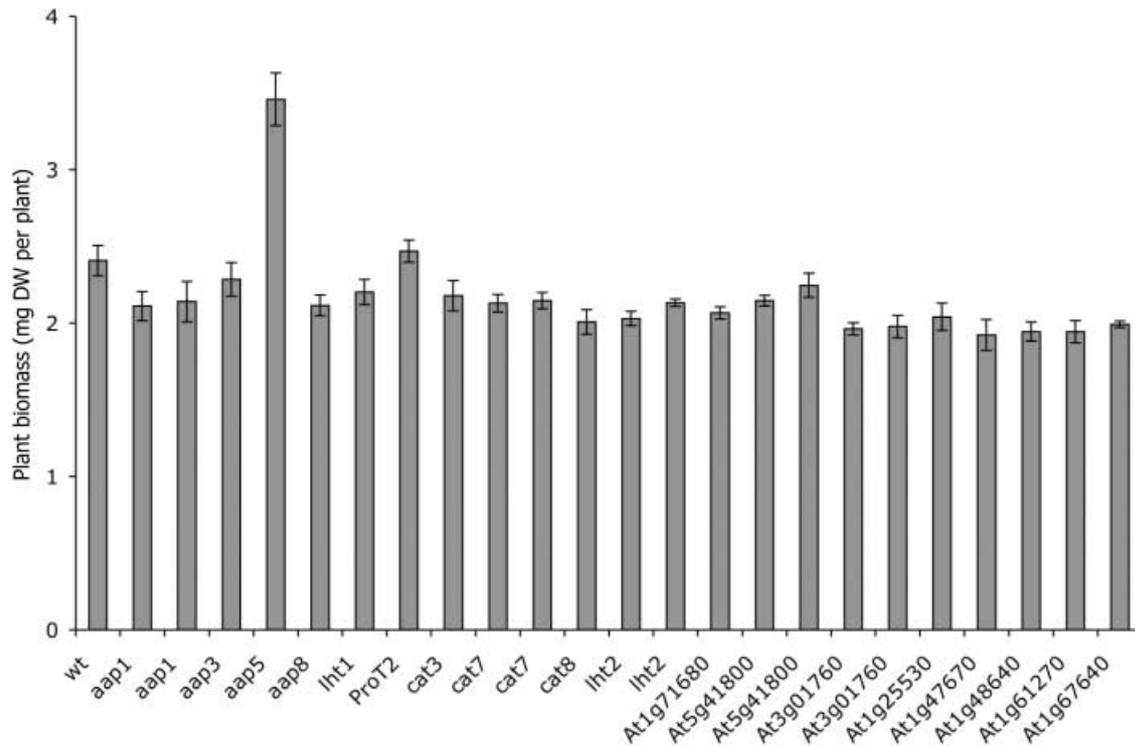


Fig. 1 Results of screening experiment on high concentrations of L-arginine (L-Arg). Plant biomass of *Arabidopsis* wild-type (wt) and 23 SALK T-DNA insertion lines, grown on 3 mM nitrate and 1 mM L-Arg for 17 d. Bars represent mean values \pm SE, $n = 4$.

and $90 \pm 5.6\%$ (average \pm SE), respectively, compared with wild-type (numbers in this paragraph refer to average values for the two replicate lines). However, uptake of L-His, the third cationic amino acid, was not significantly affected. Regarding the other tested amino acids, only minor effects of the *AAP5* mutation were found. By contrast, the *LHT1* mutant line showed reduced uptake of L-Asn and L-Pro (56 ± 6.6 and $78 \pm 3.0\%$, average \pm SE, respectively, lower than in wild-type plants), in addition to previously described reductions in capacity to take up L-His, L-Gln, L-Ser, Gly and L-Ala (Svennerstam *et al.*, 2007). The double mutant displayed reduced uptake of all neutral and basic amino acids tested compared with wild-type plants. In this line, total uptake of amino acids was reduced by $78 \pm 2.3\%$ (average \pm SE) compared with wild-type plants. Comparisons of the double mutant with the single mutant lines revealed no significant additive effects of the two mutations. Uptake rates of CCCP-pretreated plants were not significantly different from zero for all tested amino acids, except L-Ala, L-Lys and L-Gln, for which negative uptake (i.e. efflux) occurred (data not shown).

Uptake of ^{15}N amino acids

To evaluate the effects of lack of the *AAP5* transporter on long-term acquisition of amino acids from root media, an experiment was designed in which plants were grown on

media containing 3 mM nitrate and 0.03 mM of ^{15}N -labelled amino acids. Rates of amino acid acquisition, expressed as μmol of absorbed amino acid per g dry weight plant, differed significantly both between individual amino acids and between plant types (Fig. 5). For wild-type plants, the amino acids acquired in the largest quantities were L-Ala ($64 \mu\text{mol g}^{-1}$ DW), followed by L-Lys, L-Gln and L-Arg (39 , 37 and $36 \mu\text{mol g}^{-1}$ DW, respectively), then L-Glu at the somewhat lower concentration of $20 \mu\text{mol g}^{-1}$ DW. Comparisons between different plant types revealed similar patterns to those found in the amino acid uptake assay. Compared with rates found in wild-type plants, uptake rates of L-Arg and L-Lys were 86 ± 0.7 and $82 \pm 1.0\%$ (average \pm SE) lower, respectively, on average in the two *AAP5* mutant lines; for L-Ala, the rates of acquisition were slightly ($19 \pm 3.5\%$, average \pm SE) lower in *aap5-2* plants but not significantly different in *aap5-1* plants, and their rates of L-Gln and L-Glu acquisition were not significantly different. For the *LHT1* mutant line, significant reductions in the acquisition of (average \pm SE) L-Gln ($73 \pm 1.5\%$), L-Ala ($71 \pm 1.4\%$) and L-Glu ($83 \pm 1.0\%$) were recorded. For this line, small but significant reductions in L-Arg and L-Lys acquisition (21 ± 4.0 and $27 \pm 4.5\%$, respectively) were also observed, although no corresponding reductions were seen in the amino acid uptake assay (Fig. 4). The *lht1-5*aap5-1* double-mutant line showed the greatest reductions in uptake of all tested amino acids.

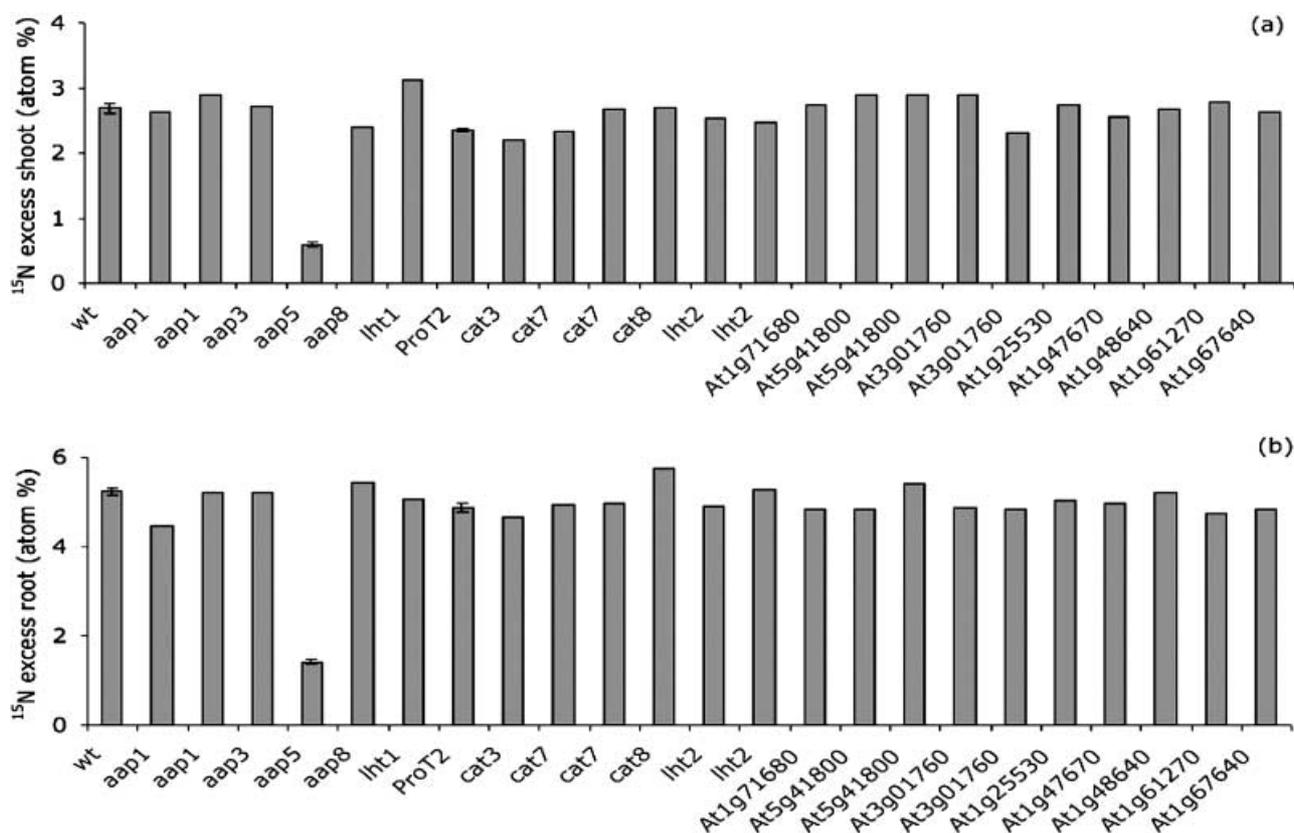


Fig. 2 Results of screening on low concentrations of L-arginine (L-Arg). ^{15}N excess in Arabidopsis wild-type (wt) and 23 SALK T-DNA insertion lines, grown on $30\ \mu\text{M}$ ^{15}N -labelled L-Arg and $3\ \text{mM}$ nitrate. (a) Shoot atom % ^{15}N excess. (b) Root atom % ^{15}N excess. Bars without SE represent values obtained for single samples, each consisting of five pooled plants. Bars \pm SE are replicated ($n = 4$, each sample consisting of five pooled plants).

Growth experiments in axenic culture

Earlier studies (Svennerstam *et al.*, 2007) have revealed that L-Gln is the amino acid that promotes growth of Arabidopsis most efficiently, so we compared the growth of the mutants with wild-type plants on a medium in which L-Gln was the sole N source. The biomass of *aap5-1* plants did not differ significantly from that of wild-type plants when tested on $0.5\ \text{mM}$ L-Gln, whereas growth of *aap5-2* was slightly increased on this N source (Fig. 6). By contrast, growth of both *lht1-5* and the double mutant line was reduced by *c.* 47% compared with that of wild-type plants. In the presence of nitrate, *aap5-2* and *lht1*aap5*, but not *aap5-1*, plants grew more strongly than wild-type plants.

Growth on soil

Wild-type and mutant plants were grown in soil to investigate phenotypic effects of the mutations. After 23 d of growth, no significant differences between wild-type and mutant lines were found, but after 34 d *lht1-5* plants were significantly smaller than wild-type plants. At the final harvest, *aap5-1*

plants were significantly larger, and *lht1-5* and *lht1*aap5* plants significantly smaller, than wild-type plants (Fig. 7). No other apparent effects on growth and development (such as time of flowering) were recorded for the *AAP5* mutant lines.

Discussion

Cationic amino acids, such as L-Arg and L-Lys, are prominent compounds in the soil amino acid pool of various ecosystems (Keilland, 1995; Nordin *et al.*, 2001; Henry & Jefferies, 2002) and plant roots are known to be capable of effectively scavenging such compounds from the soil (Öhlund & Näsholm, 2001; Persson *et al.*, 2003). The results presented here suggest that the amino acid transporter AAP5 plays an important direct and/or indirect role in this process. Earlier studies of plant amino acid transporters have shown that they generally display broad affinities and mediate the transport of a range of amino acids of different classes. Comparisons of root uptake profiles of plants carrying mutations in the amino acid transporters LHT1 and AAP5 in the present study indicate that they are largely complementary, and that there is a limited overlap between the two transporters' root uptake activities (Fig. 4).

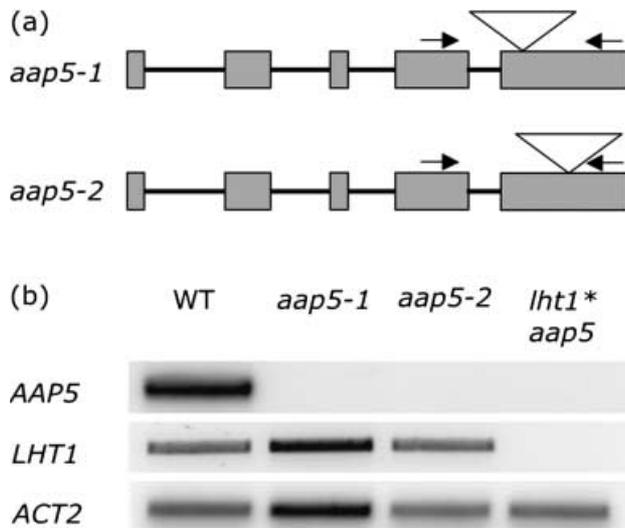


Fig. 3 Characterization of Arabidopsis mutant lines. (a) Location of the T-DNA insert (marked with triangles) in each *AAP5* mutant line, determined by sequencing. Positions of the primers used for RT-PCR in (b) are indicated by arrows. (b) Results of RT-PCR reactions using gene-specific primers spanning the targeted TDNA insertion sites in *aap5-1*, *aap5-2* and *lht1* aap5* mutants showing that the *aap5-1*, *aap5-2* and *lht1* aap5* plants lack full-length *AAP5* transcripts, and the *lht1* aap5* plants also lack full-length *LHT1* transcripts. Specific primers for the constitutively expressed Arabidopsis actin gene *ACT2* were used as controls. WT, wild type.

AAP5 is expressed throughout the plant; high transcript abundances have been found in mature leaves, stems, flowers and also roots (Fischer *et al.*, 1995, 2002). Brady *et al.* (2007) presented microarray expression profiles for nearly all cell types of the Arabidopsis root. At least 28 of the putative amino acid transporters showed differential expression between the different cell types. *AAP5* was found to be preferentially expressed in the root cortex, supporting a role of this transporter in amino acid uptake. *LHT1* and *AAP1*, both shown to mediate root amino acid uptake (Hirner *et al.*, 2006; Lee *et al.*, 2007; Svennerstam *et al.*, 2007), were expressed in epidermis, cortex and endodermis, corroborating functions in root amino acid uptake. However, *LHT1* and *AAP1*, as well as *AAP5*, were also found to be expressed in the phloem. Thus, although localized to the root cortex, it cannot be excluded that *AAP5*, as well as *LHT1* and *AAP1*, have additional functions within the Arabidopsis root, functions that may affect root uptake of amino acids. It is also conceivable that *AAP5* has functions in photosynthesizing or reproductive tissues, and therefore it is possible that the observed decreases in L-Arg and L-Lys uptake capacities are caused by changes in internal transport of amino acids. Comparisons between various AAPs (*AAP1*–*6*) with respect to their substrate profiles and kinetic characteristics have revealed that *AAP3* and *AAP5* are the only transporters displaying high affinities for basic amino acids (Fischer *et al.*, 2002). However, *AAP3* was shown

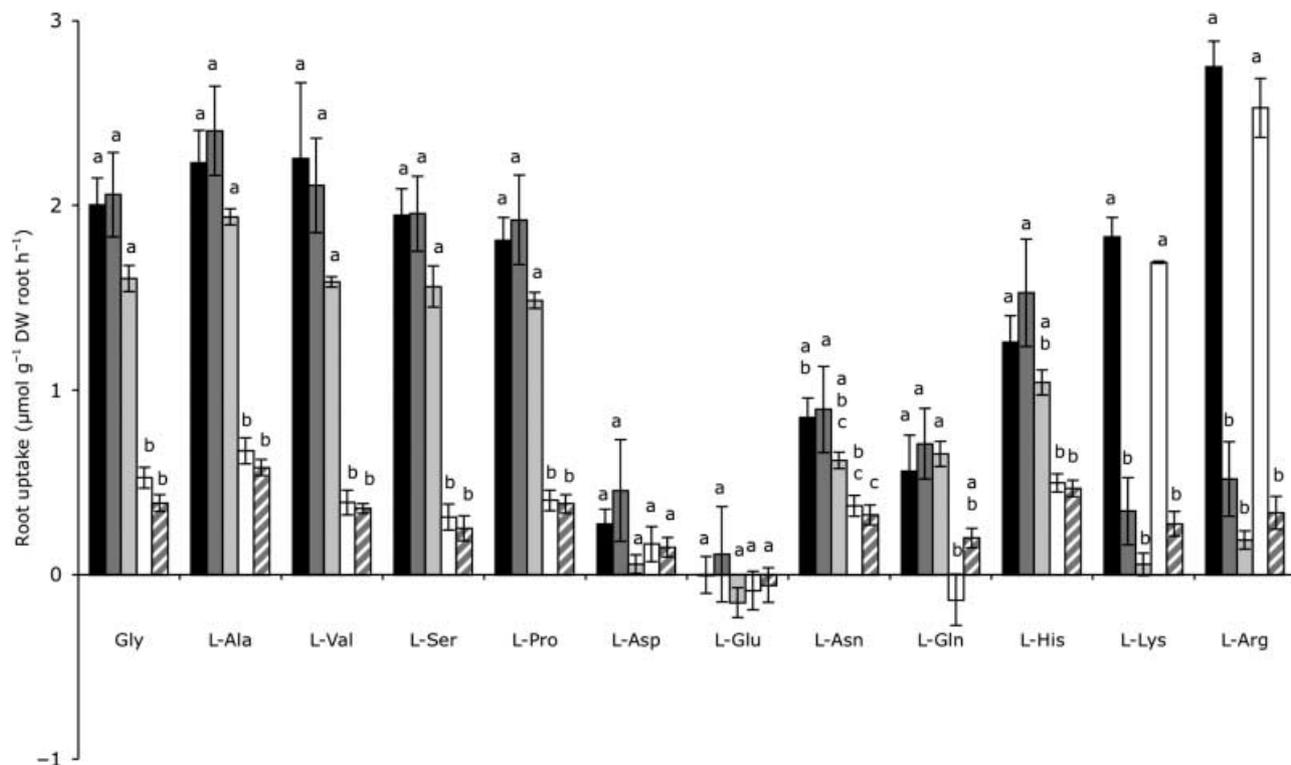


Fig. 4 Effects of amino acid transporter mutations on root amino acid uptake. Sterile-grown Arabidopsis wild-type (black bars), *aap5-1* (grey bars), *aap5-2* (light grey bars), *lht1-5* (white bars) and *lht1* aap5* (hatched bars) plants were incubated in solutions containing a mixture of 12 amino acids, each at a concentration of 10 μM , and their rates of depletion were measured. Bars represent mean values \pm SE, $n = 5$. Different letters above bars indicate significant differences between genotypes at $P < 0.05$.

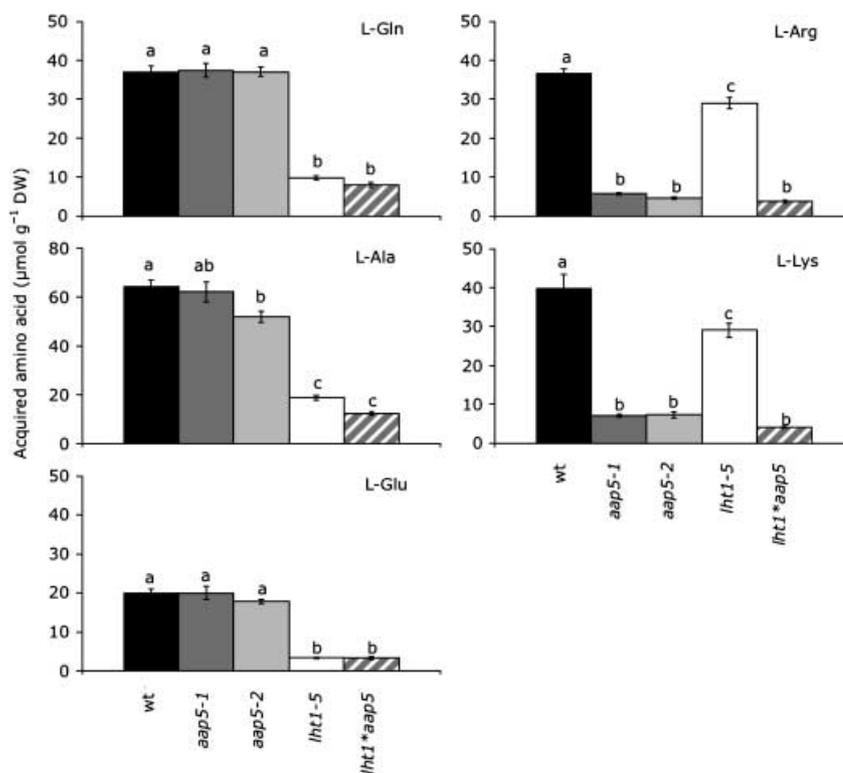


Fig. 5 Amounts of amino acids acquired during cultivation by Arabidopsis wild-type (black bars), *aap5-1* (grey bars), *aap5-2* (light grey bars), *lht1-5* (white bars) and *lht1*aap5* (hatched bars) plants grown for 20 d on N-free half-strength MS agar media in which all N sources were replaced with 3 mM nitrate and 30 µM of labelled L-glutamine (L-Gln), L-glutamine (L-Glu), L-alanine (L-Ala), L-lysine (L-Lys) or L-arginine (L-Arg). Bars represent mean values ± SE, *n* = 5. Different letters above bars indicate significant differences between genotypes at *P* < 0.05.

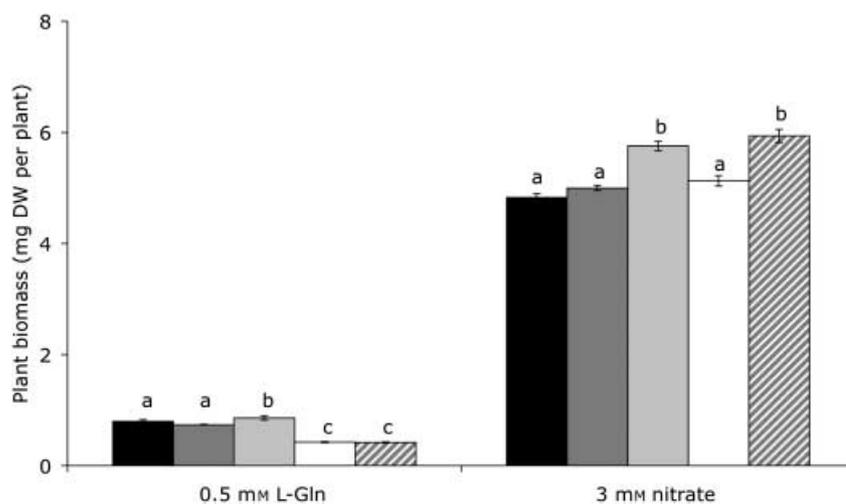


Fig. 6 Plant biomass of Arabidopsis wild-type (black bars), *aap5-1* (grey bars), *aap5-2* (light grey bars), *lht1-5* (white bars) and *lht1*aap5* (hatched bars) plants after 21 d on N-free half-strength MS agar media amended with either 0.5 mM L-glutamine (L-Gln) or 3 mM nitrate. Bars represent mean values ± SE, *n* ≥ 6. Different letters above bars indicate significant differences between genotypes at *P* < 0.05.

to be expressed in the stele, mediating phloem loading of amino acids (Okumoto *et al.*, 2004). In that study, no effects of loss of AAP3 on growth on amino acids were recorded. Accordingly, in our screens, the *AAP3* mutant line did not display an altered phenotype compared with wild-type plants (Figs 1, 2). These results show that loss of an amino acid transporter with a known function in phloem loading of amino acids did not affect root uptake of amino acids.

AAP5 has been characterized by both functional complementation of yeast mutants and in oocyte expression systems (Fischer *et al.*, 1995, 2002; Boorer & Fischer, 1997). When

characterized in yeast, AAP5 displayed broad affinity, transporting anionic, neutral and cationic amino acids (Fischer *et al.*, 1995). In oocytes, detailed kinetic studies revealed that AAP5 has high affinity not only for L-Arg and L-Lys, but also for a range of other amino acids (Boorer & Fischer, 1997). In our study, root uptake of L-Lys and L-Arg rates were *c.* 10-fold lower in *aap5-1* and *aap5-2* plants than in wild-type plants (Fig. 4), suggesting that it is the most important amino acid transporter for root uptake of these amino acids in the µM range. No significant differences between the *AAP5* mutant lines and wild-type plants with respect to uptake of any of the

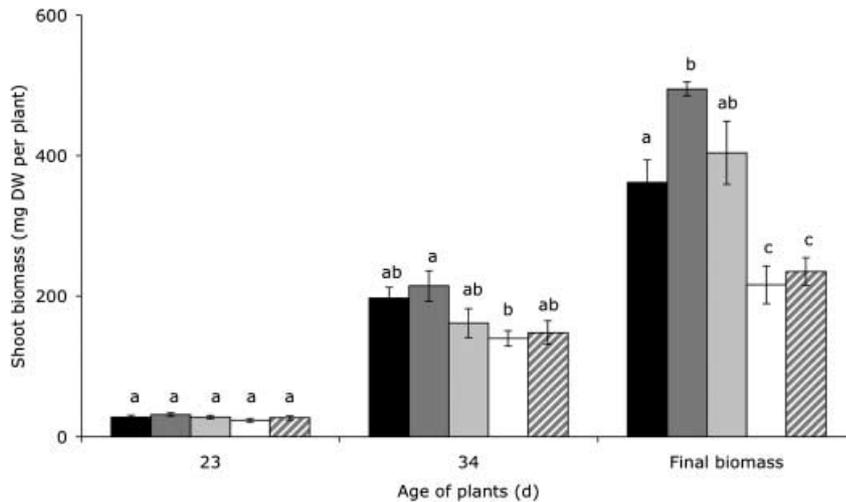


Fig. 7 Above-ground biomass of *Arabidopsis* wild-type (black bars), *aap5-1* (grey bars), *aap5-2* (light grey bars), *lht1-5* (white bars) and *lht1*aap5* (hatched bars) plants grown on soil. Bars represent mean values \pm SE, $n = 6$. Different letters above bars indicate significant differences between genotypes at $P < 0.05$.

other 10 amino acids tested, including the third basic amino acid L-His, were found. When expressed in oocytes, AAP5-mediated transport of L-His was shown to be in its neutral form; L-Lys, on the other hand, was taken up as cation (Boorer & Fischer, 1997). Thus, in the oocyte expression system, AAP5 mediates transport of both positively charged and uncharged amino acids. By contrast, in our short-term amino acid uptake assay, significant differences between wild-type and AAP5 mutants were detected only for cationic amino acids. However, the root uptake profiles of the various mutants should not be directly compared with profiles obtained in the cited yeast or oocyte studies because of at least two significant methodological differences between our study and the cited studies. Firstly, we have evaluated the effects of lack of expression of specific amino acid transporters on root uptake, while the yeast and oocyte studies were based on complementation of strains lacking specific transporters. Secondly, we used a complex mixture of amino acids as a test solution, simulating a potential root environment, and thus competitive and/or inhibitive interactions amongst the amino acids and transporters may have affected their uptake rates. By contrast, in the yeast/oocyte test studies, uptake of individual amino acids was tested and the profiles were generated in either inhibition experiments with other amino acids or through comparisons of uptake rates and/or membrane depolarization results (Boorer & Fischer, 1997).

Several earlier reports on mutants with impaired uptake of basic amino acids have been published (Bright *et al.*, 1983; Kumpaisal *et al.*, 1989; Heremans *et al.*, 1997). Heremans *et al.* (1997) studied two *Arabidopsis* mutant lines named *raec1* and *rlt11*, which displayed clear phenotypes with respect to uptake of L-Lys. They speculated that these mutants were allelic, and showed that the sites of both mutations were in chromosome 1. Their study suggested the occurrence of three uptake systems for basic amino acids: one with high affinity, one with low affinity and a third, nonsaturable system. Our results suggest that a single transporter, AAP5, is involved in the uptake of L-Arg and L-Lys at both high and low external

concentrations. Apart from this, our results are consistent with many of those presented by Heremans *et al.* (1997), suggesting that *raec1* and *rlt11* are, in fact, identical to the AAP5 mutants described in the current study. In our first test of *aap5-1* plants, we found they contained 95% less L-Arg than wild-type plants when grown on media containing 1 mM L-Arg, indicating that uptake of L-Arg is dependent on AAP5 to a significant degree even at this high L-Arg concentration, while results from the ^{15}N L-Arg screen (Fig. 2) imply that AAP5 is also important for uptake of this amino acid in the μM range. Furthermore, the results of the uptake assay (Fig. 4) and the long-term acquisition study (Fig. 5) indicate that AAP5 plays a crucial role in the uptake of basic amino acids in the μM range. Together, these findings indicate that AAP5 may be the most important transporter for root uptake of basic amino acids, in both the low- and high-affinity ranges.

To evaluate the effects of the mutations on root uptake profiles, we compared the profiles of plants representing the lines we identified in our screens, a previously characterized *LHT1* mutant line (Svennerstam *et al.*, 2007), a double mutant line (*lht1*aap5*) and wild-type counterparts. In yeast complementation analyses, Hirner *et al.* (2006) found that *LHT1* has broad specificity, with activity for neutral and acidic amino acids, but not for the basic amino acids L-Lys and L-Arg, while transport of L-His was recorded, although at slower rates than for the other tested amino acids. The results we obtained on amino acid uptake in the *LHT1* mutant are consistent with these findings. As described earlier, we did not detect any significant effects of this mutation on the root uptake of neutral and acidic amino acids in the AAP5 mutant lines (Fig. 4). It is possible, however, that such a lack of effect on root uptake rates could have resulted from increased expression of other amino acid transporters in the AAP5 mutant line (cf. Liu & Bush, 2006) that masked possible effects of the loss of AAP5 on the root uptake of neutral and acidic amino acids. However, uptake rates of all neutral and acidic amino acids were similar in the *lht1-5* and *lht1*aap5*

plants, while uptake rates of the basic amino acids were similar in *aap5-1*, *aap5-2* and *lht1*aap5* plants. Thus, the uptake profile of the double mutant reflected the summed effects seen in the profiles of the two individual mutant lines, and no significant synergistic effects were detected. This suggests that, at least for these two transporters, compensatory regulation did not occur and these two transporters do not display significant overlapping affinity spectra regarding root uptake.

Results from the long-term (^{15}N -labelling) amino acid acquisition experiment largely corroborated the findings of the short-term uptake study, with some notable exceptions. Firstly, in the long-term experiment, but not the short-term uptake assay, small but significant effects of the *LHT1* mutation on the acquisition of L-Lys and L-Arg were found (Fig. 5). In addition, in the ^{15}N study, a small but significant effect on acquisition of L-Ala in *aap5-2* was found. Thus, the results of the long-term tests of the mutant lines indicate that the activities of the two tested transporters overlap slightly more than the results of the short-term study suggest. In the long-term uptake study, L-Glu uptake was significantly affected in the *lht1-5* line, suggesting that this transporter also plays a role in the uptake of acidic amino acids, in accordance with findings presented by Hirner *et al.* (2006), but conflicting with results from both the short-term uptake study in this report and Svennerstam *et al.* (2007). However, it should be noted that solutions containing a range of amino acids were used in the short-term study, while media containing single amino acids were used in the long-term study, and this difference may have been responsible for some of the divergence in results.

L-Glutamine is the amino acid that promotes growth of Arabidopsis most efficiently (Svennerstam *et al.*, 2007; Forsum *et al.*, 2008), so we tested the growth of the mutant lines on this N source. As previously shown, *LHT1* mutants displayed retarded growth when N was administered as L-Gln (Svennerstam *et al.*, 2007). However, the growth of *AAP5* mutants was not affected (Fig. 6) and the growth of *lht1*aap5* plants did not significantly differ from that of the *LHT1* mutants, suggesting that there are no additive effects of the two mutations regarding growth on L-Gln as the sole N source. In contrast to *LHT1* mutants, *AAP5* mutants did not display a retarded growth phenotype when cultivated on soil (Fig. 7). For *LHT1* mutants, growth reductions were displayed at the stage of flower initiation (Svennerstam *et al.*, 2007), suggesting a critical role of this transporter during this developmental stage. The lack of phenotype of the *AAP5* mutant suggests either a functional redundancy in processes other than that of root absorption of cationic amino acids or that such effects were too small to be detected in the general growth assessment carried out in this experiment.

Some phenotypic differences between the two *AAP5* mutant lines were observed. For example, *aap5-2* displayed slightly higher plant biomass than wild-type and *aap5-1* when grown on 0.5 mM L-Gln or 3 mM nitrate (Fig. 6). By contrast, *aap5-1* had a higher shoot biomass than wild-type and *aap5-2* when grown on soil (Fig. 7). We can only speculate about the reasons

for this discrepancy, but it is possible that partial transcripts, and thus partial proteins, still remain in any of the mutant lines. Still, the differences between the two lines were minor compared with the clear differences observed between the *AAP5* mutant lines and wild-type or *LHT1* mutant lines, and therefore we conclude that even if residual activities of *AAP5* were present in the mutant lines, the main conclusions about the *AAP5* function in root acquisition of cationic amino acids is well founded.

Earlier studies have suggested that *AAP1* may be active in root amino acid uptake (Bick *et al.*, 1998; Lee *et al.*, 2007). It is also conceivable that several other transporters have functions in root amino acid uptake. For example, one of the proline transporters (ProT2) has been shown to be expressed in root epidermis (Grallath *et al.*, 2005) and may thus mediate root uptake of compounds such as L-Pro and GABA. In addition, various members of the cationic amino acid transporter (CAT) family have been found to be expressed in roots (Su *et al.*, 2004). Thus, it cannot be excluded that any of the CAT transporters have functions in root uptake of cationic amino acids. The strong effects of loss of *AAP5* on root uptake of L-Arg and L-Lys do, however, suggest that activity of this transporter is crucial for Arabidopsis acquisition of these compounds.

In a review of various studies of root amino acid uptake, Kinraide (1981) postulated that the observed profiles and interactive effects of tested amino acids could be explained by the presence of two major transport systems: a general system with the capacity to transport all of the major amino acids, and a specific system responsible for taking up cationic amino acids. In the present study, we have shown that *LHT1* and *AAP5* together account for a large fraction of root amino acid uptake capacity; the double mutant lines' overall uptake rate of amino acids from the mixed solution was 78% lower than that of wild-type plants (Fig. 4). Thus, although several other transporters may mediate some root amino acid uptake, our data suggest that *LHT1* and *AAP5* may be the most important components of the root amino acid uptake system.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 T-DNA insertion lines of known and putative amino acid transporter encoding genes used in screening experiments, screening for transporters active in cationic amino acid uptake

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