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

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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 apoplast, 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 apoplast, 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 (Kinrade, 1980; Despeguel 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
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 mycorrhizized, 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 14C-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 parenchymatous 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 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 14C-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) 3SS 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 apoplastic wash fluids showed that amino acid concentration is increased in the apoplasms 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 white 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, [14C]-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
remaining transport activity in \textit{lnh1} may be mediated by other members of the LHT subfamily, although \textit{LHT2} was not found in roots but rather in the tapetum (Lee and Tegeder, 2004). To test whether partial inhibition of \textit{LHT1} may already limit amino acid uptake, heterozygous \textit{LHT1/lnh1-1} plants were analyzed. The heterozygous plants displayed reduced \textit{LHT1} mRNA levels and were retarded in growth on Asp as the sole N source, demonstrating that \textit{LHT1} activity is limiting (Figure 1B; see Supplemental Figure 2 online). Thus, the analysis of insertion mutants suggests that \textit{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.

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

(A) Growth of the T-DNA insertion line \textit{lnh1/lnh1} and \textit{P}_{\text{35S}}-\textit{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 \textit{P}_{\text{35S}}-\textit{LHT1} plants grew larger than wild-type plants. Heterozygous plants (\textit{lnh1/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, homoygous T-DNA insertion lines showed a reduced uptake rate for Asp (\textit{lnh1/lnh1}, 0.31 pmol h\textsuperscript{-1} seedling\textsuperscript{-1}; \textit{Col-0}, 1.69 pmol h\textsuperscript{-1} seedling\textsuperscript{-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 \textit{P}_{\text{35S}}-\textit{LHT1} plants (7.28 pmol h\textsuperscript{-1} seedling\textsuperscript{-1}). Error bars represent SE (\textit{n} = 3). Linear uptake rates for radiolabeled Glu were 1.06 pmol h\textsuperscript{-1} seedling\textsuperscript{-1} for \textit{lnh1/lnh1} and 3.83 pmol h\textsuperscript{-1} seedling\textsuperscript{-1} for \textit{Col-0}, and rates for Gln were 1.75 pmol h\textsuperscript{-1} seedling\textsuperscript{-1} for \textit{lnh1/lnh1} and 10.8 pmol h\textsuperscript{-1} seedling\textsuperscript{-1} for \textit{Col-0} (1n = 10 seedlings, \textit{n} = 3, error bars indicate SE). Overexpression enhanced the uptake rate in all cases but was less pronounced for Gln. Uptake rates of \textsuperscript{14}C-labeled amino acids determined for \textit{P}_{\text{35S}}-\textit{LHT1} seedlings were 12.0 pmol h\textsuperscript{-1} seedling\textsuperscript{-1} for Glu and 22.2 pmol h\textsuperscript{-1} seedling\textsuperscript{-1} for Gln.
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 22D8AA (Fischer et al., 2002), which is unable to use Asn, Asp, citrulline, GABA, Glu, or Pro as the sole nitrogen source. The yeast mutant 22D8AA 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 (P35S-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 silique, with the strongest signals in roots and leaves and weaker signals in stems, silique, 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 [14C]Gln to Roots, Protoplast Uptake with [14C]Pro, and Size Distribution of Protoplasts.

(A) [14C]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 [14C]Gln was strongly reduced. Error bars represent SD (n = 3).

(B) Time-dependent uptake of [14C]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 NH₄SO₄ 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 14C-labeled Pro, His, and Glu in the yeast mutant 22Δ8AA (Figure 4B). The Km value of LHT1 for Pro was ~10 μM, with a Vmax of ~80 nmol·g protein⁻¹·min⁻¹; for Glu, the Km was ~14 μM and the Vmax was ~100 nmol·g protein⁻¹·min⁻¹; for His, the Km was ~360 μM and the Vmax 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 Km 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 Km values for Pro and Glu are in the same range as the Km 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, [¹⁴C]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 Km 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 [¹⁴C]Gly and [¹⁴C]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 6H and 6I). 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 P₃5SG-FP-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 apoplasma.

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-[¹⁴C]Pro, L-[¹⁴C]Glu, and L-[¹⁴C]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 $K_m$ values for Pro is $13.6 \pm 0.7 \mu M$ and $V_{max} = 82$ nmol Pro g⁻¹ protein·min⁻¹; for Glu, $K_m$ is $13.6 \pm 0.7 \mu M$ and $V_{max} = 100$ nmol Glu g⁻¹ protein·min⁻¹; and for His, $K_m$ is $360 \pm 28 \mu M$ and $V_{max} = 105$ nmol His g⁻¹ protein·min⁻¹.
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 \textit{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 \textit{lht1-1} leaves (Table 2). Anion and cation analyses showed that nitrate levels were reduced and free ammonium was increased in \textit{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 \textit{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 \textit{[14C]L-Pro} was linear over 60 min, but protoplasts isolated from \textit{lht1/lht1} plants showed a drastic reduction in Pro uptake velocity (\(\sim10\%\) 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 \textit{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 \textit{lht1} knockout was generated by expression

![Figure 5. Comparison of Linear Uptake Rates of Six Amino Acids at a Concentration of 10 μM, CCCP Sensitivity, and Energy Dependence of Transport.](image)

\(^{14}\text{C}\)-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.
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 depend-

**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 \( ^{14} \text{C}-\text{labeled Asp}, \text{Glu}, \) and Gln were also strongly increased in the \( \text{P}_{35S}\text{-LHT1} \) plants. 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.
Table 1. Amino Acids in Apoplasmic Wash Fluids of lht1-lht1 and Wild-Type Plants

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Col-0 (n = 3)</th>
<th>lht1/lht1 (n = 3)</th>
<th>Change (%)</th>
<th>P</th>
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<tbody>
<tr>
<td>Asp</td>
<td>263.7 ± 37.9</td>
<td>220.4 ± 8.4</td>
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<td>Thr</td>
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<td>75.4 ± 53.8</td>
<td>+2.1</td>
<td>n.s.</td>
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<tr>
<td>Glu</td>
<td>414.8 ± 66.7</td>
<td>416.5 ± 3.2</td>
<td>+0.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>Gln</td>
<td>582.2 ± 85.4</td>
<td>790.3 ± 112.4</td>
<td>+35.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>Pro</td>
<td>124.2 ± 12.7</td>
<td>91.2 ± 10.2</td>
<td>-26.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>Gly</td>
<td>83.4 ± 9.4</td>
<td>105.9 ± 7.4</td>
<td>+26.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>Ala</td>
<td>702.7 ± 109.6</td>
<td>814.8 ± 113.5</td>
<td>+16</td>
<td>n.s.</td>
</tr>
<tr>
<td>Val</td>
<td>48.7 ± 10.8</td>
<td>123.9 ± 5.5</td>
<td>+154.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ile</td>
<td>22.8 ± 1.9</td>
<td>65.9 ± 9.3</td>
<td>+189.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Leu</td>
<td>17 ± 5.2</td>
<td>78.2 ± 4.8</td>
<td>+361.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phe</td>
<td>12 ± 7.4</td>
<td>64 ± 9.3</td>
<td>+433.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>GABA</td>
<td>278.6 ± 46.6</td>
<td>378.1 ± 46.5</td>
<td>+35.7</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lys</td>
<td>26.9 ± 2.6</td>
<td>62.3 ± 2.6</td>
<td>+131.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>His</td>
<td>26.0 ± 8.1</td>
<td>46.6 ± 4.2</td>
<td>+79.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Arg</td>
<td>57.6 ± 7.9</td>
<td>92 ± 21.1</td>
<td>+59.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total</td>
<td>3,280 ± 488</td>
<td>4,326 ± 546</td>
<td>+32</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

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 apoplastic wash fluid of P₃₅S-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)
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 may not be 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 Apoplastic Wash Fluids of P35S-LHT1 and Wild-Type Plants

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Col-0 (n = 3)</th>
<th>P35S-LHT1 (n = 3)</th>
<th>Change (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>263.7 ± 37.9</td>
<td>302.9 ± 49.5</td>
<td>+14.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>Thr + Ser</td>
<td>546.1 ± 42.4</td>
<td>586.3 ± 59.3</td>
<td>+7</td>
<td>n.s.</td>
</tr>
<tr>
<td>Asn</td>
<td>73.8 ± 4.3</td>
<td>31.6 ± 20.6</td>
<td>-57.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>Glu</td>
<td>414.8 ± 66.7</td>
<td>438.2 ± 41.0</td>
<td>+5.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>Gln</td>
<td>582.2 ± 85.4</td>
<td>611.9 ± 55.5</td>
<td>+5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Pro</td>
<td>124.2 ± 12.7</td>
<td>189.1 ± 7</td>
<td>-52.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Gly</td>
<td>77.9 ± 9.4</td>
<td>180.8 ± 105.1</td>
<td>+132</td>
<td>n.s.</td>
</tr>
<tr>
<td>Ala</td>
<td>669.9 ± 109.6</td>
<td>770.5 ± 107.1</td>
<td>+15.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Val</td>
<td>48.7 ± 10.8</td>
<td>36.4 ± 5.6</td>
<td>-25.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Ile</td>
<td>22.8 ± 1.9</td>
<td>15.4 ± 2.5</td>
<td>-32.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>Leu</td>
<td>17.0 ± 5.2</td>
<td>20.5 ± 3</td>
<td>+21.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>Phe</td>
<td>12.0 ± 7.4</td>
<td>19.5 ± 4.1</td>
<td>+62.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA</td>
<td>278.6 ± 46.6</td>
<td>290.5 ± 43.8</td>
<td>+4.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lys</td>
<td>26.9 ± 2.6</td>
<td>20.8 ± 6.3</td>
<td>-22.7</td>
<td>n.s.</td>
</tr>
<tr>
<td>His</td>
<td>22.5 ± 8.1</td>
<td>23.5 ± 4</td>
<td>+4</td>
<td>n.s.</td>
</tr>
<tr>
<td>Arg</td>
<td>57.6 ± 7.9</td>
<td>27.8 ± 2</td>
<td>-52</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total</td>
<td>3,237 ± 249</td>
<td>3,565 ± 317</td>
<td>+10</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

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 P35S-LHT1 plants. Student’s t test was used for statistical analysis. Values shown are μM ± SE. n.s., not significant.
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 Apoplastic 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 from the apoplasma. This hypothesis is consistent with the finding that the apoplastic 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 *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 Negretiu 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 8.25 kBq of L-[U-14C]Asp (7.66 GBq/mmol), L-[L-14C]Glu (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.

To determine the uptake of Gln into roots and shoots separately, [14C]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 CaCl2, 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 100g for 5 min in 0.16 M mannitol/0.13 M CaCl2 and finally resuspended in ~3 mL of uptake medium (0.4 M mannitol, 8 mM CaCl2, and 10 mM MES, pH 5.5). The density of the suspension was adjusted to 106 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 3H2O (168 kBq/mL) for 30 min at 22°C to allow 3H2O 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 [14C]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 micro-centrifuge tube. The samples were centrifuged (20 s, 10,000g) to wash the protoplasts from the oil phase and avoid the contamination of radioactivity. Uptake of [14C]Pro was calculated as the increase of 14C in the sample in relation to the amount of 3H as described (Brown et al., 1997). The formula used was ([14C] [dpm] in protoplast pellet/3H2O [dpm] in pellet × 14C [dpm] in 1 μL of supernatant/3H2O [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 PstI/SalI 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 SmaI/BamHI from pDR196 and cloned into Xhol (blunted with T4 DNA polymerase) and BamHI sites of pRT100 (Töpper et al., 1987). Subsequently, the cassette (CaMV 35S-ORF LHT1-CaMV polyA signal) was excised with SphI (blunted) and cloned into EcoRI/BamHI 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 (SmaI and SalI) 3’ of the intron into EcoRI/BamHI 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 Xhol/EcoRI/366 and second with antisense SmaI/SalI into pRTRNAi. Subsequently, the cassette (CaMV 35S-sense-Intron-antisense-CaMV polyA) was cloned with PstI in pCB302.

**GFP Fusion**

The PCR-amplified ORF of LHT1 was cloned behind the CaMV 35S promoter EcoRI/EcoRI/366 in front of GFP5 (565T). The linker between LHT1 and GFP was seven amino acids (GIGQDII).

**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) EcoRI site was used to split this construct into two fragments. These two fragments were cloned into XbaI/EcoRI and EcoRI/EcoRV in pBluescript SK– (Stratagene). Subsequently, the total promoter construct XbaI/EcoRV (4063 bp) was cloned into XbaI/Smal in frame with uidA (GUS) of pCB308 (Xiang et al., 1999). The expressed fusion protein consisted of 19 amino acids of LHT1 (MVQAPPHDDHQDOEKLAAD) and a linker of 6 amino acids (MGGGGL) 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 HindIII and EcoRI and cloned into the HindIII/EcoRI sites of pCR2.1-TOPO (Invitrogen). XhoI and BamHI sites were introduced between the St LS1 promoter and the ocs terminator, and a second HindIII site was introduced behind the EcoRI site by site-directed mutagenesis (Kunkel, 1985). The vector was named pA1H. The LHT1 coding region was amplified by PCR, introducing XhoI and BamHI sites at the 5’ and 3’ ends, respectively. This LHT1 fragment was cloned into the XhoI and BamHI sites of pA1H. The St LS1 promoter-LHT1-ocs terminator cassette was excised with HindIII and cloned into the HindIII site of the pPZP312 binary vector, a derivative of pZPP212 (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: 5’SstI 5′-ctcgagACCATGGTAGCTCAAGCTCC-3′; SalI 3′ primer, 5′-gtgcAC-TATGAGTAAACCTTGATACCC-3′. For GFP fusion: blunt 5′ primer, 5′-ccctccgacACCATGGTAGCTCAAGCTCC-3′; BamHI 3′ primer, 5′-gtgcAC-TGAAACCTTGATACCC-3′. For pRTRNAi vector: intron AAP6 5′ primer, 5′-ctctAGGTCAGATTCGCTATC-3′; intron BamHI 3′ primer, 5′-tctagacGGTCCCCGGTCTCCTGTATTATTTTACATC-3′. For RNAi constructs: XhoI 5′ primer, 5′-ctcggccGCGATCCGACGCAGAAAAG-3′; blunt 3′ primer, 5′-GATTGCTCTCGTAAACCC-3′. For GUS fusion, XbaI 5′ primer, 5′-tctagacGGTCCCCGGTCTCCTGTATTATTTTACATC-3′. EcoRI 3′ primer, 5′-GTTTTCGTAAACCTTGATACCC-3′; EcoRI 5′ primer, 5′-GTA-ATTGAGTCTCGTAAACCC-3′; blunt 3′ primer, 5′-GATTGCCTCTCGTAAACCC-3′. For LS1 promoter fusion: pA1H, 5′-gagcagccaaagagaagaaagagctcgaggttctgtcgaatatgatagatgcg-3′; pA2H, 5′-ctcaggcgagagcagctgtcgaatatgatagatgcg-3′; for LHT1: XhoI, 5′-gagcagccaaagagaagaaagagctcgaggttctgtcgaatatgatagatgcg-3′; BamHI, 5′-gagcgccgcatgtcgaatatgatagatgcg-3′.
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 $\text{K}_+\text{determination, competition experiments, and linear uptake rates, yeast cells were grown to } A_{600} = 0.5, \text{ washed, and resuspended in ice-cold buffer (50 mM NaPi, pH 4.5, and 0.6 M sorbitol) to a final } A_{600} = 5. \text{ Yeast cells (200 } \mu\text{L) were preincubated at 30°C with 10 } \mu\text{L 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-14C]Pro (8.58 GBq/mmol) and a 10-fold (100 } \mu\text{M) 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 M 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. Apoplastic 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 (eluant, 20 M methansulfonic acid) for cations and an IonPac AS9-HC column (eluant, 9 mM Na$_2$CO$_3$) 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 } \mu\text{g 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 } \mu\text{M}) 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**


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


