Comprehensive Screening of Arabidopsis Mutants Suggests the Lysine Histidine Transporter 1 to Be Involved in Plant Uptake of Amino Acids[1][W]

Henrik Svennerstam, Ulrika Ganeteg, Catherine Bellini, and Torgny Näsholm*

Umea Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE–901 83 Umea, Sweden (H.S., U.G., C.B., T.N.); and Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, 78026 Versailles cedex, France (C.B.)

Plant nitrogen (N) uptake is a key process in the global N cycle and is usually considered a ''bottleneck'' for biomass production in land ecosystems. Earlier, mineral N was considered the only form available to plants. Recent studies have questioned this dogma and shown that plants may access organic N sources such as amino acids. The actual mechanism enabling plants to access amino acid N is still unknown. However, a recent study suggested the Lysine Histidine Transporter 1 (LHT1) to be involved in root amino acid uptake. In this study, we isolated mutants defective in root amino acid uptake by screening Arabidopsis (*Arabidopsis thaliana*) seeds from ethyl methanesulfonate-treated plants and seeds from amino acid transporter T-DNA knockout mutants for resistance against the toxic D-enantiomer of alanine (Ala). Both ethyl methanesulfonate and T-DNA knockout plants identified as D-Ala resistant were found to be mutated in the *LHT1* gene. *LHT1* mutants displayed impaired capacity for uptake of a range of amino acids from solutions, displayed impaired growth when N was supplied in organic forms, and acquired substantially lower amounts of amino acids than wild-type plants from solid growth media. *LHT1* mutants grown on mineral N did not display a phenotype until at the stage of flowering, when premature senescence of old leaf pairs occurred, suggesting that LHT1 may fulfill an important function at this developmental stage. Based on the broad and unbiased screening of mutants resistant to D-Ala, we suggest that LHT1 is an important mediator of root uptake of amino acids. This provides a molecular background for plant acquisition of organic N from the soil.

The degree to which plants use organic nitrogen (N) compounds as N sources in different ecosystems is an important issue to resolve, with major implications for ecology, biogeochemistry, and plant physiology (Hutchinson and Miller, 1911; Virtanen and Linkola, 1946; Chapin et al., 1993; Kielland, 1994; Näsholm et al., 1998; Schimel and Bennett, 2004). Organic N, in particular amino acids, represents a significant N pool in many soils (Senwo and Tabatabai, 1998; Yu et al., 2002), suggesting the capability of plant roots to acquire such N forms could be of critical importance for plant N nutrition. Indeed, it has been shown that plant roots are capable of absorbing various amino acids from solutions (Virtanen and Linkola, 1946; Wright, 1962). Moreover, several recent studies have shown that plants may access such compounds in field settings (compare with Lipson and Näsholm, 2001). However, very little is known as yet about the processes whereby plants take up organic N compounds. A critical step in this context is to identify the proteins that mediate root absorption of amino acids.

Plants express a multitude of different amino acid transporters with overlapping specificities and affinities (Fischer et al., 1998). In the Arabidopsis (*Arabidopsis thaliana*) genome, at least 53 genes have been annotated as being, or putatively being, amino acid transporters (Wipf et al., 2002; Lalonde et al., 2004) with possible functions in intra- and intercellular transport, capture of apoplastic amino acids, and root capture of amino acids from the soil solution. A number of amino acid transporters have been found to be expressed in roots (Fischer et al., 1998). Hirner et al. (2006) studied knockout mutants and RNAi lines of six root amino acid transporters and identified the Lysine Histidine Transporter 1 (LHT1) as involved in root amino acid uptake. Our aim was to use a broad and neutral collection of mutants to identify amino acid transporters active in root uptake. Efficient screening methods for detecting mutants with defective root amino acid uptake systems were therefore critical to our approach.

Early studies of plant amino acid uptake (Felle, 1981) as well as more recent studies of amino acid transporters (Frommer et al., 1995; Boorer et al., 1996) suggest that both D- and L-forms might be absorbed and that D-Ala is taken up relatively rapidly (Felle, 1981). It has also been demonstrated that D-amino acids such as D-Ala and D-Ser are toxic to plants (Aldag and Young, 1970; Manabe and Ohira, 1981; Erikson

[1] This work was supported by grants from the Kempe Foundation, the Swedish Research Council, and the Swedish Council for Environment, Agricultural Sciences and Spatial Planning (to T.N.).

* Corresponding author; e-mail torgny.nasholm@genfys.slu.se; fax 46–90–7868165.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Torgny Näsholm (torgny.nasholm@genfys.slu.se).

[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.106.092205

et al., 2004), providing an excellent opportunity for screening mutants that have defective root amino acid absorption mechanisms.

In the study reported here, we used an unbiased forward genetics screening procedure to identify mutants resistant to D-Ala and complemented it with a reverse genetics approach, resulting in the identification of LHT1 as a mediator of D-Ala uptake. Our hypothesis was that loss of LHT1 function would affect root uptake rates of a range of amino acids naturally occurring in soil solutions of various ecosystems (Senwo and Tabatabai, 1998; Yu et al., 2002). *LHT1* mutants were subjected to a number of studies to explore the effect of the mutations on plant growth, momentaneous uptake, and long-term acquisition of amino acids.

RESULTS

Mutant Screens and Characterization of Mutants

Our screen was based on the hypothesis that plants surviving on toxic D-amino acids would have a restricted uptake of amino acids and thus carry a mutation in a gene encoding an amino acid transporter active in absorption of exogenous amino acids. Accordingly, our strategy was to screen for mutations in root absorption of amino acids by selecting seeds on plates containing toxic levels of D-amino acids.

Several seeds originating from ethyl methanesulfonate (EMS)-treated plants survived on D-Ala, and these were transplanted to soil and self propagated. The resulting seeds were reselected on D-Ala, and in this screen only one EMS line was found to be D-Ala resistant (Fig. 1A).

Only two out of the 69 T-DNA knockout lines of amino acid transporters (SALK_034566 and SALK_036871) survived on 3 mM D-Ala. These knockout lines corresponded to T-DNA insertions in the gene encoding LHT1. Based on sequencing information from SALK, we suspected that these two lines originated from the same insertion event and, therefore, only one of these lines was used for further studies (Fig. 1B). The estimated localization of the T-DNA insert was in the third exon of *LHT1* (Fig. 1C). After the first selection, the T-DNA mutant was subjected to genetic analysis. PCR analysis showed insertion homozygosity in the plants that survived D-Ala selection. Furthermore, the offspring of heterozygous parent plants segregated 3 sensitive:1 resistant on D-Ala, indicating that the mutation was recessive. Reverse transcription (RT)-PCR analysis showed no *LHT1* transcript spanning the insertion site, indicating truncated *LHT1* transcript in the T-DNA mutant (Fig. 1D).

To investigate whether the EMS and the T-DNA insertion mutants were allelic, homozygotes from the two different mutant lines were crossed. The F_1 progeny of this cross was found to be resistant to D-Ala, suggesting that the EMS and T-DNA lines were mutated in the same gene, *LHT1*. Sequencing of the *LHT1* gene of the EMS mutant line showed a single G/A nucleotide substitution, which based on the genomic sequence of LHT1 (At5g40780.1) should result in a change of the native Trp-66 codon to a stop codon (Fig. 1C). This suggests that translation of *LHT1* transcript was hindered in this mutant line. To avoid confusion with the LHT1 lines studied by Hirner et al. (2006), the EMS and T-DNA knockout lines were named *lht1-3* and *lht1-4*, respectively.

During the course of this work, another *LHT1* mutation allele conferring D-Ala resistance, the T-DNA insertion line SALK_115555 (hereafter named *lht1-5*), was obtained. The T-DNA insert of this line was found to be within the last intron of *LHT1*, and RT-PCR analysis confirmed that transcript spanning the insert site was not present in *lht1-5* (Fig. 1D). This line was

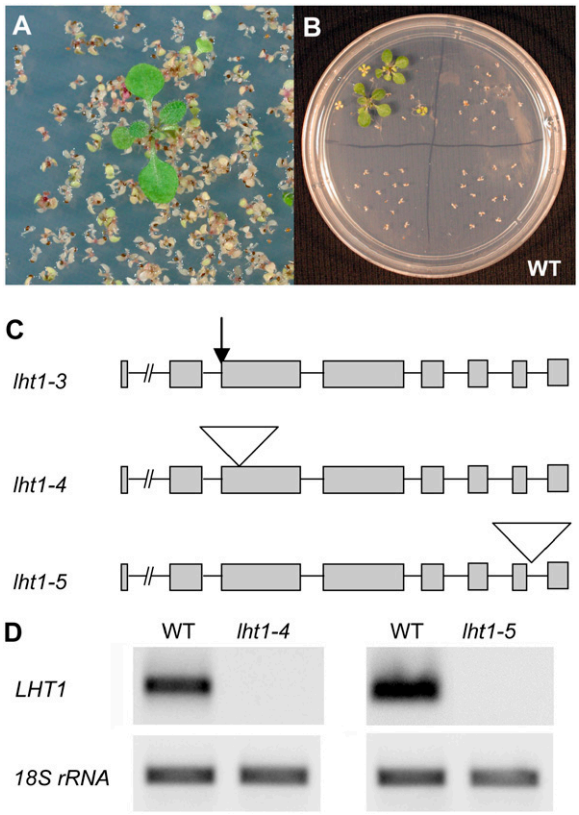

Figure 1. Characterization of Arabidopsis *LHT1* mutant lines. A and B, Identification of plants defective in amino acid uptake by screening on 3 mM D-Ala. A, Seeds from EMS-treated plants; B, segregating T2 seeds from three of the 39 studied T-DNA insertion lines and from the wild type. Upper left quarter shows an *LHT1* mutant line, and lower right quarter shows the wild type (WT). C, The *lht1-3* mutant (from the EMS mutant screen) shows a G/A substitution (marked with an arrow) at the first base of the third exon. The estimated location of the T-DNA insert in *lht1-4* is at the third exon and in *lht1-3* in the last intron (marked with triangles). D, The *lht1-4* and *lht1-5* mutants lacks full-length *LHT1* transcript. RT-PCR reactions with RNA from wild-type (WT) and *lht1-4* or *lht1-5* plants using primers spanning the insertion site are shown. 18S rRNA primers were used as control.

used together with the *lht1-4* mutant for phenotypic characterization (Fig. 2).

Phenotypic Characterization

When grown on soil, the two T-DNA knockout lines (*lht1-4* and *lht1-5*) were phenotypically indistinguishable from wild-type Arabidopsis plants and displayed the same growth rate as wild-type plants up to the point of the emergence of the first inflorescence (approximately 24 d of growth; Fig. 2). Following bolting, *lht1-4* and *lht1-5* mutant lines displayed growth inhibition and older leaves became yellow earlier than for wild-type plants. This phenotype was also noted for *lht1-3* plants (data not shown); however, dry weight measurements were not conducted using this line. The growth data suggested to us that studies on amino acid acquisition of mutants should be performed before onset of flowering.

Studies of Root Uptake of Amino Acids in LHT1 Mutants

To compare *LHT1* mutants and wild-type plants with respect to root uptake of various amino acids, an experiment was designed where roots of intact plants were submerged in a solution containing a mixture of seven different amino acids occurring in natural soils (25 μM each), and the depletion of each amino acid in this solution was measured after 5 h of incubation. D-Ala was included to assess the reduction in uptake caused by the *LHT1* mutation. This study was thus not designed to establish actual rates of amino acid absorption of plants, but rather to compare the wild type and mutants with respect to this process. No differences in uptake rates of amino acids were found between the two mutant lines *lht1-3* and *lht1-4*, but compared to wild-type plants both mutant lines were strongly affected with respect to root absorption of a range of amino acids and displayed significantly lower uptake rates of all tested amino acids except for L-Glu and L-Lys. The strongest effect of the mutation was on uptake of L-Ser, D-Ala, and Gly, for which uptake rates averaged over the two mutant lines were reduced by 100%, 92%, and 84%, respectively. For L-Gln and L-His, the average reduction in uptake of the *lht1-3* and *lht1-4* lines was 46% and 42%, respectively (Fig. 3).

Plant Growth on Amino Acids

Since *LHT1* mutants showed impaired uptake of amino acids from solutions, we wanted to study the effect of the different *LHT1* mutations on plant growth. Therefore, an experiment was conducted in which a significant share of available N was supplied as amino acids. In these tests, the *lht1-3* and *lht1-4* lines were used. Growth of mutants did not differ from that of wild-type plants when N was supplied as nitrate only or when supplied as a mixture of nitrate and L-Gln (Fig. 4). However, when N was administered as L-Gln,

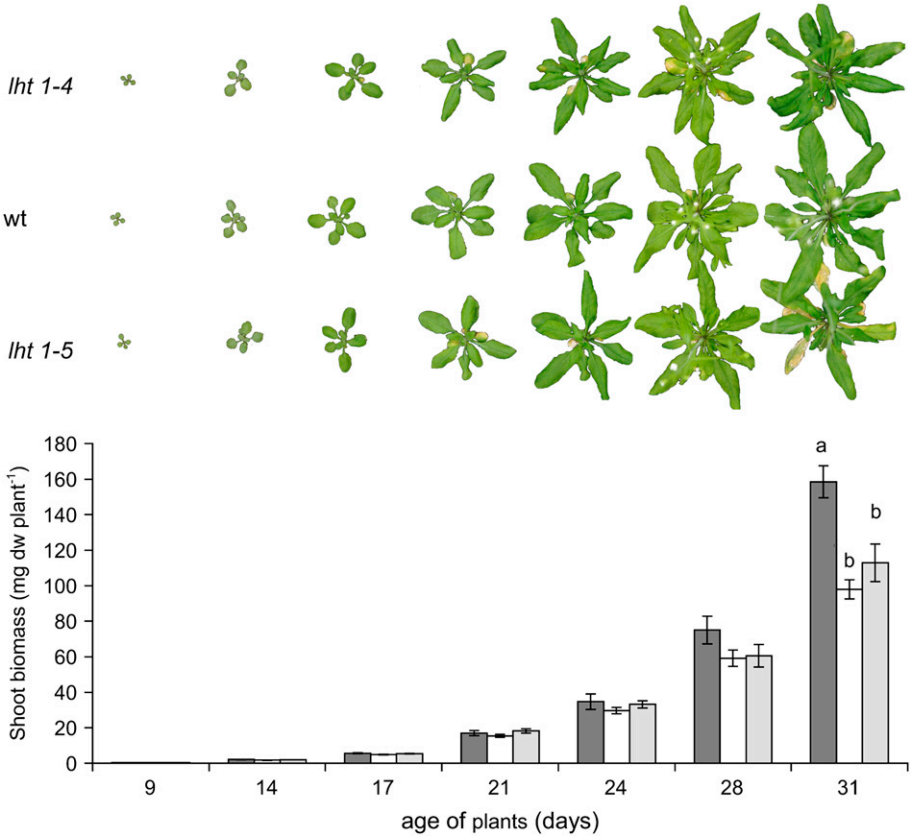

Figure 2. A, Plant morphology of wild-type (wt) and *lht1-4* and *lht1-5* plants. B, Shoot biomass of wild-type (dark gray bars), *lht1-4* (white bars), and *lht1-5* (light gray bars) plants. Bars represent mean values ± SE, $n = 5$. Different letters above bars indicate significant differences between genotypes at $P < 0.05$.

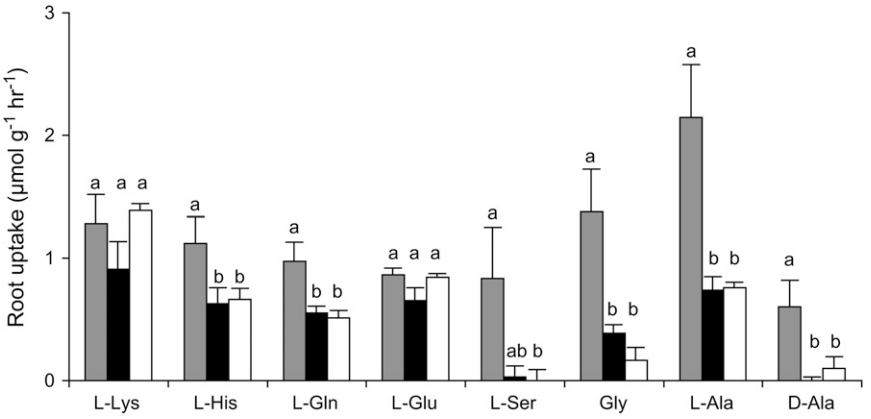

Figure 3. The effect of mutation in *LHT1* on amino acid uptake by plant roots. Sterile-grown wild-type (dark gray bars), *lht1-3* (black bars), and *lht1-4* (white bars) plants were incubated in solutions containing a mixture of eight different amino acids, each at a concentration of 25 μM, and the rate of depletion of the amino acids from this solution was measured. Bars represent mean values ± SE, $n = 5$. Different letters above bars indicate significant differences between genotypes at $P < 0.05$.

only the average biomass at harvest of the two mutant lines was significantly lower and only 68% of that of wild-type plants. Similarly, growth of mutants was significantly decreased as compared to the wild type when N was supplied as a mixture of the four amino acids L-Arg, L-Asn, L-Ser, and L-Ala, or when N was supplied as a combination of these four amino acids and nitrate. Growth, as averaged over the two mutant lines, was 56% and 68% of that of wild-type plants in the amino acid mixture and in the amino acid mixture combined with nitrate.

Acquisition of Amino Acids during Growth

To study how lack of *LHT1* expression affected plant amino acid acquisition during growth, an experiment in which small amounts of ^{15}N-labeled amino acids (0.03 mM) supplied together with larger amounts of nitrate (3 mM) to the growth media was conducted. For wild-type plants, N derived from amino acids was 1.7%, 2.1%, and 1.8% for L-Ala, L-Gln, and L-Lys, respectively (Fig. 5). The relative share of amino acid N in the growth media was 1% in the L-Ala treatment and 2% for the L-Gln and L-Lys treatments, and thus uptake of amino acids was similar to or higher than the respective share in the growth media. Fractions of N derived from amino acids did not differ significantly between *lht1-3* and *lht1-4* mutants. Plant N derived from L-Ala and L-Gln differed clearly between the wild type and *LHT1* mutants, while no such difference could be detected for L-Lys. In the two mutant lines, the average fraction of total plant N derived from uptake of amino acid was 0.5%, 0.6%, and 1.7% for L-Ala, L-Gln, and L-Lys, respectively. Hence, amino acid acquisition in mutants was on average reduced by 71% and 73% for L-Ala and L-Gln, respectively, but not significantly reduced for L-Lys.

DISCUSSION

For long it has been known that plants have the capacity to absorb amino acids from solutions (Virtanen and Linkola, 1946; Wright, 1962; Chapin et al., 1993). More recent studies have also shown that plants may acquire N from amino acids under field conditions and that such N sources may be present in concentrations similar to those of inorganic N, at least in unmanaged systems (Lipson and Näsholm, 2001). Root uptake of amino acids has been shown to be energy dependent and to be regulated by external and internal cues, indicating that this is an active process mediated by specific transporters. Plants express a multitude of different amino acid transporters with overlapping specificities and affinities (Fischer et al., 1998). The localization and regulation of transporters may therefore be the primary differentiating parameters that allow for different transporters to carry out specific functions. Our data show that a member of the ATF family of amino acid transporters, *LHT1*, has an important function in the process of root amino acid acquisition. This is in line with a recent publication by Hirner et al. (2006), who reported that screening of six knockout and RNAi mutant lines, affected in amino acid transporters, resulted in the identification of LHT1 as involved in root amino acid uptake.

Earlier studies have indicated that several amino acid transporters mediate transport of D-enantiomers of amino acids (Frommer et al., 1995; Boorer et al., 1996) but also that several D-amino acids are toxic to plants (Erikson et al., 2004). Thus, we envisaged that it would be possible to identify a transporter(s) that mediates uptake of amino acids from external sources through screening mutants for resistance against a toxic D-amino acid. By using forward genetics, an unbiased screening of mutants was performed. The disadvantage of this approach is, however, the time-consuming work of tracking the nature of a mutation that causes a specific phenotype.

We therefore used reverse genetics (T-DNA knockout lines) in parallel to the forward genetics approach. The fact that both these strategies arrived in the identification of *LHT1* as crucial for D-Ala resistance suggests that this transporter is the single most important mediator of D-Ala uptake in Arabidopsis. Because our emphasis was on identifying transporters active in root acquisition of amino acids prevalent in soil solutions of various ecosystems (compare with

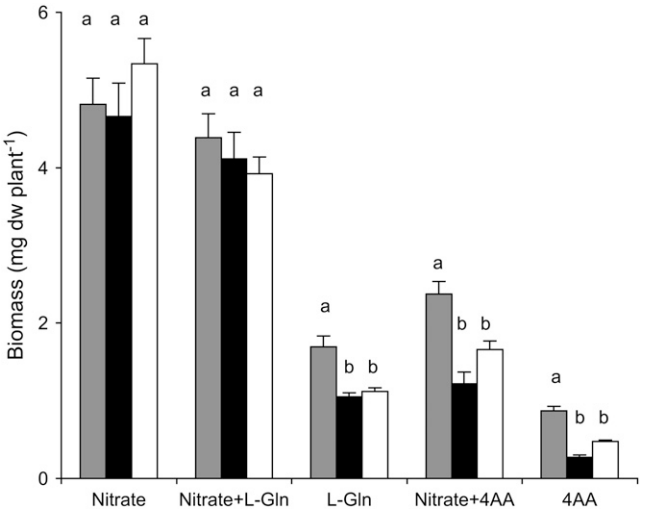

Figure 4. Arabidopsis plant growth on amino acids. Wild type (dark gray bars), *lht1-3* mutant (black bars), and *lht1-4* (white bars) mutant were grown for 20 d on N-free half-strength MS agar media amended with one of the following N sources: 3 mM NO_3^- (Nitrate); 1 mM NO_3^- + 1 mM L-Gln (Nitrate + L-Gln); 1 mM L-Gln (L-Gln); 1 mM NO_3^- plus 1 mM each of L-Ser, L-Ala, L-Asn, and L-Arg (Nitrate + 4AA); or 1 mM each of L-Ser, L-Ala, L-Asn, and L-Arg (4AA). Bars represent mean values ± SE, $n = 7$. Different letters above bars indicate significant differences between genotypes at $P < 0.05$.

Lipson and Näsholm, 2001), a thorough evaluation of the capacities of mutants to acquire amino acids was imperative.

The first study showed that rates of root uptake of Gly, L-Ser, L-Ala, L-His, and L-Gln from solution were significantly lower in the two mutant lines compared to wild-type plants, while the mutations did not affect uptake of L-Glu and L-Lys significantly (Fig. 3). These results suggest that LHT1 is active in root absorption of several of the amino acids occurring in soil solutions (Senwo and Tabatabai, 1998; Yu et al., 2002), but may have a limited role for absorption of L-Glu and L-Lys. The amino acid concentrations used by us (25 μM each amino acid) can be considered high, but still within the range of concentrations reported from studies of various ecosystems (compare with Lipson and Näsholm, 2001). The effect of the mutation on uptake rates of various amino acids did not correspond to the uptake characteristics of the LHT1 transporter displayed in the study of Chen and Bush (1997). Thus, when expressed in yeast, LHT1 exhibited a strong preference for L-Lys, L-His, and L-Glu, and lower activities against L-Ala, L-Ser, and Gly (Chen and Bush, 1997). Hirner et al. (2006) also characterized the specificity of LHT1 regarding amino acid transport using complementation of yeast strains, and found that LHT1 displayed low affinity for all basic amino acids, intermediate affinity for L-Glu and L-Asp, but high affinity for a number of aliphatic amino acids, including L-Pro, Gly, L-Ala, and L-Ser. Thus, our results from in planta studies, indicating lower affinity for L-Lys, L-His, and L-Glu but high for Gly, L-Ser, and L-Ala, are contradictory to the study by Chen and Bush (1997) but corroborate the results presented by Hirner et al. (2006).

Growth of *lht1-3* and *lht1-4* plants did not significantly differ from that of wild-type plants when N was supplied solely as nitrate (3 mM), or as a mixture of nitrate and L-Gln (1 mM + 1 mM; Fig. 4). However, when N was administered solely as 1 mM L-Gln, the mean biomass of the mutants was significantly lower as compared to wild-type plants at the time of harvest. Similarly, when N was supplied as a mixture of four L-amino acids with or without nitrate, growth was significantly reduced compared to wild-type plants. These data corroborate that growth of LHT1 mutants was impaired by a restricted uptake of L-amino acids and suggest that uptake capacities of amino acids are important determinants of plant biomass production whenever plants can access amino acids in the soil. Since LHT1 has been shown to be expressed in the shoot, we cannot rule out the possibility that loss of LHT1 and the corresponding effects on cell-to-cell transport or cycling of amino acids in the shoot could have affected growth of mutants. However, two lines of evidence corroborate that the primary effect of loss of LHT1 is that on root amino acid uptake. First, growth of mutants was only affected when N was supplied as amino acids and not when N was supplied as nitrate only (Fig. 4). Second, Hirner et al. (2006) reexpressed LHT1 in the shoot of LHT1 mutants and showed that growth of these plants was still retarded when N was administered as amino acid. When plants are cultured on agar media, nutrients such as N must be present in relatively high concentrations to assure that they are not depleted during the growth period. This is naturally problematic when studying the effects of loss of high-affinity transporters because the full effect of loss of a specific transporter may not be displayed under such conditions.

To specifically address the importance of LHT1 for plant acquisition of amino acid N during growth and

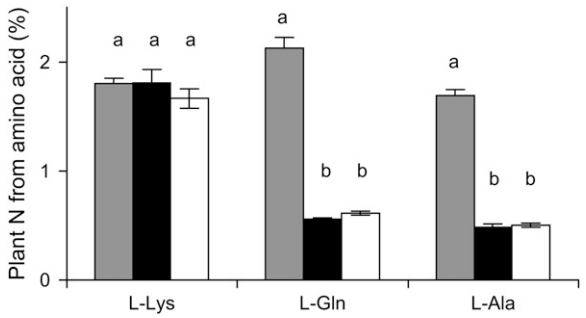

Figure 5. The fraction of Arabidopsis plant N (% of total N in the plant) derived from the amino acids L-Ala, L-Gln, and L-Lys. Wild type (dark gray bars), *lht1-3* mutant (black bars), and *lht1-4* (white bars) mutant were grown for 20 d on N-free half-strength MS agar media in which all N sources were replaced with 3 mM NO_3^- and 0.03 mM of L-^{15}N-Gln, L-^{15}N-Ala, or L-^{15}N-Lys. Bars represent mean values ± SE, $n = 5$. Different letters above bars indicate significant differences between genotypes at $P < 0.05$.

from more field-relevant concentrations of amino acids, we used an experimental design in which small amounts (0.03 mM) of ^{15}N-labeled amino acids were supplied together with larger amounts of nitrate. In this way, the effect of the mutation on the rate of amino acid acquisition from submillimolar substrate concentrations could be assessed and separated from the growth effects of amino acid N. During 3 weeks of growth, *LHT1* plants acquired less than 30% of the amounts of N absorbed by wild-type plants supplied with either L-Ala or L-Gln, but acquisition of L-Lys was not affected (Fig. 5). Hence, both the assessment of root absorption rates in aqueous media and the assessment of plant acquisition of amino acids from solid media suggest that LHT1 may have a critical role for uptake of amino acids such as L-Ala and L-Gln but not of L-Lys.

The results presented here, as well as results implicating that LHT1 is expressed in the rhizodermis of lateral roots and emerging roots and with the subcellular localization to the plasmamembrane (Alexandersson et al., 2004; Hirner et al., 2006), suggest that LHT1 is an important element of the mechanisms enabling plants to acquire N in the form of amino acids from the soil solution. It is equally obvious that transporters other than LHT1 should have similar functions because amino acid uptake was not totally abolished in *LHT1* mutants (Figs. 3–5). In addition, other transporters must also be present to mediate absorption of basic amino acids such as L-Lys. Meta-analysis using publicly available data at Genevestigator (www.genevestigator.ethz.ch; Zimmermann et al., 2004) suggested several of the known and hypothetical amino acid transporters to be expressed in roots. Moreover, earlier studies of the Pro transporter 2 indicated that this transporter may enable Arabidopsis to grow on the nonprotein amino acid γ-amino butyric acid (Breitkreuz et al., 1999). Similarly, AAP1 was shown to be expressed in root epidermal cells, suggesting a role of this transporter in root amino acid uptake (Bick et al., 1998). Some amino acid transporters may display low activity against D-amino acids (Fischer et al., 2002), suggesting that our screen based on the toxicity of D-Ala would not be able to pick up such mutants.

In this study, plants grown on agar or in nutrient solutions were not phenotypically different from wild-type plants. Hence, growth of mutants and wild-type plants did not differ when N was supplied as nitrate. When grown on soil, however, *LHT1* mutants displayed a characteristic phenotype with yellowing of older leaves at the stage of initiation of flowering. Earlier characterization of LHT1 suggests it to be expressed in several tissues, including old leaves (Chen and Bush, 1997). The recent study by Hirner et al. (2006) shows, in accordance, that LHT1 is expressed in leaves and is active in uptake of amino acids in leaf mesophyll cells. Hence, loss of LHT1 could possibly have a number of effects on plants, but according to our findings such effects were not displayed until the stage of flowering and hence did not limit our possibilities to pinpoint the role of LHT1 for root amino acid uptake.

A central question within the area of plant N nutrition is the role of different N forms. Today, it is widely held that amino acids are absorbed by plant roots, but the degree to which such compounds actually contribute substantially to the N economy of plants is disputed (Jones et al., 2005). In this context it is imperative that the mechanisms through which plants absorb amino acids, as well as other compounds, are characterized.

CONCLUSION

Our results show that functional expression of *LHT1* is important for uptake of a range of amino acids by roots of Arabidopsis. This complements the picture of root N uptake through nitrate and ammonium (Glass et al., 2002) and may provide new opportunities for studies of the role of amino acids as N sources for plants. Lack of LHT1 did not significantly affect root uptake of basic amino acids such as L-Lys, suggesting that other transporters mediating uptake of such compounds should be targeted in future studies.

MATERIALS AND METHODS

Plant Material and Mutant Screening

In the mutant screen, 1 g (approximately 50,000) seeds descended from approximately 6,250 M1 parent EMS-treated Arabidopsis (*Arabidopsis thaliana*) plants (Lehle Seeds) and seeds from 69 Arabidopsis SALK T-DNA insertional knockout mutants (Nottingham Arabidopsis Stock Centre, Nottingham, UK), with mutations in 39 individual genes encoding or putatively encoding amino acid transporters, were used (see Supplemental Table S1). Mutant screens were performed on sterile agar plates containing half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), with 0.65% (w/v) agar (plant agar; Duchefa Biochemie), 0.5% (w/v) Suc, and 3 mM D-Ala, buffered to pH 5.8 with 3.6 mM MES. The two screens resulted in the isolation of two allelic mutants of *LHT1*, both resistant to D-Ala: an EMS mutant (named *lht1-3*) and a T-DNA knockout line, SALK_036871 (named *lht1-4*). An additional LHT1 T-DNA mutant, SALK _115555 (named *lht1-5*), which was also resistant to D-Ala, was also obtained during the work. These lines were used together with wild-type Arabidopsis (ecotype Colombia-0) in the following experiments.

Analysis of T-DNA Knockout Lines

Presence of the T-DNA insert was verified with PCR using *LHT1*-specific primers (5′-GAGAAACGCCGGAGATGGAAT, *lht1-4*; 5′-ATTTCAGACCAACCACAACTCTTCG, *lht1-5*) together with a T-DNA left-border primer (5′-TGGTTCACGTAGTGGGCCATCG). Homozygous plants were verified with primers spanning the T-DNA insertion site (5′-GAGAAACGCCGGAGATGGAAT and 5′-TTTCTGACTTTTCTTCCATTGATTTTT, *lht1-4*; 5′-ATTTCAGACCAACCACAACTCTTCG and 5′-TGGCGATAGGACCATCAAGAAAAGA, *lht1-5*). For RT-PCR, total RNA was prepared from 3-week-old plants. First-strand cDNA synthesis was performed using First-Strand Synthesis kit (Amersham Biosciences) as recommended by the vendor. *LHT1*-specific primer pairs on either side of the T-DNA insert were used to confirm the lack of *LHT1* transcript in the mutants (5′-GCTCAAGCTCCTCATGATGATCA and 5′-CGTAAGCAAATGCCACATCACC TAA, *lht1-4*; 5′-AGTCATCGTTGCTTACATCGTCGT and 5′-TGGCGATAGGACCATCAAGAAAAGA, *lht1-5*). Primers amplifying 18S rRNA (catalog no. 1718; Ambion) was used as control to ensure equal amount of RNA in each reaction. The control reactions were performed separately.

For plant growth and biomass analysis on soil, seeds were sown on soil:perlite (3:1) and grown in a climate chamber at 200 μmol photons m^{-2} s^{-1}, with a 16/8 h, 23°C/18°C day/night regime. Plant aboveground biomass was measured and photographs were taken every 3 to 4 d (for 31 d). Depending on plant size, one to five plants were included in each biological replicate.

Amino Acid Uptake Assay

Wild-type, *lht1-3, and lht1-4* plants were grown under sterile conditions in a climate chamber with an 8-h-light/16-h-dark (200 μmol photons m^{-2} s^{-1}) and 23°C/18°C (day/night) regime, in a nutrient solution containing 1,000 μM Gly, 1,000 μM NH_4NO_3, 600 μM K_2HPO_4, 300 μM $CaSO_4$, 550 μM $MgSO_4$, 35 μM KCl, 50 μM FeNa-EDTA, 35 μM H_3BO_3, 7 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 1 μM $CuSO_4$, and 0.05 μM $(NH_4)_6Mo_7O_{24}$, buffered to pH 5.8 with MES. After 48 d of growth, three plants of each plant type were selected for the uptake experiment, which was carried out in a climate chamber on a shaking table. The uptake solution was identical to the nutrient solution used during the plant cultivation, except that all N sources were replaced with an amino acid mixture composed of 25 μM each of D-Ala, L-Ala, L-Ser, L-Lys, L-His, L-Gln, L-Glu, and Gly. The solution also contained 0.5 mM $CaCl_2$. Root uptake rates (μmol g dry weight $root^{-1}$ h^{-1}) were determined from the decline in concentration of each amino acid in the solution during the 5-h incubation. Concentrations of amino acids in the uptake solution were derived from two sets of analyses: Concentrations of L-Ala, D-Ala, L-Ser, L-Lys, and L-His were obtained by chiral separations of amino acids (Brückner and Westhauser, 2002), while concentrations of L-Glu, L-Gln, and Gly were obtained from nonchiral amino acid analyses (Näsholm et al., 1987).

Growth Experiments

Wild-type, *lht1-3, and lht1-4* seeds were sown on N-free half-strength strength MS media amended with one of the following N sources: 3 mM NO_3^-; 1 mM NO_3^- + 1 mM L-Gln; 1 mM L-Gln; a mix of 1 mM each of L-Ala, L-Arg, L-Asn, and L-Ser; or the mix of these four amino acids and 1 mM NO_3^-. Because some amino acids may be degraded during autoclaving, amino acid solutions were sterile filtered and added to the autoclaved solutions separately. Eight replicate plates, each with three seeds, were prepared for each seed line and treatment. After sowing, plates were incubated in a cold room for 2 d and then transferred to a climate chamber under a 16-h-light/8-h-dark (200 μmol photons m^{-2} s^{-1}) and 23°C/18°C regime. After 20 d, plants were harvested, dried overnight at 60°C in an oven, and weighed.

Amino Acid Acquisition during Growth

Wild-type, *lht1-3*, and *lht1-4* seeds were sown on plates containing 0.5% Suc, 0.65% agar, the equivalent of 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-Ala or L-^{15}N-Lys (all >98% ^{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. Agar plates were incubated in a cold room for 2 d and then transferred to a climate chamber with a 16-h-light/8-h-dark (200 μmol photons m^{-2} s^{-1}) and 23°C/18°C (day/night) regime. After 17 d, plants from five randomly selected 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 from their surfaces. Plants were dried at 60°C overnight, weighed, and homogenized. Finally, samples were analyzed using a Europa Scientific isotope ratio mass spectrometer to determine total N and ^{15}N contents.

The possibility of degradation of amino acids was checked through analyzing extracts of plates in which L-Gln, L-Ala, and L-Lys had been added to a calculated concentration of 30 μM each. Eight plates were produced, four of which were directly placed in a freezer at −20°C and four of which were placed in the climate chamber used for the growth studies (see above). The recorded concentrations of L-Ala and L-Gln in plates stored in −20°C were (average ± SE) 27.8 ± 1.2 and 29.4 ± 0.9, respectively, and the corresponding concentrations in plates kept in the climate chamber were 27.1 ± 0.5 and 28.5 ± 0.3. For L-Lys, a peak resulting from an unknown substance present in the agar extract disturbed the analysis; hence, we could not determine the L-Lys concentrations in the extracts. We also analyzed the ammonium concentrations in the plate extracts. Because no increase in ammonium could be detected and because of the insignificant differences in L-Ala and L-Gln concentrations in plates exposed to the different temperatures, we conclude that any degradation of amino acids in the plates was negligible.

Treatment of Data

All data were subjected to ANOVA, and differences between the wild type and mutant lines were checked with Tukey's post hoc test.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: LHT1, At5g40780, U39782.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. T-DNA knockout lines screened for resistance toward D-Ala.

ACKNOWLEDGMENTS

Margareta Zetherström is acknowledged for skillful laboratory work, and Ove Nilsson for discussions and advice during the course of this work.

Received November 16, 2006; accepted January 28, 2007; published February 9, 2007.

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