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Arabidopsis LHT1 Is a High-Affinity Transporter for Cellular Amino Acid Uptake in Both Root Epidermis and Leaf Mesophyll [W]

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Amino acid transport in plants is mediated by at least two large families of plasma membrane transporters. *Arabidopsis thaliana*, a nonmycorrhizal species, is able to grow on media containing amino acids as the sole nitrogen source. *Arabidopsis* amino acid permease (AAP) subfamily genes are preferentially expressed in the vascular tissue, suggesting roles in long-distance transport between organs. We show that the broad-specificity, high-affinity amino acid transporter LYSINE HISTIDINE TRANSPORTER1 (LHT1), an AAP homolog, is expressed in both the rhizodermis and mesophyll of *Arabidopsis*. Seedlings deficient in LHT1 cannot use Glu or Asp as sole nitrogen sources because of the severe inhibition of amino acid uptake from the medium, and uptake of amino acids into mesophyll protoplasts is inhibited. Interestingly, *lht1* mutants, which show growth defects on fertilized soil, can be rescued when LHT1 is reexpressed in green tissue. These findings are consistent with two major LHT1 functions: uptake in roots and supply of leaf mesophyll with xylem-derived amino acids. The capacity for amino acid uptake, and thus nitrogen use efficiency under limited inorganic N supply, is increased severalfold by LHT1 overexpression. These results suggest that LHT1 overexpression may improve the N efficiency of plant growth under limiting nitrogen, and the mutant analyses may enhance our understanding of N cycling in plants.

INTRODUCTION

Quantitatively, nitrogen is the most prominent limiting factor among mineral nutrients for plant growth and development. The uptake and distribution of inorganic N in the form of nitrate and ammonium are well characterized (Williams and Miller, 2001). The inorganic nitrogen is converted into amino acids as the organic transport form either directly in the roots or after translocation to the leaves. Amino acids are then distributed via the vascular system and follow the sink path of carbohydrates to developing organs such as sink leaves, roots, or fruits. However, under certain conditions, organic nitrogen sources such as amino acids may play a crucial role in nitrogen nutrition. In ecosystems with slow mineralization rates, such as boreal forests, arctic tundra, and alpine regions, organic nitrogen is especially vital for plant growth (Meline and Nilsson, 1953; Nasholm et al., 1998). The ability to use limiting nutrients such as nitrogen is an important factor for the productivity of a plant species (McKane et al., 2002). Moreover, the partitioning of nitrogen plays a key role in the delivery of reduced nitrogen to the heterotrophic tissues (Pate, 1973; Pate and Sharkey, 1975). The amino acid content of the phloem and xylem sap seems to be tightly regulated to meet the nitrogen requirements of various organs, especially seeds (Lam et al., 1995).

The distribution of amino acids in the plant requires several transport steps across membranes. Amino acids have to cross the plasma membrane when taken up from the soil into the root. In mycorrhized plants, the plant root cells are assumed to take up amino acids that pass the fungal layer or are produced within the fungus. Amino acids synthesized in root tissue have to be exported to the shoot via the xylem. Because mature xylem elements are postapoptotic and considered to be part of the apoplasm, amino acids need to be exported across the plasma membrane to enter the xylem via facilitators, exchangers, or antiporters or by exocytosis. The concentration of amino acids in the phloem and in the mesophyll cytoplasm is significantly higher compared with the concentration found in the apoplasm, suggesting active import of amino acids into the phloem (Lohaus and Heldt, 1995). Furthermore, large amounts of amino acids fed directly to the xylem sap appear unchanged in the phloem sap, indicating that amino acids can be exchanged between xylem and phloem (Pate and Sharkey, 1975; Atkins, 2000).

Physiological studies of amino acid transport in plants have suggested the existence of multiple amino acid carriers exhibiting broad substrate specificity energized by cotransport with protons (Kinraide, 1980; Despeghel and Delrot, 1983; Mounoury et al., 1984; Wyse and Komor, 1984). Amino acid uptake into isolated plasma membrane vesicles showed complex kinetics, suggesting the existence of multiple transport systems (Li and

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Bush, 1990a, 1990b, 1992). Amino acid transporters were identified from *Arabidopsis thaliana* by the complementation of yeast mutants defective in amino acid uptake (Frommer et al., 1993, 1995; Hsu et al., 1993; Kwart et al., 1993; Fischer et al., 1995; Rentsch et al., 1996; Chen and Bush, 1997; Chen et al., 2001). Bioinformatic analysis revealed ≥53 putative amino acid carriers in the *Arabidopsis* genome (Wipf et al., 2002b). The uptake of amino acids and their transfer into the root xylem has been described (Schobert and Komor, 1990), and the corresponding amino acid transport systems have been characterized (Bick et al., 1998). Because *Arabidopsis* is not mycorrhized, it is an excellent model for determining the relevance of amino acid uptake and translocation.

Despite the relevance of organic N for plant growth, transport systems for the uptake of amino acids from soil have not yet been identified, (e.g., inhibition of the root amino acid transporter AAP3 had no effect on the uptake of ^{14}C-labeled amino acids) (Okumoto et al., 2004). The broad specific amino acid transporters characterized to date are all expressed in vascular tissue or reproductive organs, not in parenchymatic cells of roots or leaves or in the root epidermis (e.g., AAP1 and AAP2 [Hirner et al., 1998], AAP3 [Okumoto et al., 2004], and AAP6 and AAP8 [Okumoto et al., 2002]), except the high-affinity transporter LYSINE HISTIDINE TRANSPORTER2 (LHT2), which was recently localized to the tapetum (Lee and Tegeder, 2004). Transporters with a function in amino acid uptake from soil should be localized at the plasma membrane of the rhizodermis and are expected to have a high affinity for a wide spectrum of amino acids corresponding to the low concentrations of amino acids found in soil (Lipson and Nasholm, 2001).

Given the large number of characterized transporters and the availability of a large insertion mutant collection, it may be surprising that mutants defective in amino acid uptake or distribution have not been described to date. In previous studies, antisense repression of the potato (*Solanum tuberosum*) amino acid permease AAP1 expressed in source leaves led to reduced amino acid levels in tubers (Koch et al., 2003). These data suggested a role for AAP1 in phloem loading and were the first hint that repression of an amino acid transporter influences long-distance transport.

In the framework of a systematic functional analysis of the 53 predicted *Arabidopsis* amino acid transporters, LHT1 (Chen and Bush, 1997) was found to have a much higher affinity to amino acids than the transporters of the related AAP subfamily. Here, we show that mutation of a single transporter gene (*LHT1*) is sufficient to inhibit plant growth and interfere with both the uptake and distribution of amino acids in *Arabidopsis*. In contrast with the AAPs, LHT1 expression is predominantly in nonvascular tissues, including root surface and leaf mesophyll. Mutants lacking *LHT1* mRNA show drastic developmental and metabolic phenotypes. Uptake studies with ^{14}C-labeled amino acids demonstrated that the amino acid uptake rate in *lht1* insertion lines was reduced to 15 to 27% of the rate found in the wild type. Overexpression of LHT1 under the control of the cauliflower mosaic virus (CaMV) 35S promoter led to improved growth when plants were grown on amino acids as a sole source of nitrogen. Uptake experiments with mesophyll protoplasts combined with metabolic analysis of the apoplasmic wash fluids showed that amino acid concentration is increased in the apoplasm of the mutants. Finally, growth inhibition of mutants grown on soil fertilized with inorganic nitrogen could be complemented by a construct expressing *LHT1* under the control of a leaf/stem-specific promoter. Together, these data strongly suggest that LHT1 is involved in the uptake of amino acids from soil and into the leaf mesophyll cells. Moreover, they show that amino acid cycling is an important factor for plant growth and development.

RESULTS

Identification of Mutants Defective in Amino Acid Uptake

Arabidopsis is able to use various amino acids as the sole source of nitrogen when grown axenically. To identify transporters for amino acid uptake from soil, various mutant lines with reduced expression of amino acid transporters were grown on media containing amino acids as a sole nitrogen source. The screen included insertion lines for *AAP3*, *AAP6*, *AAP8*, *LHT1*, and various RNA interference (RNAi) lines (*AAP2* and *AAP4*), several of which, according to GENEVESTIGATOR (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004), had been found to be expressed in roots. Only lines carrying insertions in *LHT1* showed reduced growth compared with the wild type, suggesting that LHT1 may be involved in amino acid uptake (Figure 1). Two independent homozygous *Arabidopsis* lines carrying T-DNA insertions in *LHT1* were identified in the SALK T-DNA insertion collection (Alonso et al., 2003). Sequence analysis confirmed that *lht1-1* and *lht1-2* contain T-DNA insertions in the third exon and fourth intron of the *LHT1* coding region, respectively (see Supplemental Figure 1 online). PCR of genomic DNA with primers on both sides of the insertion (*lht1-1*) and a left border primer and a downstream primer confirmed that the insertions disrupt the locus (see Supplemental Figure 2 online). Both RT-PCR and RNA gel blot analysis failed to detect *LHT1* mRNA, suggesting that both *lht1-1* and *lht1-2* are null mutants (Figure 2B; see Supplemental Figure 3 online).

When grown on either 5 mM Asp or 5 mM Glu (Figures 1B and 1C) as the sole nitrogen source, development of *lht1-1* was retarded, whereas on 5 mM γ-aminobutyrate (GABA), a slight reduction in growth and development was observed relative to the wild type. Conversely, Asn, which had a toxic effect on wild-type plants, was less toxic for the *lht1* mutants. Other proteinogenic amino acids were tested but did not sustain any growth of wild-type plants at concentrations of 5 mM (data not shown). The role of LHT1 as an amino acid uptake system in roots (at least for the amino acids tested) was confirmed and quantified by tracer uptake studies in whole seedlings. The uptake rates were reduced by 85% (Gln), 73% (Glu), and 82% (Asp) in *lht1-1* relative to the wild type (Figure 1D). Total uptake rate was highest for Gln, with 1.8 pmol·h^{-1}·seedling^{-1} in *lht1-1* and 10.8 pmol·h^{-1}·seedling^{-1} in the wild type. The uptake rates were lowest for Asp, with 0.3 pmol·h^{-1}·seedling^{-1} in *lht1-1* and 1.7 pmol·h^{-1}·seedling^{-1} for wild-type plants.

In a second experiment, [^{14}C]L-Gln uptake from the growth medium into roots and shoots was analyzed separately. The results show that both uptake via the root and transport to the shoot were strongly reduced in the mutants (Figure 3A). The

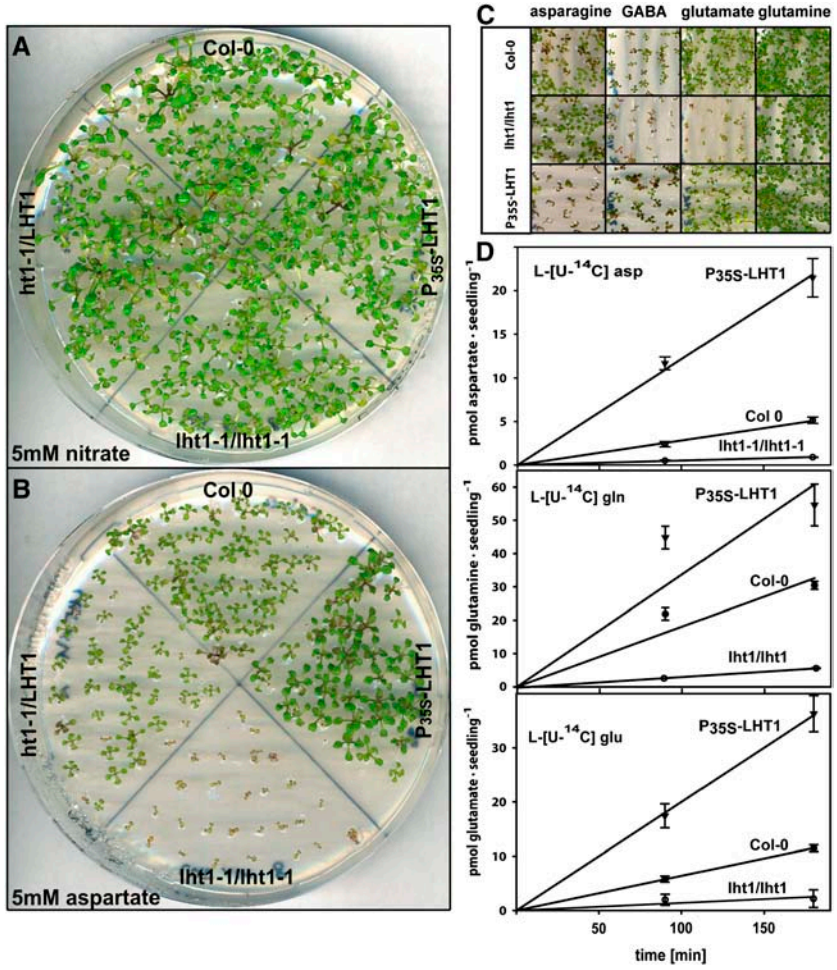

Figure 1. Growth Comparison of T-DNA Insertion Lines, Wild-Type, and P_{35S}-LHT1 Plants on Amino Acids as Sole Nitrogen Source, and Uptake Rates of ^{14}C-Labeled Amino Acids.

(A) Growth of the T-DNA insertion line *lht1/lht1* and P_{35S}-LHT1 plants on 5 mM nitrate as nitrogen source was comparable to the growth of wild-type plants.

(B) On 5 mM Asp as sole nitrogen source, growth of the T-DNA insertion line was strongly reduced and P_{35S}-LHT1 plants grew larger than wild-type plants. Heterozygous plants (*lht1*/LHT1) showed an intermediate growth reduction. No differences were observed during the first 10 d, growth differences were visible after 15 d, and photographs were taken after 21 d.

(C) Growth comparison on Asn, GABA, Glu, and Gln. On 5 mM of the amino acids indicated, no difference was observed with Gln, reduced growth of the T-DNA insertion line was visible with Glu and GABA, and the T-DNA insertion line grew better than the wild type and overexpressors with Asn, possibly as a result of a toxic effect. Photographs were taken after 21 d.

(D) Uptake rates for Asp, Glu, and Gln. In short-time uptake studies, homozygous T-DNA insertion lines showed a reduced uptake rate for Asp (*lht1/lht1*, 0.31 pmol·h^{-1}·seedling^{-1}; Col-0, 1.69 pmol·h^{-1}·seedling^{-1}). Uptake rates were reduced to 18% in the T-DNA insertion line. Uptake rates were enhanced up to 253% of the wild type for Asp in P_{35S}-LHT1 plants (7.28 pmol·h^{-1}·seedling^{-1}). Error bars represent SE ($n = 3$). Linear uptake rates for radiolabeled Glu were 1.06 pmol·h^{-1}·seedling^{-1} for *lht1/lht1* and 3.83 pmol·h^{-1}·seedling^{-1} for Col-0, and rates for Gln were 1.75 pmol·h^{-1}·seedling^{-1} for *lht1/lht1* and 10.8 pmol·h^{-1}·seedling^{-1} for Col-0 ($1n = 10$ seedlings, $n = 3$, error bars indicate SE). Overexpression enhanced the uptake rate in all cases but was less pronounced for Gln. Uptake rates of ^{14}C-labeled amino acids determined for P_{35S}-LHT1 seedlings were 12.0 pmol·h^{-1}·seedling^{-1} for Glu and 22.2 pmol·h^{-1}·seedling^{-1} for Gln.

remaining transport activity in *lht1* may be mediated by other members of the LHT subfamily, although *LHT2* was not found in roots but rather in the tapetum (Lee and Tegeder, 2004). To test whether partial inhibition of *LHT1* may already limit amino acid uptake, heterozygous LHT1/*lht1-1* plants were analyzed. The heterozygous plants displayed reduced *LHT1* mRNA levels and were retarded in growth on Asp as the sole N source, demonstrating that LHT1 activity is limiting (Figure 1B; see Supplemental Figure 2 online). Thus, the analysis of insertion mutants suggests that LHT1 functions as a nonselective amino acid transporter and represents one of the major uptake systems from the growth medium, at least for the amino acids tested.

Broad Selectivity of Yeast-Expressed LHT1

The LHT1 clone used in the original study complemented the His auxotrophy of the yeast strain JT16 (his4, hip1, can1, ura3), a mutant with reduced transport activity for basic amino acids (Chen and Bush, 1997). The substrate specificity, as determined by uptake measurements with radiolabeled amino acids and subtraction of the endogenous background mediated by a large number of endogenous transporters, had indicated that Lys and His were the best substrates, but other substrates, such as Glu, Leu, and several others, were also transported. To analyze the selectivity of LHT1, *LHT1* cDNA was isolated by PCR from ecotype Columbia (Col-0) and expressed in the multiple–amino acid permease knockout yeast strain 22Δ8AA (Fischer et al., 2002), which is unable to use Asn, Asp, citrulline, GABA, Glu, or Pro as the sole nitrogen source. The yeast mutant 22Δ8AA was transformed with vectors expressing three clones of *LHT1* isolated by RT-PCR under the control of the PMA1 promoter; AAP6 served as a positive control. On media containing 3 mM citrulline, Glu, Asp, GABA, or Pro as the sole nitrogen source,

Figure 2. Organ-Specific Expression of *LHT1*, and Phenotypes of T-DNA Insertion Lines, *lht1-RNAi* Plants, and *LHT1*-Overexpressing Plants.

(A) Two homozygous T-DNA insertion lines (*lht1-1* = SALK_034566, *lht1-2* = SALK_026389) were significantly smaller than the wild type when grown in soil. To verify the effect of the disruption of the LHT1 locus, transgenic plants expressing a hairpin construct directed against *LHT1* transcripts were generated. Two lines (*lht1-RNAi-3* and *lht1-RNAi-4*) showed the same reduced growth as both homozygous T-DNA insertion lines. Overexpression of *LHT1* (P_{35S}-LHT1) did not result in a significant phenotype.

(B) The reduction of transcript levels of *LHT1* or overexpression in 35S plants was analyzed by RNA gel blot analysis on RNA extracted from whole plants. No transcripts of *LHT1* were detectable in homozygous lines and RNAi lines.

(C) Total RNA was used as a loading control.

(D) *LHT1* transcripts in Col-0 were detected in all organs except siliques, with the strongest signals in roots and leaves and weaker signals in stems, siliques, and flowers. Plants for RNA extraction were grown in fertilized soil in the greenhouse.

(E) Total RNA from each organ was used as a loading control.

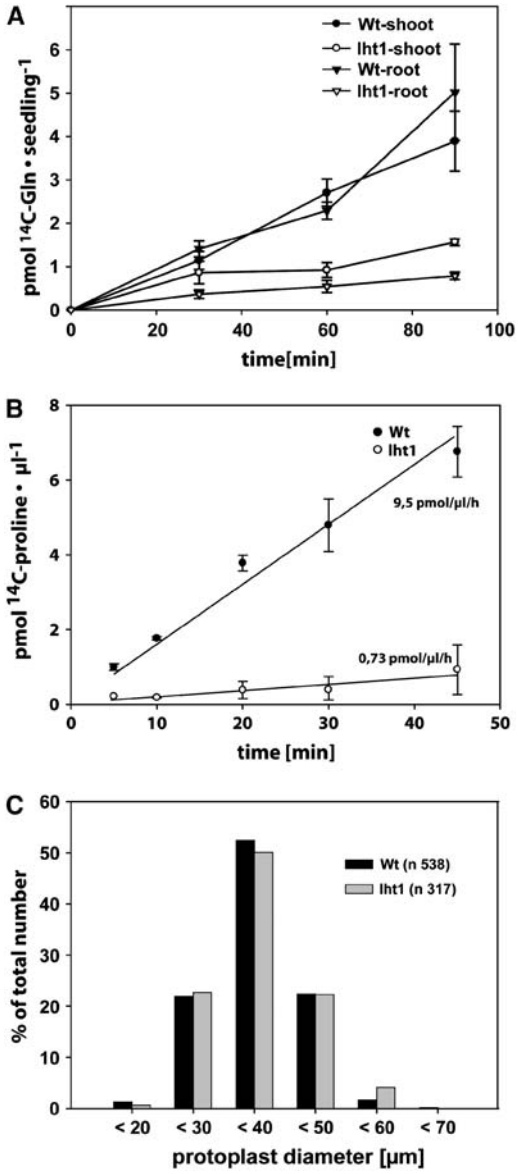

Figure 3. Feeding of [^{14}C]Gln to Roots, Protoplast Uptake with [^{14}C]Pro, and Size Distribution of Protoplasts.

(A) [^{14}C]Gln was applied to the agar, seedlings were removed, and roots and shoots were analyzed separately. Both in roots and shoots of the mutants, uptake of [^{14}C]Gln was strongly reduced. Error bars represent SD ($n = 3$).

(B) Time-dependent uptake of [^{14}C]Pro in mesophyll protoplasts of wild-type (closed circles) and *lht1*/*lht1* (open circles) plants. Mesophyll protoplasts were isolated from 10-week-old plants grown under short-day conditions. Error bars represent SD ($n = 3$).

(C) Size distribution of mesophyll protoplasts isolated from wild-type plants and mutants. Values represent numbers of protoplasts between the indicated sizes as percentages of the total number (wild type, $n = 538$; *lht1*, $n = 317$).

independent transformants expressing *LHT1.1* were able to grow, in contrast with *LHT1.3*, which carried a T402G mutation caused by a PCR error that led to a premature stop codon (Figure 4A). Cells transformed with the control plasmids grew only on 10 mM NH_4SO_4 and weakly on 1 mM Arg. On media with citrulline as the sole nitrogen source, only yeast transformed with functional *LHT1* clones showed significant growth. These data demonstrate directly that LHT1 is able to mediate the transport of a variety of amino acids.

The kinetic properties of LHT1 were determined by uptake experiments with ^{14}C-labeled Pro, His, and Glu in the yeast mutant 22Δ8AA (Figure 4B). The K_m value of LHT1 for Pro was ~10 μM, with a V_{max} of ~80 nmol·g protein^{-1}·min^{-1}; for Glu, the K_m was ~14 μM and the V_{max} was ~100 nmol·g protein^{-1}·min^{-1}; for His, the K_m was ~360 μM and the V_{max} was ~105 nmol·g protein^{-1}·min^{-1}. Pro uptake kinetics were linear over time (Figure 4C), as were the other kinetics for the determination of K_m values (data not shown). Although Glu transport was also significant compared with the previous study (Chen and Bush, 1997), the significant Pro transport activity observed here was not detected in the previous work. The K_m values for Pro and Glu are in the same range as the K_m value for Pro determined for LHT2 (13 μM) (Lee and Tegeder, 2004), and both LHT1 and LHT2 show a higher affinity toward these amino acids compared with the AAPs (Fischer et al., 2002; Okumoto et al., 2002).

To determine the specificity in further detail, [^{14}C]Pro uptake was determined at a 10-fold excess of unlabeled proteinogenic amino acids. Under the conditions tested (i.e., with a Pro concentration at the K_m for Pro [10 μM]), the strongest competitors for Pro uptake were Cys and Phe; the weakest competitors were the basic amino acids His, Lys, and Arg (Figure 4D).

Finally, to demonstrate that amino acids, which are competitors for Pro uptake, are also direct substrates for LHT1, uptake studies were performed at a concentration of 10 μM. All tested amino acids showed linear uptake kinetics, but as already shown in the competition experiments, His was transported with the lowest velocity at a concentration of 10 μM (Figure 5).

The energy dependence of the uptake was shown by omitting glucose from the uptake medium in the experiments using [^{14}C]Gly and [^{14}C]Ala as tracers, leading to a reduction of uptake activity to background levels. The addition of the protonophore CCCP (10 μM) 1 min after the start of the uptake experiment abolished amino acid uptake. These data suggest proton cotransport as the uptake mechanism (Figure 5), although secondary effects like the change in cytosolic pH and the breakdown of ATP synthesis may affect transport activity.

We also compared the properties of LHT1 in the presence and absence of sorbitol, a major difference to the protocol used compared with the previous study (Chen and Bush, 1997), but could not detect significant effects of sorbitol (data not shown).

Thus, LHT1 functions as a general amino acid permease similar to other AAPs (Okumoto et al., 2002); however, compared with all other known amino acid transporters, it displays a much higher affinity for amino acids. Both the high affinity and the broad selectivity are compatible with a function in the uptake of amino acids from soil and with the in planta results observed in *lht1* mutants.

Expression Pattern of LHT1 and Subcellular Localization

RNA gel blot analysis showed strong expression in roots, leaves, stems, flowers, and siliques (Figure 2D) (Chen and Bush, 1997). To analyze the expression pattern in more detail, promoter β-glucuronidase (GUS) studies were performed. A 2.7-kb promoter fragment containing the upstream region (up to the next open reading frame [ORF]) and including the first intron of *LHT1* was fused translationally to the GUS gene (P_{LHT1}-GUS), and *Arabidopsis* plants were transformed. Analysis of 14 independent lines showed a similar GUS pattern. Histochemical GUS studies showed that *LHT1* is expressed in roots already at early stages of development, mainly in the rhizodermis of emerging roots and in lateral roots, but not in the main root (Figures 6A and 6F). In older plants, the tips of all roots show GUS staining (Figure 6B). Expression in young leaves is weaker compared with older leaves and is detected first at the hydathodes. At later stages of leaf development, expression is found in the epidermis, whereas in mature leaves expression was found in all mesophyll cells (Figures 6D, 6E, and 6I). Expression was absent from vascular bundles (Figures 6C to 6E). In flowers, the sepals and petals were also stained, but no expression was detected in siliques and seeds (Figures 6G and 6H). The GUS expression in roots supports the assumed role of LHT1 in the uptake of amino acids from the soil. In leaves, a strong increase in GUS activity is visible during the progression of plant age (Figures 6I to 6K). The leaf expression suggests that LHT1 serves additional functions, potentially the cellular uptake of amino acids delivered from the root via the xylem or in cell-to-cell transport in source leaves. All of the GUS data fit well to the available expression data on public microarray resources and are in good agreement with the observed root expression in seedlings and the developmental change in the expression pattern.

Because amino acids are transported not only across the plasma membrane but essentially in and out of all cellular compartments, the cellular localization of the transporter was analyzed. Fluorescence microscopy of N-terminal P_{35S}-green fluorescent protein (GFP)-LHT1 fusions transiently expressed in protoplasts indicates that LHT1 is located at the plasma membrane (Figure 7). Moreover, LHT1 had been identified in a plasma membrane proteomics analysis (Alexandersson et al., 2004). Thus, LHT1 seems to be responsible for amino acid uptake from the soil and for cellular retrieval from the apoplasm.

A Second Role of LHT1 in Leaves

To test for the function of LHT1 in other tissues in addition to roots (i.e., in leaves), plants were grown in the presence of inorganic nitrogen sources in soil. On fertilized soil, homozygous *lht1* lines were significantly smaller compared with the wild type (Figure 2A). A similar phenotype was observed when the expression of *LHT1* was inhibited by RNAi (Figures 2A and 2B; see Supplemental Figure 1 online). In the heterozygous lines, in which expression of *LHT1* mRNA was reduced by ~50%, growth of the plants in soil was similar to that in the wild type (see Supplemental Figure 2 online). Because nitrogen supply should not be limiting on fertilized soil, the growth phenotype is most likely attributable to a lack of amino acid transport activity at a different site in the plant. Because LHT1 is the only amino acid transporter found to be expressed in leaf cells outside of the vascular system to date, the second crucial

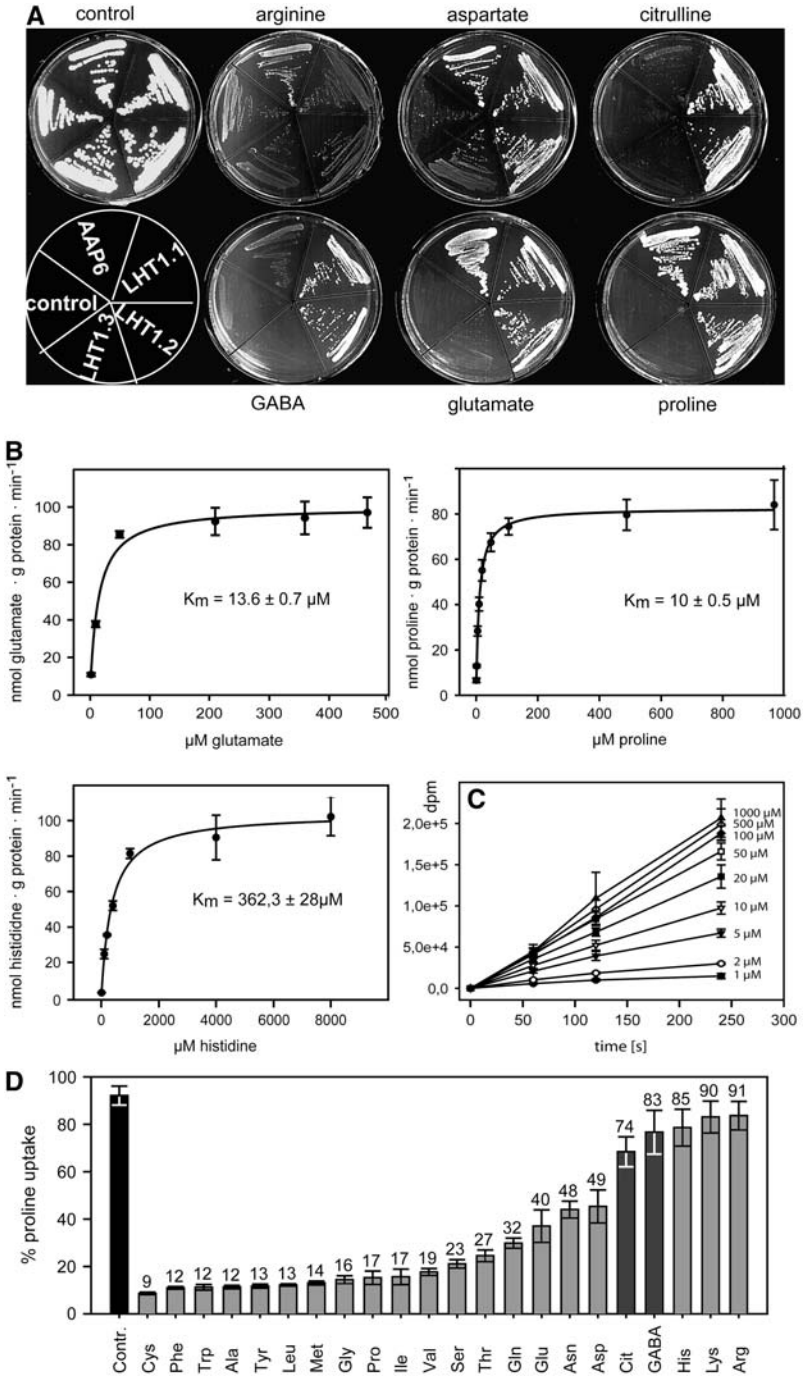

Figure 4. Biochemical Characterization of LHT1.

(A) Comparison of growth of the yeast mutant strain 22Δ8AA mediated by three PCR clones of LHT1 on different amino acids as sole nitrogen source compared with the general amino acid permease AAP6. Yeast transformed with empty vector was used as a control. LHT1.1 and LHT1.2 mediated growth comparable to AAP6 on 3 mM Asp, Pro, or Glu and mediated faster growth on 3 mM citrulline or GABA. LHT1.3, carrying a T402G mutation leading to a premature stop codon attributable to a PCR error, served as a control and did not mediate growth. Plates were scanned after 5 d except citrulline plates (7 d).

(B) Kinetic properties of LHT1. Linear uptake rates of L-[^{14}C]Pro, L-[^{14}C]Glu, and L-[^{14}C]His were determined with concentrations ranging from 1 μM to saturation. Data represent six repeats for each time point (1, 2, and 4 min) and concentration, and error bars indicate SE. The calculated K_m value of LHT1 for Pro is 10 ± 0.5 μM, and V_{max} ~ 82 nmol Pro·g^{-1} protein· min^{-1}; for Glu, K_m is 13.6 ± 0.7 μM and V_{max} ~ 100 nmol Glu·g^{-1} protein·min^{-1}; and for His, K_m is 360 ± 28 μM and V_{max} ~ 105 nmol His· g^{-1} protein·min^{-1}.

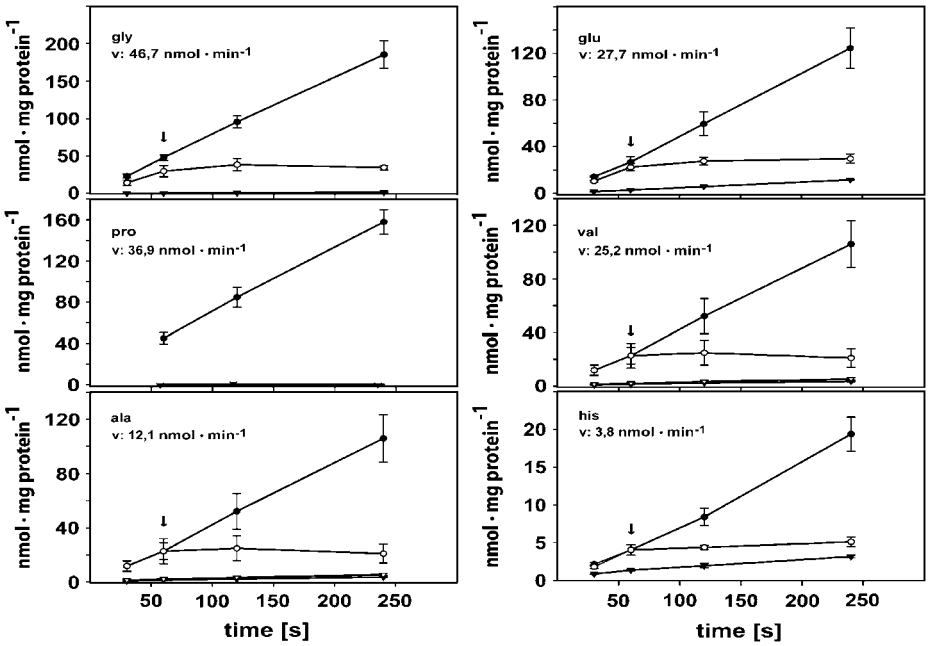

Figure 5. Comparison of Linear Uptake Rates of Six Amino Acids at a Concentration of 10 μM, CCCP Sensitivity, and Energy Dependence of Transport.

^{14}C-labeled Gly, Ala, Val, Glu, Pro, and His were used at a concentration of 10 μM, and the velocity of transport was determined. Closed circles, pDR196-LHT1; open circles, addition of CCCP (10 μM) to yeast expressing LHT1 after 1 min; open triangles, LHT1 without glucose; closed triangles, vector control (pDR196). The addition of CCCP (arrows) abolished the uptake activity of LHT1. Yeast cells expressing LHT1 without glucose displayed no uptake activity above the vector control. The uptake velocity of His was a factor 4- to 12-fold lower than the uptake rates for other amino acids, confirming the lower affinity of LHT1 toward His.

role of LHT1 is potentially in the cellular uptake of amino acids in leaf cells. The long-distance transport of amino acids between roots and leaves and the other organs is complex, because amino acids are both transported in the phloem and xylem and cycle in the plant (Cooper and Clarkson, 1989). A defect in cycling is expected to limit nitrogen use and thus growth, and one would expect amino acids to accumulate in the apoplasm of leaves. A function in leaf cell uptake is supported by increased amino acid content in the apoplasm of *lht1-1* compared with the wild type (Table 1). Given the complexity of cycling and the involvement of multiple transport steps, it is not surprising that the overall amino acid composition of the leaves was altered compared with the wild type and that the total protein content was increased in *lht1-1* leaves (Table 2). Anion and cation analyses showed that nitrate levels were reduced and free ammonium was increased in *lht1* leaves (Table 2).

Reduced Amino Acid Uptake in *lht1* Mesophyll Protoplasts

To test directly whether the loss of one amino acid transporter in mesophyll cells could lead to the increased level of amino acids in the apoplasm of *lht1* mutants, analyses of the uptake of radiotracers into protoplasts isolated from mature leaves of the mutants were performed. In both mutant and wild-type protoplasts, uptake of [^{14}C]L-Pro was linear over 60 min, but protoplasts isolated from *lht1/lht1* plants showed a drastic reduction in Pro uptake velocity (~10% compared with the wild type) (Figure 3B). Because the mutant plants are significantly smaller and uptake capacity depends on the cell surface, protoplast diameters were measured. No difference in size distribution or average size of the protoplast of a typical preparation was found when comparing wild-type and mutant protoplasts (Figure 3C). These data suggest that LHT1 is important for amino acid uptake from the apoplasm into leaf mesophyll cells.

Complementation of the Soil Phenotype by Reexpression of LHT1 in Leaves of *lht1-1* Mutants

To determine whether the two potential phenotypes (i.e., the defect in root uptake and the defect in leaf uptake) are independent, a root-specific *lht1* knockout was generated by expression

Figure 4. (continued).

(C) Linearity and concentration-dependent velocity of Pro transport. These data were used for the K_m calculation of Pro uptake.

(D) Specificity of transport. Uptake of L-[^{14}C]Pro (10 μM) into yeast cells (22Δ8AA-LHT1) was monitored in the presence of a 10-fold excess (100 μM) of final competitor concentration. Numbers indicate percentage uptake compared with control. L-[^{14}C]Pro uptake without competitor was taken as 100%. $n = 6$, and error bars indicate SE.

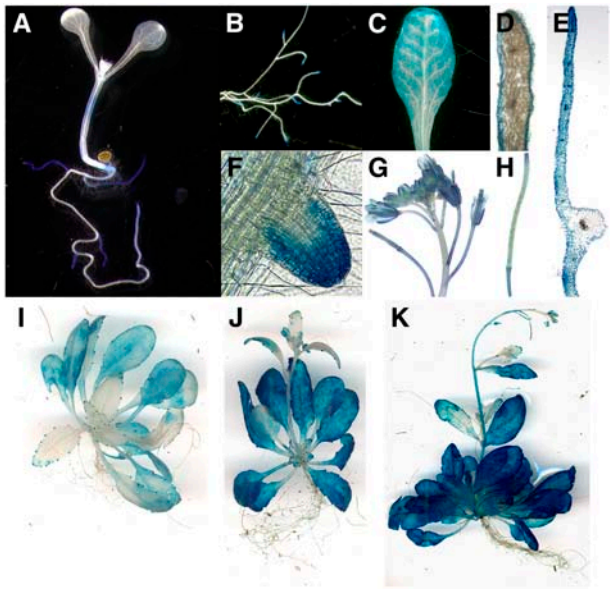

Figure 6. Tissue-Specific GUS Activity under the Control of the LHT1 Promoter.

(A) In seedlings (5 d old), strong GUS expression is visible in the lateral roots and only weak signals are visible in the cotyledons.
(B) In older plants grown in soil, GUS expression can be found at all root tips.
(C) No expression is visible in the veins of leaves.
(D) Cross section of a young leaf shows staining exclusively in the lower and upper epidermis.
(E) Cross section of an old leaf displays staining in all mesophyll cells and in the epidermis.
(F) In roots, staining is also visible in the emerging lateral roots.
(G) In flowers, GUS signal can be found in sepals and pedicels.
(H) No signal is visible in siliques or seeds.
(I) to **(K)** Staining of whole plants grown in fertilized soil after 24 **(I)**, 31 **(J)**, and 42 **(K)** d. All plants were stained for 8 h at 37°C. Signal intensity increased in leaves of older plants.

of *LHT1* in the mutant under the control of the potato stem/leaf-specific promoter St LS1 (Eckes et al., 1986). The St LS1 promoter is active in cells with fully developed chloroplasts, and reporter gene analysis showed the absence of promoter activity in all tissues without green chloroplasts (Stockhaus et al., 1989). *lht1* plants transformed with this construct developed normally in fertilized soil (Figure 9A), whereas the mutants were strongly retarded in growth. The recovery of the phenotype in soil supports the hypothesis that the phenotype in fertilized soil is attributable to reduced cell-to-cell transport and cycling of amino acids. On the other hand, the phenotype of the *lht1* mutant observed on media containing Asp as the sole nitrogen source (Figure 1B) could not be rescued by the expression of *LHT1* under the control of the St LS1 promoter (Figures 9B to 9F), indicating that the expression of *LHT1* in root tissue is necessary for Asp uptake.

Regulation of LHT1 Expression by Nitrogen

Inorganic nitrogen uptake is highly regulated by the availability of nitrogen sources (Gazzarrini et al., 1999; von Wiren et al., 2000). To analyze whether the expression of *LHT1* is regulated depending on the nitrogen supply or nitrogen status of the plant, seedlings were grown on various N sources and mRNA levels in the wild type were determined. In parallel, enzymatic GUS activity was determined in extracts from P_{LHT1}-GUS plants grown under the same conditions. Expression of *LHT1* was found to be inducible by amino acids. On control medium (2MS; see Methods) with 40 mM ammonium nitrate, *LHT1* expression was low. In the presence of other amino acids as sole N sources (5 mM), but also on 5 mM nitrate, expression of LHT1 was induced (Figure 8). The GUS activity determined in parallel experiments with P_{LHT1}-GUS correlates with this upregulation of *LHT1* mRNA levels. The data show that nitrogen supply and composition are sensed by the plant and that LHT1 expression is regulated accordingly. The regulation of expression may be a means of adapting to the local changes in organic and inorganic N supply in the root area. Further studies will be required to define the exact nature of the signal and signaling cascade and the integration with other nitrogen transporters.

Overexpression of LHT1 Leads to Enhanced Amino Acid Uptake in Roots

Given that heterozygous *lht1* mutants showed a reduction in amino acid uptake, we tested whether LHT1 activity limits organic nitrogen uptake in a noncultivated plant species, a question relevant with respect to the finding that plant species differ in their capacity to take up amino acids (Lipson and Nasholm, 2001). To test whether LHT1 is limiting for amino acid uptake, *LHT1* was overexpressed under the control of the CaMV 35S promoter. The overexpressing lines grew larger than wild-type plants on media containing Asp as the sole nitrogen source, whereas the toxicity observed in the presence of Asn was increased (Figures 1B and 1C). The uptake rates of ^{14}C-labeled Asp, Glu, and Gln were also strongly increased in the P_{35S}-LHT1

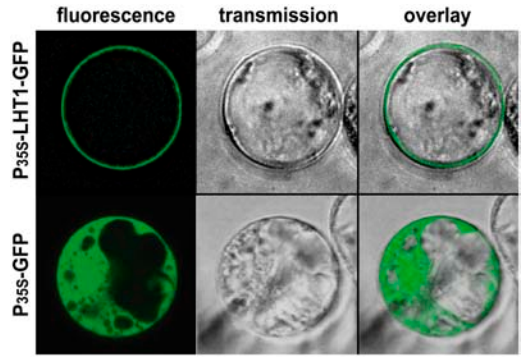

Figure 7. Subcellular Localization Using P_{35S}-LHT1-GFP Fusion Protein.

LHT1 mRNA was translationally fused with GFP and expressed transitionally in *Arabidopsis* protoplasts (P_{35S}-LHT1-GFP). Representative fluorescence microscopic images of a protoplast expressing LHT1-GFP fusion protein are shown. GFP fluorescence was detected at the plasma membrane. Protoplasts expressing GFP protein under the control of the CaMV 35S promoter were used as a control (P_{35S}-GFP). GFP fluorescence in control protoplasts was detected throughout the cytosol. The fluorescence (left) and bright-field (middle) images are overlaid at right.

Table 1. Amino Acids in Apoplasmic Wash Fluids of *lht1-lht1* and Wild-Type Plants

Amino Acids	Col-0 ($n = 3$)	*lht1/lht1* ($n = 3$)	Change (%)	P
Asp	263.7 ± 37.9	220.4 ± 8.4	−16.4	n.s.
Thr	172.6 ± 24.8	67 ± 82.1	−61.2	n.s.
Ser	373.3 ± 47.2	834.3 ± 60.4	+123.4	<0.01
Asn	73.8 ± 4.3	75.4 ± 53.8	+2.1	n.s.
Glu	414.8 ± 66.7	416.5 ± 13.2	+0.4	n.s.
Gln	582.2 ± 85.4	790.3 ± 112.4	+35.8	n.s.
Pro	124.2 ± 12.7	91.2 ± 10.2	−26.6	n.s.
Gly	83.4 ± 9.4	105.9 ± 7.4	+26.9	n.s.
Ala	702.7 ± 109.6	814.8 ± 113.5	+16	n.s.
Val	48.7 ± 10.8	123.9 ± 5.5	+154.6	<0.01
Ile	22.8 ± 1.9	65.9 ± 9.3	+189.8	<0.01
Leu	17 ± 5.2	78.2 ± 4.8	+361.3	<0.001
Phe	12 ± 7.4	64 ± 9.3	+433.6	<0.01
GABA	278.6 ± 46.6	378.1 ± 46.5	+35.7	n.s.
Lys	26.9 ± 2.6	62.3 ± 2.6	+131.6	<0.001
His	26.0 ± 8.1	46.6 ± 4.2	+79.6	<0.05
Arg	57.6 ± 7.9	92 ± 21.1	+59.9	n.s.
Total	3,280 ± 488	4,326 ± 546	+32	<0.05

Amino acids were determined after infiltration and centrifugation of leaves of 3 × 30 wild-type plants and 3 × 100 mutant plants (see Methods). The concentrations of 14 amino acids were increased, and those of 3 amino acids were decreased; the levels of 7 amino acids were increased significantly, including Ser. Total concentration of amino acids was also significantly increased in the mutants. Student's *t* test was used for statistical analysis. Values shown are μM ± SE. n.s., not significant.

plants, consistent with the observation of improved growth under limiting N conditions (Figures 1B and 1D). Partial growth inhibition was observed in heterozygous lines that expressed less *LHT1* mRNA on Asp-containing media (Figure 1B), establishing a direct correlation to the growth phenotype under limiting N conditions and the *LHT1* mRNA levels. The overexpression lines showed no obvious phenotypic differences compared with the wild type under greenhouse conditions, and amino acid levels in the apoplasmic wash fluid of P_{35S}-LHT1 plants were not altered significantly (Table 3), suggesting that LHT1 does not limit xylem unloading in leaves. The data show that overexpression of an amino acid transporter can improve plant growth under limiting N conditions.

DISCUSSION

Nitrogen is, at least quantitatively, the most important mineral nutrient. Thus, plants have developed multiple transport systems to acquire the various forms of nitrogen from the soil and to distribute them within the plant, ultimately to securely supply their seeds with nutrients for early phases of development.

LHT1 Is Involved in Amino Acid Uptake into Roots

Apart from the inorganic nitrogen forms, ammonium and nitrate, the soil may also contain significant amounts of organic nitrogen derived from the decomposition of organic matter. It had been shown that plants have the capacity to take up amino acids, and putative amino acid transporters have been localized to epidermal root cells (Soldal and Nissen, 1978; Bick et al., 1998).

In this work, LHT1, a transporter identified previously by Chen and Bush (1997), transports a broad spectrum of amino acids and is expressed in the rhizodermis of roots, making it a candidate for

Table 2. Metabolic Analysis of Leaves of the T-DNA Insertion Line Salk_034566 (*lht1/lht1*) Compared with the Wild Type (Col-0)

Analysis	Col-0 ($n = 7$)	*lht1/lht1* ($n = 15$)	Change (%)	P
Amino Acids				
Asp	18.4 ± 0.8	17.2 ± 0.67	−6.3	n.s.
Thr	8.2 ± 0.4	8.9 ± 0.31	+8.9	n.s.
Ser	12.5 ± 0.9	23.7 ± 0.79	+89.2	<0.001
Asn	4.5 ± 0.32	12.8 ± 0.71	+186.2	<0.001
Glu	41.7 ± 1.6	33.9 ± 1.3	−18.7	<0.01
Gln	30.5 ± 2.4	23.6 ± 1.0	−22.7	<0.01
Pro	7.6 ± 0.3	2.7 ± 0.19	−64.1	<0.001
Gly	0.79 ± 0.06	0.83 ± 0.06	+3.9	n.s.
Ala	5.9 ± 0.18	5.8 ± 0.25	−2.6	n.s.
Citrulline	0.56 ± 0.04	0.39 ± 0.04	−29.9	<0.05
Val	2.86 ± 0.12	4.3 ± 0.13	+52.0	<0.001
Cys	0.48 ± 0.04	0.81 ± 0.03	+69.4	<0.001
Met	0.09 ± 0.01	0.11 ± 0.01	+19.7	<0.05
Ile	0.50 ± 0.04	0.81 ± 0.05	+60.6	<0.001
Leu	0.62 ± 0.05	0.88 ± 0.05	+41.8	<0.01
Tyr	0.16 ± 0.03	0.19 ± 0.02	+17.9	n.s.
Phe	0.42 ± 0.04	1.6 ± 0.08	+274.9	<0.001
GABA	0.42 ± 0.05	0.4 ± 0.02	−1.7	n.s.
Ethanolamine	1.34 ± 0.12	1.7 ± 0.09	+26.9	<0.05
Lys	0.56 ± 0.04	0.95 ± 0.04	+69.8	<0.001
His	0.51 ± 0.03	1.0 ± 0.04	+103.1	<0.001
Arg	1.85 ± 0.19	3.7 ± 0.3	+101.3	<0.01
Total	145.2 ± 4.7	151.3 ± 4.8	+4.20	n.s.
Anions				
Fluoride	27.8 ± 2.0	25.3 ± 1.06	−9.1	n.s.
Chloride	41.7 ± 3.5	43.7 ± 0.89	+4.8	n.s.
Nitrate	1604.4 ± 42.6	777.7 ± 22.2	−51.5	<0.001
Phosphate	137.5 ± 4.8	171.1 ± 5.4	+24.5	<0.01
Sulfate	193.7 ± 6.7	219.3 ± 5.6	+13.2	<0.05
Total	2,005.2 ± 49.1	1,237.2 ± 26.8	−38.3	<0.001
Cations				
Sodium	44.5 ± 1.5	43.1 ± 2.1	−3.2	n.s.
Ammonium	81.8 ± 20.6	235.2 ± 25.7	+187.5	<0.001
Potassium	936.7 ± 28.7	885.6 ± 25.1	−5.5	n.s.
Magnesium	211.3 ± 5.7	132.4 ± 3.2	−37.7	<0.001
Calcium	1110.2 ± 26.8	637.5 ± 15.2	−42.6	<0.001
Total	2,384.6 ± 53.3	1,933.9 ± 50.9	−18.9	<0.001
Soluble protein	102.2 ± 3.4	118.4 ± 2.4	+15.8	<0.01

Rosette leaves were pooled and homogenized in liquid nitrogen, and samples were freeze-dried before analysis. Sixteen of 22 determined amino acids (including GABA) were significantly changed. The strongest changes among anions and cations were reduced nitrate and increased ammonium content in *lht1/lht1* plants. Total protein content was significantly increased (+15%) in the insertion lines. Student's *t* test was used for statistical analysis. Values shown are nmol/mg dry weight ± SE except for soluble protein, which is μg/mg dry weight. Also for soluble protein, $n = 5$ for Col-0 and $n = 6$ for *lht1/lht1*. n.s., not significant.

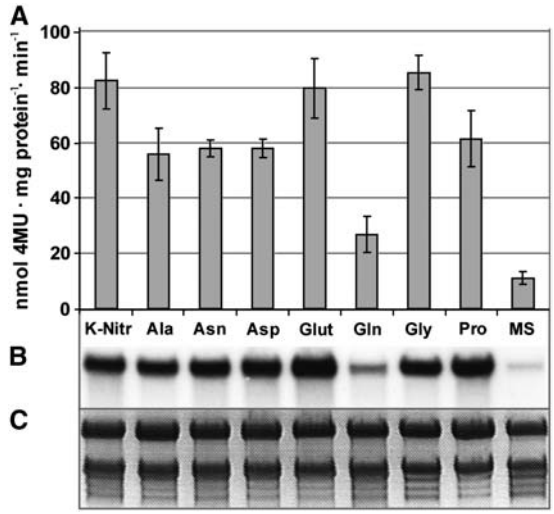

Figure 8. GUS Activity under the Control of the LHT1 Promoter and *LHT1* mRNA Levels in Seedlings Grown on Various N Sources.

(A) LHT1 promoter-GUS seeds were sown on the indicated amino acids or nitrate as N source (all at 5 mM), and GUS activity was determined after 14 d as described. GUS activity was induced on all amino acids and nitrate as N sources compared with MS medium containing 40 mM ammonium nitrate. Induction was lowest on plates containing 5 mM Gln (1n = five seedlings, $n = 3$, error bars represent SE). 4MU, 4-methylumbelliferone.
(B) Corresponding expression levels of *LHT1* determined by RNA gel blot analysis. Wild-type (Col-0) seedlings were grown as described above, and RNA was extracted. The level of *LHT1* mRNA is in good correlation with the determined GUS activity and was lowest for plants grown on MS medium (40 mM ammonium nitrate). On Gln, only weak induction of *LHT1* is visible.
(C) Total RNA was used as a loading control.

an uptake carrier for amino acids. The growth of mutants defective in LHT1 function is retarded, and radiotracer studies provide direct evidence for reduced uptake activity.

Moreover, the growth of *lht1* mutants on Asp as the sole nitrogen source was not complemented when the *LHT1* gene was expressed only in leaf tissue (Figures 9B to 9F), whereas the phenotype was complemented by expressing the *LHT1* gene under the control of the 35S promoter (Figure 1B). These results indicate that LHT1 is responsible for the uptake of amino acids into root tissue, which may occur either at the root surface or from the apoplasm of the cortex.

The kinetic properties of LHT1 and its high affinity toward amino acids are consistent with ecological data. Free amino acid levels in soils are 20 to 100 μM (Lipson and Nasholm, 2001). The function in cellular uptake is further supported by the finding that LHT1 is sensitive to protonophores and that the activity depends on the presence of glucose as an energy source (Chen and Bush, 1997; this work). Together, the biochemical properties of LHT1 and its localization in lateral roots are all in agreement with a function in amino acid uptake from the soil. The direct correlation of the expression level of *LHT1*, amino acid uptake, and growth is supported by the reduced growth of heterozygous plants on Asp as a nitrogen source and the better growth of *LHT1*-overexpressing plants. The capacity to take up amino acids even in a species naturalized over a broad latitudinal and climatic range may increase competitiveness, especially under limiting N conditions. It has been shown that the natural variance in uptake capacity varies within the species of a family, dependent probably on adaptation to different climatic environments, resulting in a higher or lower concentration of organic N in soil (Raab et al., 1999). This can be highly relevant for plant growth because the ability to use limiting nutrients such as amino acids is an important factor for the competitive productivity of a plant species. In arctic regions with high amounts of organic N in the soil, the use of limiting N determines the dominance of a species (McKane et al., 2002). Moreover, plants compete with microorganisms for organic nitrogen in soil (Lipson et al., 1999), and high-affinity uptake mechanisms and specialization can help them to survive in N-limited regions.

The data showing enhanced amino acid uptake by plants overexpressing LHT1 open new research areas, such as whether the overexpressing plants are more competitive in wild populations and can outcompete other plants and microorganisms under limiting N supply. In addition, the enhanced uptake capacity for organic N may be one step toward improving the nitrogen use efficiency of crop plants, expanding agriculture into areas where cultivation has not been possible and decreasing nitrogen leaching and ground water contamination.

In contrast with *Arabidopsis*, most plants are mycorrhized. Mycorrhiza contribute to nitrogen supply and transfer nitrogen across the fungus–root interface, presumably in the form of amino acids (Wipf et al., 2002a). Thus, it is conceivable that in mycorrhized species, LHT1 homologs may be involved in the transfer of fungus-derived amino acids to the plant. In addition, it has been shown that mineralization of organic N is not a prerequisite for the uptake of nitrogen in boreal forests (Nasholm et al., 1998) and that intact amino acids are taken up directly from the soil (Meline

Table 3. Amino Acids in Apoplasmic Wash Fluids of P_{35S}-LHT1 and Wild-Type Plants

Amino Acids	Col-0 ($n = 3$)	P_{35S}-LHT1 ($n = 3$)	Change (%)	P
Asp	263.7 ± 37.9	302.9 ± 49.5	+14.9	n.s.
Thr + Ser	546.1 ± 42.4	586.3 ± 59.3	+7	n.s.
Asn	73.8 ± 4.3	31.6 ± 20.6	−57.2	n.s.
Glu	414.8 ± 66.7	438.2 ± 41.0	+5.6	n.s.
Gln	582.2 ± 85.4	611.9 ± 55.5	+5	n.s.
Pro	124.2 ± 12.7	189.1 ± 7	+52.3	<0.01
Gly	77.9 ± 9.4	180.8 ± 105.1	+132	n.s.
Ala	669.9 ± 109.6	770.5 ± 107.1	+15.1	n.s.
Val	48.7 ± 10.8	36.4 ± 5.6	−25.1	n.s.
Ile	22.8 ± 1.9	15.4 ± 2.5	−32.4	n.s.
Leu	17 ± 5.2	20.5 ± 3	+21.2	n.s.
Phe	12 ± 7.4	19.5 ± 4.1	+62.9	n.s.
GABA	278.6 ± 46.6	290.5 ± 43.8	+4.3	n.s.
Lys	26.9 ± 2.6	20.8 ± 6.3	+22.7	n.s.
His	22.5 ± 8.1	23.5 ± 4	+4	n.s.
Arg	57.6 ± 7.9	27.8 ± 2	−52	<0.01
Total	3,237 ± 249	3,565 ± 317	+10	n.s.

Amino acids were determined after infiltration and centrifugation of leaves. Total concentration of amino acids was not significantly changed; only Pro concentration was significantly increased in the P_{35S}-LHT1 plants. Student's t test was used for statistical analysis. Values shown are μM ± SE. n.s., not significant.

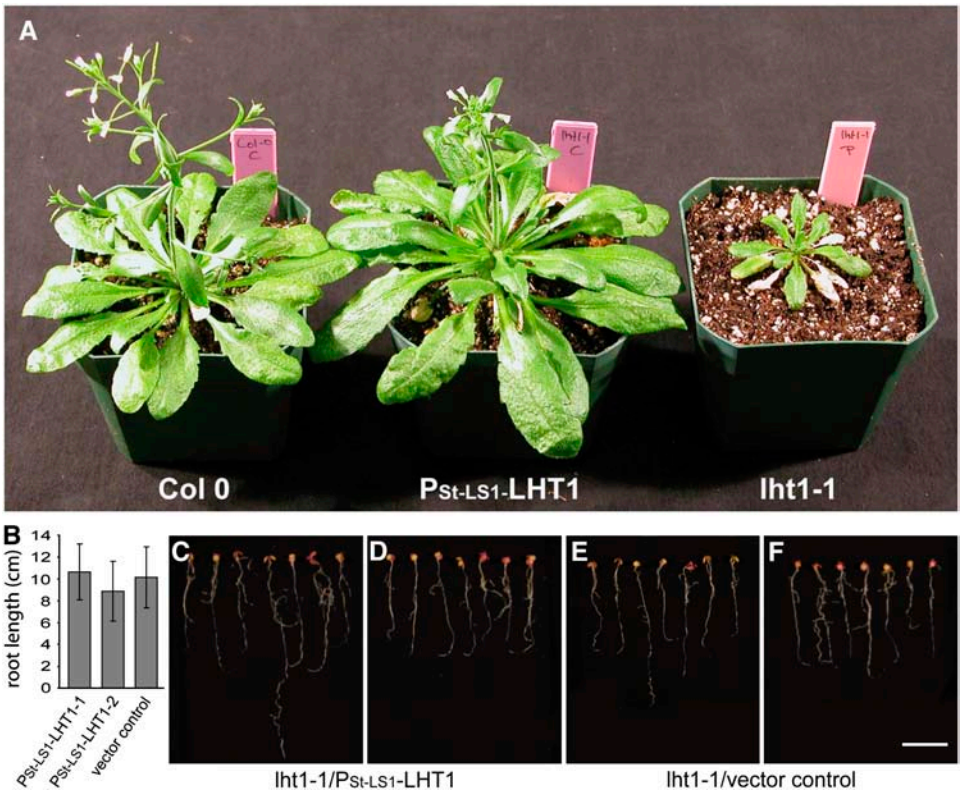

Figure 9. Expression of *LHT1* under the Control of the Potato LS1 Promoter in the *lht1* Mutant Complements the Growth Phenotype in Soil but Not on Media Supplemented with Asp as the Sole Nitrogen Source.

(A) *lht1* mutants transformed with LHT1 cDNA under the control of a leaf-specific promoter, $P_{St\text{-}LS1}$-LHT1, grew normally in fertilized soil, whereas *lht1* mutants were retarded in growth.

(B) to **(F)** Expression of *LHT1* under $P_{St\text{-}LS1}$-LHT1 does not complement the growth phenotype of the *lht1* mutant in axenic culture on media containing 5 mM Asp as the sole nitrogen source.

(B) Root length of seedlings after growth on agar plates containing 5 mM Asp and glufosinate for 28 d. Expression of $P_{St\text{-}LS1}$-LHT1 in *lht1* mutants did not improve growth compared with *lht1* mutants transformed with the vector control.

(C) and **(D)** Two independent lines of *lht1*/$P_{St\text{-}LS1}$-LHT1 plants grown on 5 mM Asp.

(E) and **(F)** Two independent lines of *lht1* plants expressing vector control grown under the same conditions as the lines in **(C)** and **(D)**. Note that no phenotypic difference can be observed between **(C)** and **(D)**, and **(E)** and **(F)**. Bar = 1 cm.

and Nilsson, 1953). Trees, although most are mycorrhized, are able to use supplied organic N as a sole nitrogen source, and no growth differences are observed on amino acids compared with inorganic forms of nitrogen (Ohlund and Nasholm, 2001). LHT1 homologs may be important for amino acid uptake in these species as well, because database searches show that LHTs are present not only in other dicotyledons but also in monocotyledonous species (rice [*Oryza sativa*], Os HT [Liu et al., 2005]; easter lily [*Lilium longiflorum*], accession number BAA04838.1). The availability of LHT1 as a probe may provide a useful starting point for studying N efficiency in ecosystems in which organic N plays an important role in plant growth and species development.

A Potential Role for LHT1 in Apoplasmic Retrieval in Leaves and Amino Acid Cycling

Apart from its role in amino acid uptake from the substrate, LHT1 seems to play a second role in amino acid distribution. In soil fertilized with inorganic nitrogen, plant growth was severely inhibited. Because the major nitrogen sources, ammonium and nitrate, were present under these conditions, it is not likely that the inhibition of growth is attributable to reduced amino acid uptake. The expression of LHT1 in leaf mesophyll and epidermis suggested a role for LHT1 in the uptake of amino acids into leaf cells from the apoplasm. This hypothesis is consistent with the finding that the apoplasmic wash fluids of the *lht1* mutants are enriched in amino acid content (Table 1). Mesophyll protoplasts isolated from leaves of the mutants display a strong reduction in amino acid uptake (Figure 3B). Moreover, reintroduction of LHT1 into the *lht1* mutant under the control of the stem/leaf-specific promoter St LS1 was sufficient to suppress the growth deficiency phenotype.

Amino acids are transported from leaves to roots in the phloem and from roots to shoots via the xylem. On average, each 1 mol of N absorbed cycles once before being used for growth (Cooper and Clarkson, 1989). Little exchange has been

observed between N in the transport pools and that in the bulk of shoots and roots (Atkins, 2000). Thus, inhibition of uptake from the apoplasm reduces cell-to-cell transport and the cycling of amino acids within the plant. A defect in amino acid cycling is expected to limit nitrogen use and thus growth. Because extracellular pathogens such as epiphytic fungi and bacteria are thought to use amino acids for their nutrition, the presence of a retrieval system for amino acids from the apoplasm may also represent an important passive defense mechanism to keep the amino acid levels in the apoplasm low (Struck et al., 2004). It is thus suggested that LHT1 plays a role in taking up amino acids delivered to the mesophyll (e.g., via the xylem) and in keeping amino acid levels low in the cell wall space around leaf cells. The fact that the amino acid levels in the apoplasmic wash fluids of the overexpressing plants is not further reduced compared with those in control plants (Table 3) suggests that the available LHT activity is not limiting in the wild type. The ~30% increase in amino acid accumulation in the apoplast of *lht1* mutants may be considered low in that LHT1 would be the only importer for amino acids from the apoplasm; however, it is expected that other processes, such as phloem loading and regulatory effects, limit the extracellular levels in *lht1* mutants. The observation that a mesophyll amino acid transporter is required for normal plant growth suggests that cycling is of central importance. Pulse-labeling studies in these mutants may provide novel insights into the complex cycling processes (Atkins, 2000).

The expression profile of *LHT1* in leaves is compatible with the uptake into the mesophyll and epidermal cells, whereas other transporters are probably involved directly in the uptake of xylem-borne amino acids around the xylem, such as AAP6 (Okumoto et al., 2002). Nevertheless, the phenotype conferred by *lht1* in fertilized soil and the altered levels of various amino acids (Table 2) show that this disruption interferes with a complex network of amino acid transport and metabolism. At least 53 putative transporters for amino acids have been identified. Most transporters analyzed to date show broad selectivity. Considering the large number of putative amino acid transporters, combined with possible colocalization, spatial and temporal overlapping expression, or specialized function in subcellular compartments, concerted effort will be required to analyze the complete cycling system. In some cases, the disruption of transporter genes leads to no visible phenotype, as in AAP3 (Okumoto et al., 2004). Double or triple knockouts may help determine how these transporters function in the network of amino acid distribution within the plant. With 53 putative amino acid transporters (Wipf et al., 2002b), however, a combinatorial analysis of all triple knockout mutants would involve the analysis of 23,426 combinations (n choose k, $\binom{n}{k} = \frac{n!}{k!(n-k)!}$ n = 53, k = number of *knock out* combinations). This demonstrates that a complete understanding of amino acid transport requires concerted action to examine the functions of all amino acid transporters. In addition, novel approaches will be required to obtain insights regarding the spatial and temporal distribution of amino acids in mutant leaves (Looger et al., 2005).

METHODS

Plant Material, Growth, Transformation, and Transport Measurements

Arabidopsis thaliana plants (ecotype Col-0) were grown in growth chambers (14 h of light, 21.5°C) on Murashige and Skoog (MS) medium (40 mM ammonium nitrate; Murashige and Skoog, 1962) supplemented with 2% glucose (2MS) or 2MS without nitrogen (2MS-N) supplemented with 5 mM of each nitrogen source. Plant material used for RNA gel blot analysis, amino acid HPLC, and promoter-GUS expression was cultured in the greenhouse (16 h of light, 25°C) in soil. Transformation was performed under vacuum infiltration with *Agrobacterium tumefaciens* strains pGV2260 and pGV3101 (Bechtold and Pelletier, 1998). The suspension culture of tobacco (*Nicotiana tabacum*) Bright Yellow-2 cell line was grown as described (Merkle et al., 1996). Protoplasts from tobacco Bright Yellow-2 cultures were prepared as described (Merkle et al., 1996). Transient transformation of the protoplasts with polyethylene glycol was performed according to the protocol of Negrutiu et al. (1992). For confocal laser scanning microscopy, protoplasts were incubated overnight at 24°C in the dark after transformation and observed with a Leica DM RE microscope.

T-DNA insertion lines (Alonso et al., 2003) provided by the Salk Institute Genomic Analysis Laboratory were ordered at the ABRC. The T-DNA insertions of SALK_034566 (exon 3) and SALK_026389 (intron 4) were confirmed by sequencing PCR products with pROK2-specific left border primer.

For transport measurements, seedlings were grown for 14 d on 2MS (2% sucrose, 40 mM ammonium nitrate) and transferred in 1 mL of 2MS-N supplemented with 9.25 kBq of L-[U-^{14}C]Asp (7.66 GBq/mmol), L-[U-^{14}C]Glu (8.81 GBq/mmol), or L-[U-^{14}C]Gln (8.85 GBq/mmol). Samples for linear uptake rates were removed after 90 and 180 min, transferred to 8 mL of ice-cold buffer (2MS-N, 10 mM Asp, 10 mM Glu, and 10 mM Gln), filtered, and washed twice with 8 mL of buffer. The seedlings were transferred to 1 mL of tissue solubilizer (*p*-diisobutyl-cresoxyethoxyethyl dimethylbenzylammonium hydroxide; Packard Instrument), incubated overnight, and neutralized with 1 mL of acetic acid, and radioactivity was determined by liquid scintillation spectrometry. The measurements represent means of three parallel experiments, and each sample consisted of 10 seedlings.

To determine the uptake of Gln into roots and shoots separately, [^{14}C]Gln (92.5 kBq in 4 mL of water) was applied carefully to the plates without contacting the leaves and allowed to diffuse for 60 min. Seedlings were removed at the indicated time points, and roots and shoots were separated and treated as described above.

Protoplast Work: Mesophyll Protoplasts, Viability Assay, Size Measurements, and Uptake

Mesophyll protoplasts were prepared from 10-week-old plants grown under short-day conditions (8 h of light, 16 h of dark). Two pots of wild-type plants and six to eight pots for *lht1* plants were used. Abaxial sides of leaves were peeled and collected in glass Petri dishes in mannitol solution (0.5 M mannitol). The mannitol solution was replaced with cell wall–digesting medium (1% [w/v] Cellulase [Onozuka R10], 0.25% Macerozyme (Duchefa), 0.4 M mannitol, 8 mM $CaCl_2$, and 10 mM MES, pH 5.5) and incubated for 3 h at 28°C without shaking. The resulting suspension was carefully filtered subsequently through nylon mesh (120 and 80 μm). Protoplasts were sedimented and washed twice at 100*g* for 5 min in 0.16 M mannitol/0.13 M $CaCl_2$ and finally resuspended in ~3 mL of uptake medium (0.4 M mannitol, 8 mM $CaCl_2$, and 10 mM MES, pH 5.5). The density of the suspension was adjusted to 10^6 cells/mL. The viability of the preparation was determined with fluorescein diacetate (Sigma-Aldrich) as described by Lasat et al. (1998). The viability of a typical preparation ranged from 89 to 93%.

To measure the diameter of the protoplasts, diameters were determined from digital images of the suspension and measured with the freeware tool ImageJ (http://rsb.info.nih.gov/ij/) using the squares of a hemacytometer (250 μm × 250 μm) as the calibration standard.

Uptake experiments were performed as described previously with slight modifications (Brown et al., 1997; Cosio et al., 2004). Protoplasts were preincubated with ^3H_2O (168 kBq/mL) for 30 min at 22°C to allow ^3H_2O to reach equilibrium inside and outside the protoplasts, enabling the calculation of the protoplast volume as described (Fieuw and Willenbrink, 1991; Brown et al., 1997).

Uptake experiments were started by adding [^{14}C]Pro (84 kBq/mL final concentration) in uptake buffer. At the time points indicated, 100 μL of the suspension was removed and placed on top of a discontinuous gradient of 150 μL of silicon oil (AR200; Fluka) on top of 30 μL of 40% Percoll medium (uptake solution with 40% Percoll, pH 5.5) in a 400-μL microcentrifuge tube. The samples were centrifuged (20 s, 10,000*g*) to wash the protoplasts through the oil and separate from the feeding solution. The tips containing the protoplasts were cut off and placed in scintillation vials, and radioactivity was counted via liquid scintillation spectrometry. One microliter of the supernatant was taken in parallel for the determination of radioactivity. Uptake of [^{14}C]Pro was calculated as the increase of ^{14}C in the sample in relation to the amount of ^{3}H as described (Brown et al., 1997). The formula used was {(^{14}C [dpm] in protoplast pellet/^3H_2O [dpm] in pellet × ^{14}C [dpm] in 1 μL of supernatant/^3H_2O [dpm] in 1 μL of supernatant)} × external Pro concentration (nmol/μL) to calculate the amount of Pro taken up (nmol) and the protoplast volume (μL).

DNA Work

Yeast Expression

The ORF of *LHT1* was amplified by PCR from first-strand cDNA of RNA extracted from leaves of Col-0 and cloned with *Pst*I/*Sal*I in the yeast expression vector pDR196. Sequences of the two independent functional clones were verified by sequencing the PCR products and found to be identical to that of At5g40780.1.

35S Construct

The ORF of *LHT1* was excised with *Sma*I/*Bam*HI from pDR196 and cloned into *Xho*I (blunted with T4 DNA polymerase) and *Bam*HI of pRT100 (Töpfer et al., 1987). Subsequently, the cassette (CaMV 35S-ORF LHT1-CaMV polyA signal) was excised with *Sph*I (blunted) and cloned into *Ecl*136II of the binary vector pCB302 (Xiang et al., 1999).

pRTRNAi, a Vector to Generate Double-Stranded RNA

The second intron of *AAP6* flanked by 5 bp (length, 844 bp) was amplified by PCR on genomic DNA and cloned inclusive of two new sites (*Sma*I and *Sal*I) 3′ of the intron into *Ecl*136II/*Xba*I of pRT100.

Construct to Induce Posttranscriptional Gene Silencing

A 374-bp fragment (ORF positions 49 to 422) of *LHT1* was amplified by PCR and cloned first with sense *Xho*I/*Ecl*136II and second with antisense *Sma*I/*Sal*I into pRTRNAi. Subsequently, the cassette (CaMV 35S-sense-Intron-antisense-CaMV polyA) was cloned with *Pst*I in pCB302.

GFP Fusion

The PCR-amplified ORF of *LHT1* was cloned behind the CaMV 35S promoter *Ecl*136II/*Bam*HI in front of GFP5 (S65T). The linker between *LHT1* and GFP was seven amino acids (GIQGDII).

Promoter-GUS Fusion

The promoter GUS construct consisted of promoter (2740 bp upstream of the start codon), exon 1 (30 bp), intron 1 (1275 bp), and 28 bp of exon 2. An internal (intron 1) *Eco*RI site was used to split this construct into two fragments. These two fragments were cloned into *Xba*I/*Eco*RI and *Eco*RI/*Eco*RV in pBluescript SK− (Stratagene). Subsequently, the total promoter construct *Xba*I/*Eco*RV (4063 bp) was cloned into *Xba*I/*Sma*I in frame with *uidA* (GUS) of pCB308 (Xiang et al., 1999). The expressed fusion protein consisted of 19 amino acids of LHT1 (MVAQAPHDDHQDDEKLAAA) and a linker of 6 amino acids (MGGQSL) fused to *uidA*. PCR was performed on genomic DNA, and correct cloning was confirmed by sequencing.

St LS1 Promoter-LHT1 Construct

The St *LS1* promoter-*ocs* terminator cassette from pA9 (Eckes et al., 1986) was digested with *Hin*dIII and *Eco*RI and cloned into the *Hin*dIII/*Eco*RI sites of pCR2.1-TOPO (Invitrogen). *Xho*I and *Bam*HI sites were introduced between the St *LS1* promoter and the *ocs* terminator, and a second *Hin*dIII site was introduced behind the *Eco*RI site by site-directed mutagenesis (Kunkel, 1985). The vector was named pAH1. The *LHT1* coding region was amplified by PCR, introducing *Xho*I and *Bam*HI sites at the 5′ and 3′ ends, respectively. This *LHT1* fragment was cloned into the *Xho*I and *Bam*HI sites of pAH1. The St *LS1* promoter-*LHT1*-*ocs* terminator cassette was excised with *Hin*dIII and cloned into the *Hin*dIII site of the pPZP312 binary vector, a derivative of pPZP212 (U10462) carrying the *basta* resistance gene. The correct sequence was confirmed.

Primers

Sequence-specific primers are shown in uppercase letters, and restriction sites are underlined. For yeast expression: *Pst*I 5′ primer, 5′-ctgcagACCATGGTAGCTCAAGCTCC-3′; *Sal*I 3′ primer, 5′-gtcgacTTATGAGTAAAACTTGTATCC-3′. For GFP fusion: blunt 5′ primer, 5′-ccccctgcagACCATGGTAGCTCAAGCTCC-3′; *Bam*HI 3′ primer, 5′-ggatccTGAGTAAAACTTGTATCCTTTTGC-3′. For pRTRNAi vector: intron *AAP6* 5′ primer, 5′-ctcCTAGGTCAGATTCGCTATC-3′; intron *AAP6* 3′ primer, 5′-tctagagtcgacccccccgggTTCCTCCTGTTTATCTTTTAACATC-3′. For RNAi constructs: *Xho*I 5′ primer, 5′-ctcgagGCAGCAGCGAGACAAAAAG-3′; blunt 3′ primer, 5′-GATTTGCCTCCAGTGACC-3′. For GUS fusion, *Xba*I 5′ primer, 5′-tctagaCCTTCATGTTCTAAACTATATG-3′; *Eco*RI 3′ primer, 5′-GTTTTCGTAAGAATTCAATTAC-3′; *Eco*RI 5′ primer, 5′-GTAATTGAATTCTTACGAAAAC-3′; blunt 3′ primer, 5′-atcGCTGCTGCTAATTTCTCATC-3′. For St LS1-promotor fusion: pAH1, 5′-gagagcaaaagaagaaaaaggctcgagtttggatcccctgctttaatgagatatgcg-3′; pAH2, 5′-ctatagggcgaattgggaagcttatatctgcagaattc-3′. For LHT1: XhO1, 5′-gagctcgagatggtagctcaagctcctcatgatg-3′; BamH1, 5′-gagggatccTTATGAGTAAAACTTGTATCCTTTTGC-3′.

RNA Work

Total RNA was isolated from mature leaves, stems, and other organs, and 20 μg of total RNA was separated on 1.2% formaldehyde agarose gels (Riesmeier et al., 1994). Hybridization was performed at 65°C in 0.25 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA for 16 h using the cDNA of *LHT1* as a probe. Filters were washed twice with 2× SSC/0.1% SDS (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.2× SSC/0.1% SDS for 20 min at 65°C and exposed to x-ray film (Hyperfilm Amersham)

Yeast, Transformation, Competition, and Transport Measurements

The yeast strain 22Δ8AA (Fischer et al., 2002) was transformed (Dohmen et al., 1991) with three independent cDNA clones of *LHT1*, one cDNA clone of *AAP6*, and the vector pDR196. Transformants were selected on

media lacking uracil supplemented with 10 mM ammonia sulfate. Complementation was performed on Arg (1 mM), Asp, citrulline, GABA, Glu, and Pro (3 mM each) as sole nitrogen source. For K_m determination, competition experiments, and linear uptake rates, yeast cells were grown to $A_{600} = 0.5$, washed, and resuspended in ice-cold buffer (50 mM NaPi, pH 4.5, and 0.6 M sorbitol) to a final $A_{600} = 5$. Yeast cells (200 μL) were preincubated at 30°C with 10 μL of glucose (1 M) before the start of the experiment. Samples for linear uptake rates were removed after 60, 120, and 240 s and transferred to 4 mL of ice-cold buffer, filtered on glass fiber filters, and washed twice with 4 mL of buffer. Radioactivity was determined by liquid scintillation spectrometry. Endogenous uptake activity of yeast transformed with pDR196 was subtracted as background. Competition experiments were performed with 18.5 kBq of L-[U-^{14}C]Pro (8.58 GBq/mmol) and a 10-fold (100 μM) excess of the respective amino acids. The measurements represent means of three parallel experiments.

Analytical Methods

Liquid nitrogen–ground leaves were freeze-dried before amino acid extraction. Five milligrams of powder was extracted once in 80% methanol and once in 20% methanol. Both extracts were pooled and lyophilized. Pellets were resuspended in lithium buffer (0.7% lithium acetate and 0.6% LiCl; Pickering Laboratories) with 0.2 mM norleucine as an internal standard. Amino acids were separated by HPLC on a cation-exchange column (high-efficiency fluid column, 3 mm × 150 mm; Pickering Laboratories) using lithium buffer as an eluant. Amino acids were derivatized with ninhydrin before photometric detection.

Apoplasmic wash fluids were collected using the infiltration–centrifugation method (Lohaus et al., 2001) as described by Pilot et al. (2004). For one run, rosette leaves corresponding to 4 g fresh weight (leaves of ~100 mutant plants and ~30 wild-type plants) were collected.

Anion and cations were extracted from 5 mg of freeze-dried material with 1 mL of deionized water at 95°C for 5 min. Ion composition of leaf material was determined by HPLC (DX120 ion chromatography; Dionex) with an IonPac CS12A column (elunt, 20 M methansulfonic acid) for cations and an IonPac AS9-HC column (eluant, 9 mM Na_2CO_3) for anions using a suppressed conductivity detection system and an AS40 automated sampler (Dionex).

Soluble protein concentrations were determined according to Bradford (1976).

Enzymatic GUS Assay

GUS activity was determined using a modified fluorometric assay according to Jefferson et al. (1987) in a microplate fluorescence reader (Tecan-Safire). Proteins were extracted from five seedlings grown for 14 d on 2MS-N supplemented with the respective N source, and 20 μg of protein was used in the assay with 4-methylumbelliferyl-β-glucoronide as a substrate. The linearity of the reaction was monitored over 30 min, and a calibration curve using 4-methylumbelliferone (0 to 75 μM) was used for quantification.

Accession Numbers

Sequence data discussed in this article can be found in the GenBank/EMBL data libraries under the following accession numbers: LHT1, At5g40780, U39782; AAP6, At5g49630; LHT2, At1g24400; potato AAP1, Y09825.

Supplemental Data

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

Supplemental Figure 1. Maps of Genomic Structure, T-DNA Insertion, and DNA Constructs for GUS, RNAi, and GFP.

Supplemental Figure 2. Verification of T-DNA Insertion and Reduced RNA Levels in Heterozygous Plants.

Supplemental Figure 3. Absence of *LHT1* Transcripts in *lht1/lht1* Plants Compared with Col-0 Determined by RT-PCR.

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REFERENCES

Alexandersson, E., Saalbach, G., Larsson, C., and Kjellbom, P. (2004). Arabidopsis plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. Plant Cell Physiol. **45,** 1543–1556.

Alonso, J.M., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301,** 653–657.

Atkins, C.A. (2000). Biochemical aspects of assimilate transfers along the phloem path: N-solutes in lupins. Aust. J. Plant Physiol. **27,** 531–537.

Bechtold, N., and Pelletier, G. (1998). *In planta Agrobacterium* mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol. Biol. **82,** 259–266.

Bick, J.A., Neelam, A., Hall, J.L., and Williams, L.E. (1998). Amino acid carriers of *Ricinus communis* expressed during seedling development: Molecular cloning and expression analysis of two putative amino acid transporters, RcAAP1 and RcAAP2. Plant Mol. Biol. **36,** 377–385.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Brown, M.M., Hall, J.L., and Ho, L.C. (1997). Sugar uptake by protoplasts isolated from tomato fruit tissues during various stages of fruit growth. Physiol. Plant. **101,** 533–539.

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

Chen, L., Ortiz-Lopez, A., Jung, A., and Bush, D.R. (2001). ANT1, an aromatic and neutral amino acid transporter in Arabidopsis. Plant Physiol. **125,** 1813–1820.

Cooper, H.D., and Clarkson, D.T. (1989). Cycling of amino nitrogen and other nutrients between shoots and roots in cereals: A possible mechanism integrating shoot and root in the regulation of nutrient uptake. J. Exp. Bot. **40,** 753–762.

Cosio, C., Martinoia, E., and Keller, C. (2004). Hyperaccumulation of cadmium and zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. Plant Physiol. **134,** 716–725.

Despeghel, J., and Delrot, S. (1983). Energetics of amino acid uptake by *Vicia faba* leaf tissue. Plant Physiol. **71,** 1–6.

Dohmen, R.J., Strasser, A.W., Honer, C.B., and Hollenberg, C.P. (1991). An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. Yeast **7,** 691–692.

Eckes, P., Rosahl, S., Schell, J., and Willmitzer, L. (1986). Isolation and characterization of a light-inducible, organ-specific gene from

potato and analysis of its expression after tagging and transfer into tobacco and potato shoots. Mol. Gen. Genet. **205,** 14–22.

Fieuw, D., and Willenbrink, J. (1991). Isolation of protoplasts from tomato fruits (*Lycopersicon esculentum*): First uptake studies. Plant Sci. **76,** 9–17.

Fischer, W.N., Kwart, M., Hummel, S., and Frommer, W.B. (1995). Substrate specificity and expression profile of amino acid transporters (AAPs) in Arabidopsis. J. Biol. Chem. **270,** 16315–16320.

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

Frommer, W.B., Hummel, S., and Riesmeier, J.W. (1993). Expression cloning in yeast of a cDNA encoding a broad specificity amino acid permease from *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **90,** 5944–5948.

Frommer, W.B., Hummel, S., Unseld, M., and Ninnemann, O. (1995). Seed and vascular expression of a high-affinity transporter for cationic amino acids in Arabidopsis. Proc. Natl. Acad. Sci. USA **92,** 12036–12040.

Gazzarrini, S., Lejay, L., Gojon, A., Ninnemann, O., Frommer, W.B., and von Wiren, N. (1999). Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into Arabidopsis roots. Plant Cell **11,** 937–948.

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

Hsu, L.C., Chiou, T.J., Chen, L., and Bush, D.R. (1993). Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant. Proc. Natl. Acad. Sci. USA **90,** 7441–7445.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6,** 3901–3907.

Kinraide, T. (1980). Electrical evidence for different mechanisms of uptake of basic, neutral and acidic amino acids in oat. Plant Physiol. **65,** 1085–1089.

Koch, W., Kwart, M., Laubner, M., Heineke, D., Stransky, H., Frommer, W.B., and Tegeder, M. (2003). Reduced amino acid content in transgenic potato tubers due to antisense inhibition of the leaf H^+/amino acid symporter StAAP1. Plant J. **33,** 211–220.

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82,** 488–492.

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

Lam, H.M., Coschigano, K., Schultz, C., Melo-Oliveira, R., Tjaden, G., Oliveira, I., Ngai, N., Hsieh, M.H., and Coruzzi, G. (1995). Use of Arabidopsis mutants and genes to study amide amino acid biosynthesis. Plant Cell **7,** 887–898.

Lasat, M.M., Baker, A.J., and Kochian, L.V. (1998). Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. Plant Physiol. **118,** 875–883.

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

Li, Z.C., and Bush, D.R. (1990a). ΔpH-dependent amino acid transport into plasma membrane vesicles isolated from sugar beet leaves. I. Evidence for carrier-mediated, electrogenic flux through multiple transport systems. Plant Physiol. **94,** 268–277.

Li, Z.C., and Bush, D.R. (1990b). ΔpH-dependent amino acid transport into plasma membrane vesicles isolated from sugar beet leaves. II. Evidence for multiple aliphatic, neutral amino acid symports. Plant Physiol. **96,** 1338–1344.

Li, Z.C., and Bush, D.R. (1992). Structural determinants in substrate recognition by proton-amino acid symports in plasma membrane vesicles isolated from sugar beet leaves. Arch. Biochem. Biophys. **294,** 519–526.

Lipson, D.A., and Nasholm, T. (2001). The unexpected versatility of plants: Organic N use and availability in terrestrial ecosystems. Oecologia **128,** 305–316.

Lipson, D.A., Raab, T.K., Schmidt, S.K., and Monson, R.K. (1999). Variation in competitive abilities of plants and microbes for specific amino acids. Biol. Fertil. Soils **29,** 257–261.

Liu, D., Gong, W., Bai, Y., Luo, J.C., and Zhu, Y.X. (2005). *OsHT*, a rice gene encoding for a plasma-membrane localized histidine transporter. J. Integr. Plant Biol. **47,** 92–99.

Lohaus, G., and Heldt, H.W. (1995). Further studies of the phloem loading process in leaves of barley and spinach. The comparison of metabolite concentrations in the apoplasmic compartment with those in the cytosolic compartment and in the sieve tubes. Bot. Acta **108,** 270–275.

Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M., and Hermann, M.K. (2001). Is the infiltration-centrifugation technique appropriate for the isolation of apoplasmic fluid? A critical evaluation with different plant species. Physiol. Plant. **111,** 457–465.

Looger, L.L., Lalonde, S., and Frommer, W.B. (2005). Genetically encoded FRET sensors for visualizing metabolites with subcellular resolution in living cells. Plant Physiol. **38,** 555–557.

McKane, R.B., Johnson, L.C., Shaver, G.R., Nadelhoffer, K.J., Rastetter, E.B., Fry, B., Giblin, A.E., Kielland, K., Kwiatkowski, B.L., Laundre, J.A., and Murray, G. (2002). Resource-based niches provide a basis for plant species diversity and dominance in arctic tundra. Nature **415,** 68–71.

Meline, E., and Nilsson, H. (1953). Transfer of labelled nitrogen from glutamic acid to pine seedlings through the mycelium of *Boletus variegatus* (Sw.) Fr. Nature **171,** 134.

Merkle, T., Leclerc, D., Marshallsay, C., and Nagy, F. (1996). A plant in vitro system for the nuclear import of proteins. Plant J. **10,** 1177–1186.

Mounoury, G., Delrot, S., and Bonnemain, J. (1984). Energetics of threonine uptake by pod wall tissues of *Vicia faba*. Planta **161,** 178–185.

Murashige, T., and Skoog, G. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–496.

Nasholm, T., Ekblad, A., Nordin, A., Giesler, R., Hogberg, M., and Hogberg, P. (1998). Boreal forest plants take up organic nitrogen. Nature **392,** 914–916.

Negrutiu, I., Hinnisdaels, S., Cammaerts, D., Cherdshewasart, W., Gharti-Chhetri, G., and Jacobs, M. (1992). Plant protoplasts as genetic tool: Selectable markers for developmental studies. Int. J. Dev. Biol. **36,** 73–84.

Ohlund, J., and Nasholm, T. (2001). Growth of conifer seedlings on organic and inorganic nitrogen sources. Tree Physiol. **21,** 1319–1326.

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

Okumoto, S., Schmidt, R., Tegeder, M., Fischer, W.N., Rentsch, D., Frommer, W.B., and Koch, W. (2002). High affinity amino acid transporters specifically expressed in xylem parenchyma and developing seeds of Arabidopsis. J. Biol. Chem. **277,** 45338–45346.

Pate, J.S. (1973). Uptake, assimilation and transport of nitrogen compounds by plants. Soil Biol. Biochem. **5,** 109–119.

Pate, J.S., and Sharkey, P.J. (1975). Xylem to phloem transfer of solutes in fruiting shoots of legumes, studied by a phloem bleeding technique. Planta **12,** 11–26.

Pilot, G., Stransky, H., Bushey, D.F., Pratelli, R., Ludewig, U., Wingate, V.P., and Frommer, W.B. (2004). Overexpression of GLUTAMINE DUMPER1 leads to hypersecretion of glutamine from hydathodes of Arabidopsis leaves. Plant Cell **16,** 1827–1840.

Raab, T.K., Lipson, D.A., and Monson, R.K. (1999). Soil amino acid utilization among species of the Cyperaceae: Plant and soil processes. Ecology **80,** 2408–2419.

Rentsch, D., Hirner, B., Schmelzer, E., and Frommer, W.B. (1996). Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. Plant Cell **8,** 1437–1446.

Riesmeier, J.W., Willmitzer, L., and Frommer, W.B. (1994). Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. EMBO J. **13,** 1–7.

Schobert, C., and Komor, E. (1990). Transfer of amino acids and nitrate from the roots into the xylem of *Ricinus communis* seedlings. Planta **181,** 85–90.

Soldal, T., and Nissen, P. (1978). Multiphasic uptake of amino acids by barley roots. Physiol. Plant. **43,** 181–188.

Stockhaus, J., Schell, J., and Willmitzer, L. (1989). Correlation of the expression of the nuclear photosynthetic gene ST-LS1 with the presence of chloroplasts. EMBO J. **8,** 2445–2451.

Struck, C., Mueller, E., Martin, H., and Lohaus, G. (2004). The *Uromyces fabae* UfAAT3 gene encodes a general amino acid permease that prefers uptake of in planta scarce amino acids. Mol. Plant Pathol. **5,** 183–189.

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J., and Steinbiss, H.H. (1987). A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res. **15,** 5890.

von Wiren, N., Gazzarrini, S., Gojon, A., and Frommer, W.B. (2000). The molecular physiology of ammonium uptake and retrieval. Curr. Opin. Plant Biol. **3,** 254–261.

Williams, L., and Miller, A. (2001). Transporters responsible for uptake and partitioning of nitrogenous solutes. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52,** 659–688.

Wipf, D., Benjdia, M., Tegeder, M., and Frommer, W.B. (2002a). Characterization of a general amino acid permease from *Hebeloma cylindrosporum*. FEBS Lett. **528,** 119–124.

Wipf, D., Ludewig, U., Tegeder, M., Rentsch, D., Koch, W., and Frommer, W.B. (2002b). Conservation of amino acid transporters in fungi, plants and animals. Trends Biochem. Sci. **27,** 139–147.

Wyse, R.E., and Komor, E. (1984). Mechanism of amino acid uptake by sugarcane suspension cells. Plant Physiol. **76,** 865–870.

Xiang, C., Han, P., Lutziger, I., Wang, K., and Oliver, D.J. (1999). A mini binary vector series for plant transformation. Plant Mol. Biol. **40,** 711–717.

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