

Original Article

Amino acid transporter mutants of *Arabidopsis* provides evidence that a non-mycorrhizal plant acquires organic nitrogen from agricultural soil

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ABSTRACT

Although organic nitrogen (N) compounds are ubiquitous in soil solutions, their potential role in plant N nutrition has been questioned. We performed a range of experiments on *Arabidopsis thaliana* genetically modified to enhance or reduce root uptake of amino acids. Plants lacking expression of the Lysine Histidine Transporter 1 (LHT1) displayed significantly lower contents of ¹³C and ¹⁵N label and of U-¹³C₅, ¹⁵N₂ L-glutamine, as determined by liquid chromatography–mass spectrometry when growing in pots and supplied with dually labelled L-glutamine compared to wild type plants and LHT1-overexpressing plants. Slopes of regressions between accumulation of ¹³C-labelled carbon and ¹⁵N-labelled N were higher for LHT1-overexpressing plants than wild type plants, while plants lacking expression of LHT1 did not display a significant regression between the two isotopes. Uptake of labelled organic N from soil tallied with that of labelled ammonium for wild type plants and LHT1-overexpressing plants but was significantly lower for plants lacking expression of LHT1. When grown on agricultural soil plants lacking expression of LHT1 had the lowest, and plants overexpressing LHT1 the highest C/N ratios and natural $\delta^{15}\text{N}$ abundance suggesting their dependence on different N pools. Our data show that LHT1 expression is crucial for plant uptake of organic N from soil.

INTRODUCTION

Small organic N compounds including amino acids are abundant in soils (Weintraub & Schimel, 2005; Kielland *et al.*, 2007; Rothstein, 2009; Inselsbacher & Näsholm, 2012), and laboratory studies have shown that a range of different plant species, both mycorrhizal and non-mycorrhizal, can access such compounds (Chapin *et al.*, 1993; Kielland, 1994; Persson & Näsholm, 2001). Even more complex organic N forms can be used, including both peptides and protein (Komarova *et al.*, 2008; Paungfoo-Lonhienne *et al.*, 2008). Experiments using

dually (¹³C, ¹⁵N) labelled amino acids (Näsholm *et al.*, 1998) and peptides (Wilkinson *et al.*, 2015) injected into soil have provided clues that plants exploit this potential N source in the field, circumventing mineralization and potentially reducing their reliance on mineral N forms for their nutrition. However, several authors have argued that plants cannot access significant amounts of such N forms because they are weaker competitors for organic N than are soil microbes (Hodge *et al.*, 2000; Jones *et al.*, 2005; Jones *et al.*, 2013; Kuzyakov & Xu, 2013). The use of dually labelled amino acids to infer plant uptake of organic N compounds might be inconclusive if alternative pathways of tracer absorption bring in both ¹³C and ¹⁵N after mineralization (Rasmussen *et al.*, 2010). The addition of label may also elevate concentrations of amino acids in soil, conditions that would favour plants over microbes (Jones *et al.*, 2005) and claims have been made that this may even result in passive uptake of labelled compounds.

Nitrogen uptake by plants is, however, an actively regulated process, mediated by numerous membrane transporters (Nacry *et al.*, 2013). The Lysine Histidine Transporter 1 (LHT1; Hirner *et al.*, 2006) in the model plant *Arabidopsis thaliana* was the first transporter shown to be involved in root amino acid uptake from soil. LHT1 was later deemed a key constituent of the root organic N uptake mechanism in *A. thaliana*, facilitating uptake of neutral and acidic (but not basic) amino acids at field-relevant concentrations (Svennerstam *et al.*, 2011). The demonstration of tangible mechanisms whereby plants absorb organic N is an important part of supporting the role of organic N uptake in soils. The mechanism can also be manipulated with molecular tools by specifically targeting the unresolved question of whether or not plants access organic N from soil. Plant genotypes differing in their capacity to absorb amino acids would offer a critical test of this possibility; plants with impaired amino acid uptake capacities should show much lower ¹³C:¹⁵N from dual-labelled tracers than unimpaired plants. If the ratio of absorbed ¹³C:¹⁵N did not differ between impaired and unimpaired plants, this would indicate that ¹³C was absorbed independently of ¹⁵N after mineralization of the dual-labelled amino

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acid and thus suggest earlier studies claiming that plants can access organic N in the form of amino acids from soil are unsubstantiated.

To that end, both knock-out (KO) mutants and plants overexpressing the *LHT1* gene have been produced and characterized with regard to root uptake and growth phenotypes. *LHT1*-KO plants display impaired root uptake capacities of a range of amino acids, including L-glutamine (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007, 2011). Moreover, growth on L-glutamine in sterile media was tested and found to be significantly lower for *LHT1*-KO plants than for wild type plants, while plants overexpressing the *LHT1* gene displayed enhanced growth on a range of amino acids (L-alanine, L-glutamic acid, L-glutamine and L-asparagine; Forsum *et al.*, 2008). Interpretation of results from physiological studies using mutants is often confounded by the potential for pleiotropic effects. The present study focused on the question whether altered expression of an amino acid transporter gene would affect the plant's capacity to acquire such compounds from the soil. Potential effects of the *LHT1* mutation on uptake of other N sources such as NH_4^+ and NO_3^- cannot be ruled out, but such effects would be manifested in altered rates of ^{15}N -labelled N, but not in ^{13}C -labelled C acquisition. Thus, we argue that using soil grown plants of amino acid transporter mutants and dual, isotopic labelling provides a stringent test of the hypothesis that plants cannot acquire organic N compounds from soil.

Organic N compounds absorbed by plants are rapidly metabolized, and thus a fraction of the carbon (C) that is acquired by the plant will be lost through respiration (Näsholm *et al.*, 2009). This limits the use of the dual labelling technique to short-term studies and unfortunately other direct indices of organic N nutrition are not known. However, circumstantial evidence of organic N nutrition may be envisioned. Hence, organic N and inorganic N should theoretically have different effects on the C-status and natural abundance of ^{15}N of plants.

Plant organic N uptake should result in enhanced C-nutrition both because of the C absorbed together with the N in the organic N molecules and because assimilation of N acquired in organic forms would in general have lower metabolic costs of assimilation (Penning De Vries *et al.*, 1974; Bloom *et al.*, 1992; Zerihun *et al.*, 1998). Together, this would be expected to result in a positive effect of organic N uptake on plant C/N ratios (Franklin *et al.*, 2016). Moreover, transformation of N compounds in soil occurs through a chain of reactions in which each step discriminates against the heavier (^{15}N), naturally occurring N isotope leading to more isotopically enriched soil amino acids (Ostle *et al.*, 1999) compared to NH_4^+ and NO_3^- . Thus, a positive effect of organic N on $\delta^{15}\text{N}$ abundance of soil-grown plants may be expected. This theoretical shift in ^{15}N natural abundance would not, however, confound the results of labelling studies because labelling would result in shifts in ^{15}N that are orders of magnitude greater than variations in $\delta^{15}\text{N}$ abundance.

We report on a series of experiments with *A. thaliana* genotypes differing in *LHT1* expression, aimed to critically challenge the potential role of organic N in plant N nutrition. Three types of experiments were performed: (I) *A. thaliana*

genotypes differing in *LHT1* expression were cultivated on agricultural soil and subjected to a minute pulse of dual-labelled L-glutamine and harvested at short time intervals to study the impact of *LHT1* expression differences on the dynamics of organic N acquisition. Then, (II) different soil-cultivated *A. thaliana* genotypes were charged with either dual-labelled L-glutamine or $^{15}\text{NH}_4^+$ to assess the impact of *LHT1* expression differences on ^{13}C and ^{15}N labelling patterns and to enable comparison of plant acquisition of the two N sources. Finally, (III) the different genotypes were grown on agricultural soil to study the effect of *LHT1* expression on plant C and N status and on natural ^{15}N abundance.

MATERIALS AND METHODS

U $^{13}\text{C}_5$, $^{15}\text{N}_2$ L-glutamine and $^{15}\text{NH}_4^+$ labelling experiments of different *LHT1* genotypes

Seeds of *A. thaliana* (Columbia-0; wild type), the *LHT1* KO mutant *lht1-5* (Svennerstam *et al.*, 2007) and *LHT1* overexpressor *35S-LHT1-2* (Svennerstam *et al.*, 2011) were sown in 125 mL pots filled with 100 g of sieved (<2 mm) topsoil collected from a conventionally managed agricultural field within the Rönneby Research Facilities, Swedish University of Agricultural Sciences, Umeå, Sweden (63°49'N, 20°16'E). The soil is a fine, silty sand with low clay content (4% clay, 58% silt, 38% fine sand), a pH of 6.0 and C/N ratio of 14.8 (Mulder *et al.*, 2002). Three plants per pot (Fig. S1) were grown in short-day conditions: 9/15 h light ($120 \mu\text{mol m}^{-2} \text{s}^{-1}$)/dark cycles with 22/18 °C day/night temperatures. Plants were watered twice per week.

Three separate experiments were conducted with the three genotypes under the conditions described in the previous paragraph. In the first labelling experiment (I), 32 days after sowing, 5 mL of 100 μM dual-labelled L-glutamine (U- $^{13}\text{C}_5$, $^{15}\text{N}_2$ with 98 atom % ^{13}C , 98 atom % ^{15}N supplied from Larodan fine chemicals—www.larodan.se) was carefully applied to the soil surface of each of 20 pots per genotype. In this experiment, plant roots were harvested at 15 min and at 60 min after the applications of dual-labelled L-glutamine. In the second labelling experiment (II), 32 days after sowing, either 5 mL of water (control), 100 μM ^{13}C , ^{15}N L-glutamine or 100 μM $^{15}\text{NH}_4^+$ was carefully applied to the soil surface of each of six pots per genotype. Plant roots and shoots were harvested 60 min after the applications. In both experiments, roots were carefully excavated from the soil, washed in tap water and surface-attached label was removed by immersing roots thrice in 0.5 mM CaCl_2 . Harvested plants were separated into roots and shoots, which were either freeze dried 48 h (experiment I) or oven-dried at 65 °C overnight (experiment II). For both experiments, the ^{13}C and ^{15}N contents of plant material were determined using a Delta V Isotope ratio mass spectrometer (IRMS; Thermo Fisher Scientific).

In the first experiment, additional analyses on plants were performed using liquid chromatography–mass spectrometry (LC–MS). Root amino acids were extracted from freeze dried samples and analysed with an Agilent 6540 UHD Accurate Mass QTOF LC/MS using an electrospray ionization

(Dual AJS ESI) probe following the method described by Czaban *et al.* (2016). The gradient elution buffers were A (H_2O , 0.1% formic acid) and B (acetonitrile, 0.1% formic acid), and the column used for separation of amino acids was a Phenomenex C_{18} column (2.1 mm \times 100 mm, 1.7 μm). The samples were derived with a Waters AccQ-TagTM Ultra Derivatization kit for amino acid analysis. $\text{U-}^{13}\text{C}_5\text{-}^{15}\text{N}_2\text{-L-glutamine}$ was identified based on retention time and the m/z 324.135, which is the mass of $\text{U-}^{13}\text{C}_5\text{-}^{15}\text{N}_2\text{-L-glutamine}$ plus the attached AccQ-TagTM Derivatized glutamine was detected on the mass spectrometer equipped with a jet stream electrospray source operating in positive ion mode. The jet-stream gas temperature was 300 °C with a gas flow of 8 L h^{-1} , and a sheath gas temperature equal to 350 °C and flow rate of 11 L h^{-1} , nebulizer pressure was set to 40 psi. The capillary voltage was set to 4 kV, and nozzle voltage was set to 0 V. Reference mass correction was achieved by the reference mass m/z 922.0097. The concentration of glutamine was calculated from a standard calibration curve based on the peak area of the sum of all glutamine isotopologues in the standards. The detectable isotopologues were monoisotopic glutamine (m/z 317.124), glutamine +1 and glutamine +2. The concentration of $\text{U-}^{13}\text{C}_5\text{-}^{15}\text{N}_2\text{-L-glutamine}$ in the extracts was then calculated from the total concentration of glutamine (sum of all isotopologues in each sample) multiplied with the proportion of m/z 324.135 to the sum of all isotopologues of glutamine in the samples. All peak areas were normalized by the internal standard, norvaline, present in the samples and standards.

Fluxes of soluble N compounds in pots during labelling experiment II were assessed by microdialysis (Inselsbacher *et al.*, 2011; Fig. S1). Briefly, microdialysis probes (CMA 20) equipped with a polyarylethersulphone membrane (10 mm length, 500 μm outer and 400 μm inner diameter) with a 20 kDa molecular weight cut-off were carefully inserted into the root-zone to a depth of 1 cm. Two probes per pot were mounted in four pots in the treatment including labelled L-glutamine, resulting in a total of eight probes. High-purity deionized water was used as perfusate, delivered by two syringe infusion pumps (CMA 4004) at a flow rate of 5 $\mu\text{L min}^{-1}$. Dialysates were collected at 30 min intervals in two refrigerated microfraction collectors (CMA 470) before, during and after labelling. Samples were immediately analysed for ammonium, nitrate and individual amino acids as described previously (Inselsbacher *et al.*, 2011), and N fluxes were expressed as $\text{nmol m}^{-2} \text{s}^{-1}$ (Inselsbacher & Näsholm, 2012).

The third experiment (III) aimed at assessing the impact of N source on C and N contents and of $\delta^{15}\text{N}$ *A. thaliana* plants. The three genotypes (LHT1 KO plants, wild type plants and 35S-LHT1 plants; $N = 25$ per genotype) were grown in sieved topsoil collected from the same field site and under the same short day conditions as in the labelling experiments (I and II) described above. After 31 days of growth, shoots were harvested and dried at 65 °C overnight, ground to a fine powder and their C, N and $\delta^{15}\text{N}$ contents were determined using a DeltaV Isotope ratio mass spectrometer and a Flash EA 2000 Elemental Analyzer (both supplied by Thermo Fisher Scientific). To aid the interpretation of this experiment, the impact of N source on plant C and N contents was studied by

cultivating wild type *A. thaliana* plants on sterile media. Plants were grown from seeds on sterile agar media with either 3 mM nitrate or 1.5 mM L-glutamine (corresponding to 3 mM N) as N sources for 21 days. Shoots harvested from five plates ($N = 5$) representing each N treatment were then harvested, dried and their C and N contents were determined. Previously described protocols were used for this experiment (Cambui *et al.*, 2011).

CALCULATIONS AND STATISTICAL TREATMENT OF DATA

Root and shoot contents of ^{13}C and ^{15}N derived from tracers are reported in units of $\mu\text{mol } ^{13}\text{C}$ -labelled C and $\mu\text{mol } ^{15}\text{N}$ -labelled N per gram dry mass of tissue. These were calculated using the formulas:

$$\mu\text{mol } ^{13}\text{C} - \text{labeled C} = \left(\text{atom}\% ^{13}\text{C}_{\text{ls}} - \text{atom}\% ^{13}\text{C}_{\text{nls}} \right) / \left(\text{atom}\% ^{13}\text{C}_{\text{tr}} \right) * \mu\text{moles C}_{\text{ls}}$$

and:

$$\mu\text{mol } ^{15}\text{N} - \text{labeled N} = \left(\text{atom}\% ^{15}\text{N}_{\text{ls}} - \text{atom}\% ^{15}\text{N}_{\text{nls}} \right) / \left(\text{atom}\% ^{15}\text{N}_{\text{tr}} \right) * \mu\text{moles N}_{\text{ls}}$$

where the subscript 'ls' denotes labelled sample, 'nls' denotes non-labelled sample and 'tr' denotes tracer. (Note that in the current experiment, where the tracer is 98% label, this simplified equation is applicable but with lower rates of labelling, or when using depleted rather than enriched tracers, the denominator would be expected to be $\text{atom}\%_{\text{tr}} - \text{atom}\%_{\text{nls}}$ to take into account natural abundance.)

Statistical analyses of the data were performed using the software JMP (vers. 10.0.0; www.jmp.com). Analysis of variance followed by Tukey's HSD test was used for comparisons of genotypes with respect to contents of C and N and ^{13}C -labelled C and ^{15}N -labelled N in experiment I, II and III.

RESULTS

In labelling experiment I, we studied potential differences between LHT1 genotypes in acquisition labelled L-glutamine 15 and 60 min after addition to pots. Root concentrations of N derived from the isotopically labelled organic N source differed between genotypes, being c. 50% lower in LHT1-KO plants and 50% higher in 35S-LHT1 plants compared to wild type plants at the 15 min harvest (Fig. 1a). At this harvest, also concentrations of ^{13}C derived from the dual-labelled organic N source differed significantly for the different LHT1 genotypes (Fig. 1b). Thus, LHT1-KO plants displayed ^{13}C -labelled C contents three times lower and 35S-LHT1 plants two times higher than wild type plants. The differences between genotypes in rates of acquisition of ^{15}N and ^{13}C after 15 min were also apparent, and of similar magnitude, after 60 min (Fig. 1a,b). Regressions of root ^{13}C -labelled C versus root ^{15}N -labelled N for the different genotypes revealed significant relationships for wild type and 35S-LHT1 plants but not for LHT1-KO plants (Fig. 2). Slopes of regressions were higher at the 15 min harvest than for

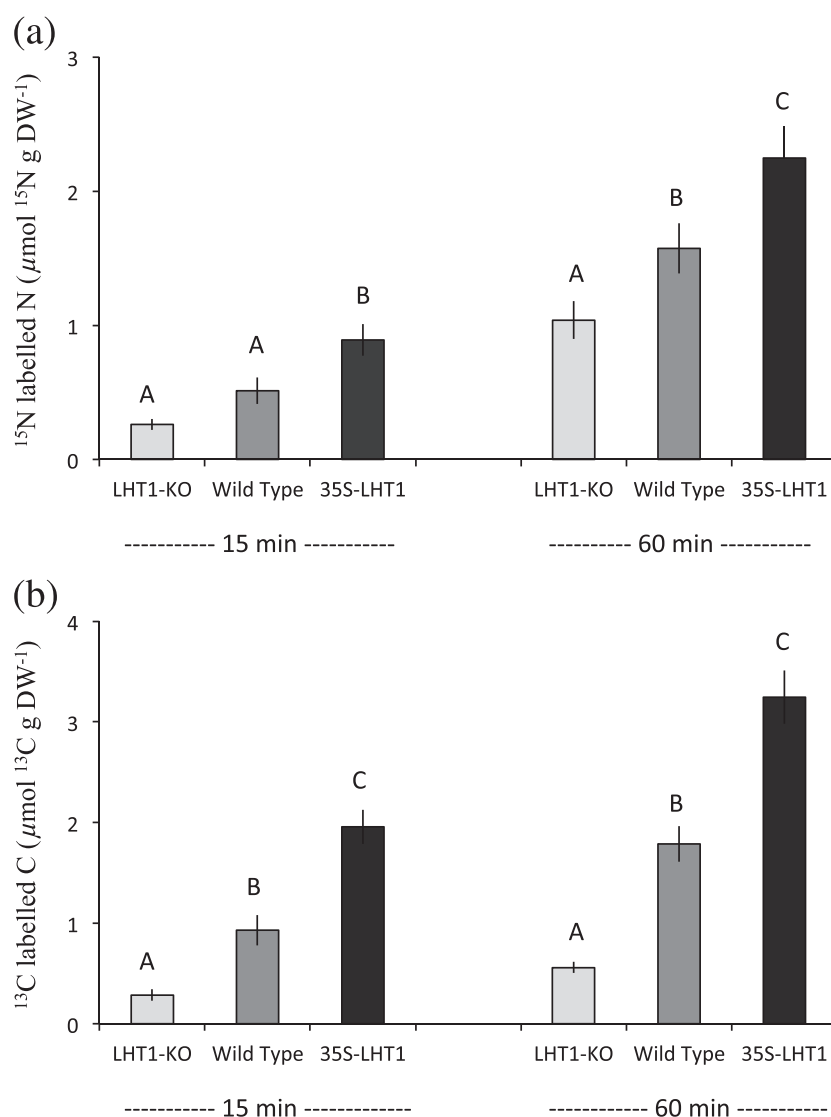


Figure 1. Time dependence of label acquisition for different Arabidopsis genotypes. Root contents of ^{15}N -labelled N (a) and ^{13}C -labelled C (b) 15 and 60 min after adding 5 mL of a 100 μM solution of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine to soil in pots supporting *A. thaliana* Lysine Histidine Transporter 1 (*LHT1*) knock-out mutants (LHT1-KO), wild type plants (Wild Type) and *LHT1*-overexpressing (35S-LHT1) plants. Bars represent mean values \pm SE, $N=8-12$. Different capital letters indicate a difference between genotypes within each harvest.

the 60 min harvest for both wild type (1.5 versus 0.9) and 35S-LHT1 (1.4 versus 1.1) plants. Analyses of the same plant materials using LC-MS revealed significant differences between plant genotypes as well (Fig. 3). Intensities of m/z 324.135, corresponding to the derivative of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine, were significantly lower for LHT1-KO plants and higher for 35S-LHT1 plants compared to wild type plants (Fig. 3 & Fig. S2). Those differences corresponded to clear differences in contents of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine between genotypes. Thus, compared to wild type plants, LHT1-KO and 35S-LHT1 plants displayed contents of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine c. 10 times lower and two times higher, respectively. Increased proportions of the +1 and +2 isotopologues were detected for all genotypes at 60 min following addition of the tracer (Table 1). No other significant changes either in glutamine or glutamate isotopologues were detected. Amounts of ^{15}N corresponding to the measured contents of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$

L-glutamine were calculated and compared to amounts of ^{15}N -labelled N, measured by IRMS. This approach showed that at the first harvest root contents of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine accounted for 24, 106 and 84% of the ^{15}N -labelled N in LHT1-KO plants, wild type plants and 35S-LHT1 plants, respectively (Fig. 4). Corresponding fractions at the 60 min harvest were 4, 25 and 51%.

In labelling experiment II, we studied genotypic differences in rates of uptake of L-glutamine and of NH_4^+ . LHT1-KO mutants subjected to labelled L-glutamine contained significantly less of ^{13}C -labelled C and ^{15}N -labelled N in roots compared to wild type and 35S-LHT1 plants (Fig. 5). Wild type plants showed similar ^{15}N labelling for the two N sources, indicating similar N uptake from L-glutamine or NH_4^+ . In contrast, 35S-LHT1 plants displayed higher rates of ^{15}N labelling from L-glutamine and LHT1-KO plants displayed higher rates of ^{15}N labelling from NH_4^+ (Fig. 5a). Root ^{13}C -labelled C was

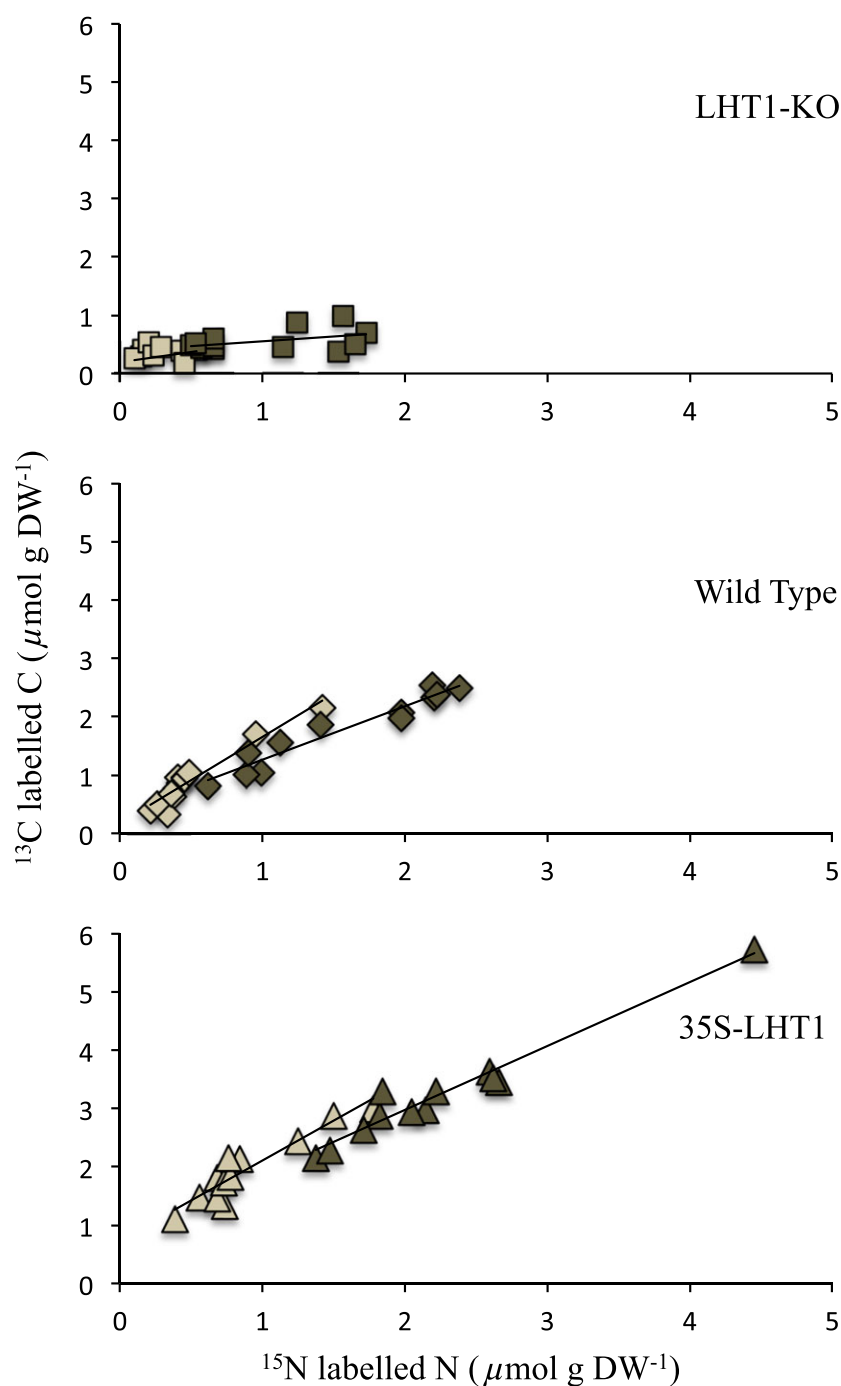


Figure 2. Dynamics of root acquisition of ^{13}C and ^{15}N for the three different LHT1 genotypes. Root contents of ^{13}C -labelled C versus ^{15}N -labelled N 15 min (light gray symbols) and 60 min (dark gray symbols) after adding 5 mL of a 100 μM solution of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine to soil in pots supporting *A. thaliana* Lysine Histidine Transporter 1 (*LHT1*) knock-out mutants (top), wild type plants (middle) and *LHT1*-overexpressing (35S-*LHT1*) plants (bottom). Each symbol represents one value from three plants per pot. The regression lines for *LHT1* overexpressing (15 min: $y = 1.4x + 0.7$, $r^2 = 0.87$; 60 min: $y = 1.1x + 0.8$, $r^2 = 0.96$) and wild type plants (15 min: $y = 1.5x + 0.2$, $r^2 = 0.92$; 60 min: $y = 0.9x + 0.3$, $r^2 = 0.93$) were significant, but not for *LHT1* knock-out plants. [Colour figure can be viewed at wileyonlinelibrary.com]

significantly higher for 35S-LHT1 plants and wild type plants than for LHT1-KO plants (Fig. 5b).

Wild type plants and plants overexpressing the LHT1 gene also increased ^{13}C -labelled C in proportion to ^{15}N -labelled N in roots (Fig. 6), and LHT1-KO plants showed little variation and no correlation. Slopes of regression of ^{13}C -labelled C

versus ^{15}N -labelled N in wild type and 35S-LHT1 plants were 0.50 and 0.83, respectively, while LHT1-KO plants demonstrated no acquisition of intact L-glutamine.

Rates of isotope labelling for all genotypes and for both N-forms (L-glutamine and NH_4^+) were much lower in shoots than in roots (Fig. S3a). No differences in contents of

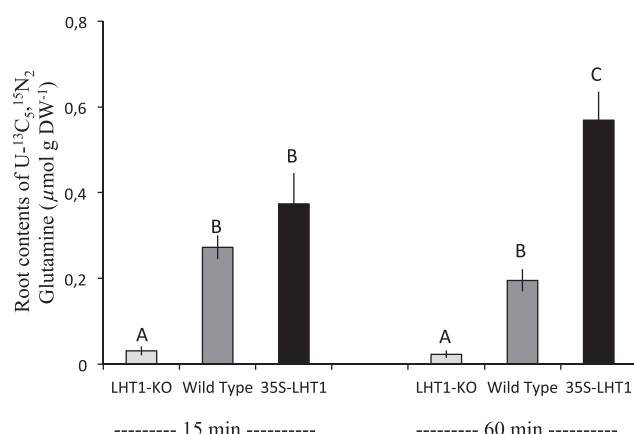


Figure 3. LC-MS quantification of root contents of labelled glutamine. Root contents of U-¹³C₅, ¹⁵N₂ L-glutamine 15 min and 1 h after adding 5 mL of a 100 μM solution of labelled L-glutamine to soil in pots supporting *A. thaliana* Lysine Histidine Transporter 1 (*LHT1*) knock-out mutants (LHT1-KO), wild type plants (Wild Type) and *LHT1*-overexpressing (35S-LHT1) plants. Bars represent mean values ± SE, *N* = 8–12. Different capital letters indicate a difference between genotypes within each harvest.

Table 1. Root contents of glutamine isotopologues at the start of the experiment, 15 min and 1 h after adding 5 mL of a 100 μM solution of labelled L-glutamine to soil in pots supporting *A. thaliana* Lysine Histidine Transporter 1 (*LHT1*) knock-out mutants (LHT1-KO), wild type plants (Wild Type) and *LHT1*-overexpressing (35S-LHT1) plants. Values are expressed as percent of individual glutamine isotopologues to the content of monoisotopic glutamine and represent mean values ± SE, *N* = 8–12. Different lower case letters indicate differences between harvests (0, 15 and 60 min) within each genotype; different upper case letters indicate differences between genotypes within each harvest

Harvest	Genotype	Gln +1	Gln +2	Gln +7
0 min	LHT1-KO	16.8 ± 0.2 ^{aA}	2.3 ± 0.1 ^{aA}	0.02 ± 0.0 ^{aA}
	Wild type	16.9 ± 0.2 ^{aA}	2.4 ± 0.1 ^{aA}	0.02 ± 0.0 ^{aA}
	35S-LHT1	17.0 ± 0.3 ^{aA}	2.3 ± 0.1 ^{aA}	0.01 ± 0.0 ^{aA}
15 min	LHT1-KO	17.0 ± 0.1 ^{aA}	2.3 ± 0.2 ^{aA}	0.1 ± 0.0 ^{aA}
	Wild type	17.1 ± 0.1 ^{aA}	2.4 ± 0.1 ^{aA}	0.4 ± 0.1 ^{bB}
	35S-LHT1	17.7 ± 0.3 ^{bA}	2.5 ± 0.1 ^{bA}	0.5 ± 0.1 ^{bB}
60 min	LHT1-KO	18.6 ± 0.5 ^{bA}	2.7 ± 0.1 ^{bA}	0.1 ± 0.1 ^{aA}
	Wild type	19.7 ± 0.8 ^{bA}	3.3 ± 0.2 ^{bA}	0.5 ± 0.1 ^{bB}
	35S-LHT1	19.1 ± 0.3 ^{bA}	2.9 ± 0.1 ^{bA}	0.7 ± 0.1 ^{bB}

¹⁵N-labelled N of shoots were detected for the three genotypes when L-glutamine was the source of label while slightly higher values of shoot ¹⁵N were detected in 35S-LHT1 plants compared to LHT1-KO plants and with wild type plants taking the intermediary position when ¹⁵NH₄⁺ was the source of label. Shoot contents of ¹³C-labelled C were higher for 35S-LHT1 than for LHT1-KO plants and again with wild type plants taking the intermediary position (Fig. S3b). For wild type plants and 35S-LHT1 plants root contents of ¹⁵N-labelled N were 2–3 times higher in experiment II compared to experiment I (compare Figs 1a & 5a). We do not know the cause of this difference, but we note that root biomass was considerably higher

in experiment I compared to experiment II. Thus, root biomass was 5.8 ± 0.3 and 1.2 ± 0.1 for wild type plants at experiment I and II, respectively, while corresponding values for 35S-LHT1 plants were 6.6 ± 0.7 and 1.8 ± 0.2. Hence, a greater dilution of tracer in the larger root biomass of plants in experiment I is a plausible cause of the discrepancy in rates of ¹⁵N-labelled N.

In labelling experiment II, we also used microdialysis to study the alteration of soil N fluxes following addition of labelled L-glutamine. Our measurements revealed that addition of labelled L-glutamine induced diffusive fluxes that increased transiently from 1.7 to 4.1 nmol m⁻² s⁻¹ (Fig. 7), corresponding to a calculated increase in soil solution concentration from ca. 1.0 to 2.4 μM. Simultaneous monitoring of fluxes of other N sources showed that nitrate and ammonium each accounted for approximately 25% of the total measured N flux in the soil, and total amino acids for ca. 50% (Fig. 7). In addition to the slight increase in glutamine following addition of label, a strong increase in nitrate fluxes was recorded (Fig. 7).

In experiment III, plants of differing genotypes were grown in agricultural soil. Here, 35S-LHT1 plants had higher C concentration, C/N ratios and δ¹⁵N values but lower N concentrations compared to the other two genotypes (Table 2). Differences between wild type plants and LHT1-KO plants were small with slightly higher C/N ratios and δ¹⁵N values for wild type plants.

DISCUSSION

Amino acids are present in soil solutions of various ecosystems along with other organic and inorganic N forms (Kielland *et al.*, 2007; Rothstein, 2009; Inselsbacher & Näsholm, 2012; Warren, 2013; Brackin *et al.*, 2015). Although plants have been shown to absorb amino acids from soil and although molecular mechanisms underpinning this process have been defined, the prospect of plant organic N nutrition is still debated (Jones *et al.*, 2005; Kuzyakov & Xu, 2013). The general notion is that plants growing on infertile soils and forming ectomycorrhiza or ericoid mycorrhiza are capable of acquiring organic N, but arbuscular mycorrhizal and in particular non-mycorrhizal plants are not considered to use such N forms (Read & Perez-Moreno, 2003). Our series of experiments targeted a non-mycorrhizal weed, and experiments were carried out in a fertile, agricultural soil thus providing a critical test of plant organic N nutrition outside the range of conditions normally regarded as relevant for this process. The experiments also extended traditional dual labelling methodology with novel approaches via use of plant genotypes with distinct differences in uptake capacities for amino acids for monitoring and via measurements of *in situ* soil fluxes of N compounds via a miniaturized dialysis system (microdialysis; Inselsbacher *et al.*, 2011).

The three genotypes of *A. thaliana* (LHT1-KO, Wild type, 35S-LHT1) had been characterized with respect to uptake kinetics of a variety of amino acids in an earlier study (Svennerstam *et al.*, 2011). The current study used L-glutamine as a tracer, and at a L-glutamine concentration of 2 μM, the genotypes' root uptake rates differed across a 20-fold range (calculated rates under the experimental conditions: 0.02, 0.1 and

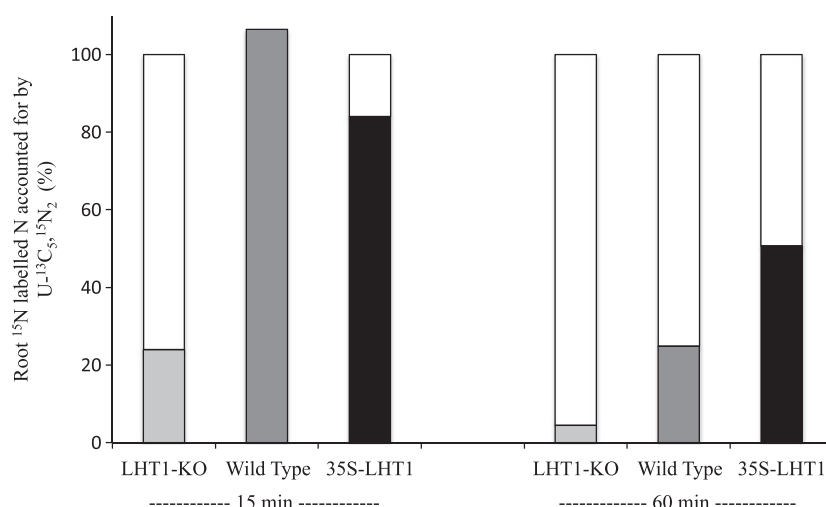


Figure 4. Root contents of ^{15}N -labelled N accounted for by root contents of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine. Contents of ^{15}N -labelled N corresponding to average concentrations of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine (as measured by LC-MS; Fig. 3) for the three different genotypes were compared to corresponding average contents of ^{15}N -labelled N as measured by IRMS (Fig. 1).

$0.4 \mu\text{mol L-glutamine g}^{-1}$ root DW h^{-1} for LHT1-KO, wild type, 35S-LHT1, respectively; Svennerstam *et al.*, 2011). Thus, this experiment set out to investigate if this range in root uptake capacities for one of the most abundant amino acids in soil solutions (Werdin-Pfisterer *et al.*, 2009; Jämtgard *et al.*, 2010; Inselsbacher & Näsholm, 2012) would be manifested also when plants are in competition for amino acids against soil microbes.

The short incubation times (15 and 60 min) used in the experiments were motivated by rapid metabolism of absorbed, labelled amino acids observed in earlier studies (cf. Näsholm *et al.*, 2009). The limited increase in ^{13}C (Figs 1b & 5b) and absence of relationship between ^{13}C and ^{15}N isotopes in roots of LHT1-KO mutants (Figs 2 & 6) indicate that these plants could only access labelled N after L-glutamine had been mineralized into $^{15}\text{NH}_4^+$ and possibly $^{15}\text{NO}_3^-$. In contrast, the substantial increase in ^{13}C contents and significant positive relationships between ^{13}C -labelled C and ^{15}N -labelled N in roots of both wild type plants and 35S-LHT1 plants (Figs 1–6) suggest that these genotypes acquired intact L-glutamine from soil. The higher levels of ^{15}N -labelled N and ^{13}C -labelled C in 35S-LHT1 plants compared to wild type plants indicate that the root capacity for amino acid uptake is an important trait for plant organic N nutrition. Amino acid transporters are known to display high specificity for amino acids (Näsholm *et al.*, 2009). Hence, we argue that the genotypic differences in root ^{13}C -labelled C and ^{15}N -labelled N detected in our study should be linked to differences in root uptake of the amino acid L-glutamine and not to pleiotropic effects leading, for example, to altered uptake of inorganic N and organic acids. The predominance of the +7 ion in the isotopic patterns of root samples from wild type- and 35S-LHT1 plants but not in LHT1-KO plants (Table 1) corroborates the notion that our approach of using amino acid transporter mutants can be used to assess the relevance of plant organic N nutrition. This notion is further strengthened by the genotypic differences in the degree to which LC-MS derived estimates of ^{15}N -labelled N corresponded to IRMS derived uptake estimates of ^{15}N -labelled N (Fig. 4).

In experiment 1, slopes of linear regression of ^{13}C -labelled C versus ^{15}N -labelled N in roots of the wild type plants and 35S-LHT1 plants were significantly lower than 2.5, corresponding to the stoichiometric influx of ^{15}N and ^{13}C that is 100% labelled N uptake as intact L-glutamine and no loss of ^{13}C through internal metabolism of absorbed, labelled L-glutamine (Fig. 2). Notably, these slopes declined significantly between the 15 and 60 min harvest. Thus, this deviation from 2.5 suggests that rapid internal metabolism of absorbed, labelled L-glutamine followed by loss of $^{13}\text{CO}_2$ did occur as well as uptake of $^{15}\text{NH}_4^+$ and/or $^{15}\text{NO}_3^-$ produced through decomposition of labelled L-glutamine in soil. However, this is at least partly contradicted by the results of LC-MS analysis of isotopologues of glutamine, showing that most of the root ^{15}N -labelled N in wild type plants and 35S-LHT1 plants (but not in the LHT1-KO plants) was accounted for by root $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine (Fig. 4; see below).

A relatively large range of root levels of both ^{15}N -labelled N and ^{13}C -labelled C was found in both labelling experiments when $^{13}\text{C}, ^{15}\text{N}$ L-glutamine was the source of label (Figs 1–6). This range was, however, smaller for LHT1-KO plants than for the two other genotypes (Figs 1–6). The source of this variation is not known, but the similarly large range of ^{15}N -labelled N when $^{15}\text{NH}_4^+$ was the source of label (Fig. 5) points to the possibility of uneven distribution of the tracer in the pots.

We also employed LC-MS analysis of root extracts of seedlings charged with labelled L-glutamine in parallel with the experiment using IRMS-analysis to study the contents of ^{13}C and ^{15}N in the different LHT1 genotypes. This enabled an additional test of the fundamental question of plant organic N uptake from soil by tracing the specific compound supplied to pots into plant roots. Clear differences between genotypes in root contents of the molecular ion corresponding to $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine (m/z 324.135) were found, with LHT1-KO plants again deviating strongly and displaying close to background contents while wild type and 35S-LHT1 plants

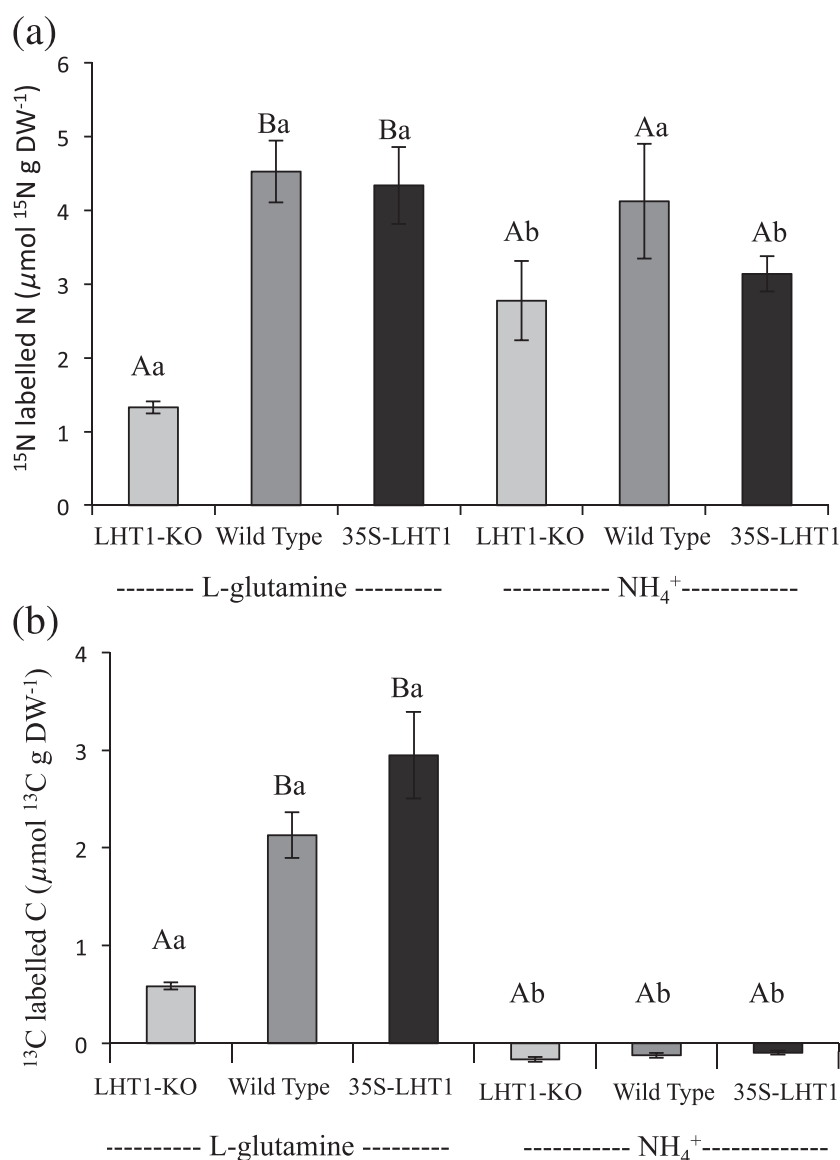


Figure 5. Comparison of label acquisition from isotopically labelled L-glutamine and ammonium for different Arabidopsis genotypes. Root contents of ^{15}N -labelled N (a) and ^{13}C -labelled C (b) 1 h after adding 5 mL of a 100 μM solution of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine or 5 mL of a 100 μM solution of $^{15}\text{NH}_4^+$ to soil in pots supporting *A. thaliana* Lysine Histidine Transporter 1 (*LHT1*) knock-out mutants (LHT1-KO), wild type plants (Wild Type) and *LHT1*-overexpressing (35S-LHT1) plants. Bars represent mean values \pm SE, $N = 6$. Different lowercase letters indicate a difference between L-glutamine and NH_4^+ treated plants within each genotype, different capital letters indicate a difference between genotypes within each nitrogen treatment.

displayed significantly higher contents of labelled L-glutamine (Fig. 3). We then compared root ^{15}N contents corresponding to $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine as measured by LC-MS with root ^{15}N -labelled N contents as measured by IRMS (Fig. 4). At the 15 min harvest, 106 and 85% of the root ^{15}N -labelled N were accounted for by $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine in wild type and 35S-LHT1 plants, respectively, but for LHT1-KO plants, only 24% of ^{15}N -labelled N was accounted for by $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine. The fraction of root ^{15}N -labelled N accounted for by $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine declined for all genotypes between the first and second harvest, while clear differences between genotypes were still discernable; only 4% of ^{15}N -labelled N was present as $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine in LHT1-KO plants while corresponding values for wild type and 35S-LHT1 plants were 25 and 51% (Fig. 4). The high

agreement in root ^{15}N contents between IRMS and LC-MS analyses for wild type plants and 35S-LHT1 plants at the 15 min harvest is at odds with the linear regression analysis of isotopes (Fig. 2), the former suggesting most of the ^{15}N -labelled N was contained in intact molecules of the labelled source, the latter suggesting rapid internal metabolism and/or labelled N was also absorbed as $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$. Two observations suggest that the linear regression analysis underestimated the $^{13}\text{C}:^{15}\text{N}$ ratio: Firstly, ratios of isotopes as calculated from the mean contents of ^{13}C and ^{15}N (Fig. 1) are significantly higher than those resulting from the linear regression analysis. Secondly, significant, positive intercepts were recorded in the linear regression analysis and forcing the regressions through the origin resulted in significant higher values of slopes. It should be noted that the linear regression method was

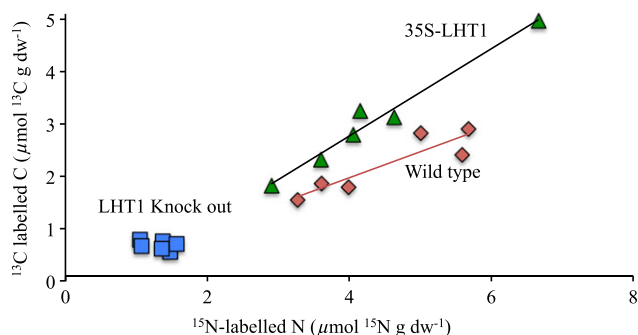


Figure 6. Root contents of ^{13}C -labelled C versus ^{15}N -labelled N 60 min after adding 5 mL of a 100 μM solution of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine to soil in pots supporting *A. thaliana* Lysine Histidine Transporter 1 (*LHT1*) knock-out mutants (gray squares), wild type plants (dark-gray diamonds) and *LHT1*-overexpressing (*35S-LHT1*) plants (black triangles). Each symbol represents one value from three plants per pot. The regression lines for *LHT1* overexpressing ($y = 0.8x - 0.7$, $r^2 = 0.97$) and wild type plants ($y = 0.5x - 0.1$, $r^2 = 0.82$) were significant, but not for *LHT1* knock-out plants.

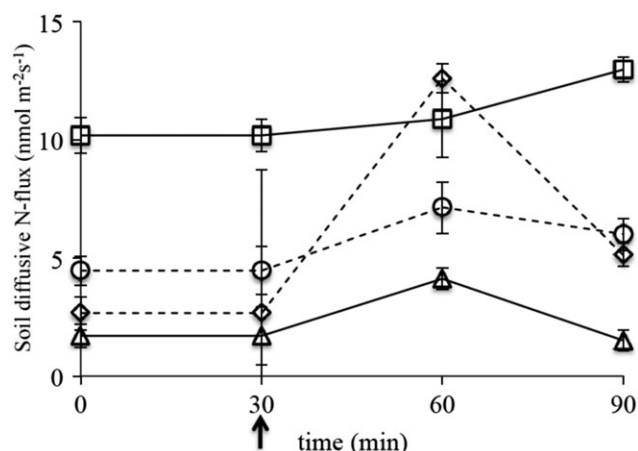


Figure 7. Soil diffusive fluxes of glutamine (triangles solid line), total amino acids (squares, solid line), ammonium (circles, dotted line) and nitrate (diamonds, dotted line) following injection of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine. Fluxes were measured through microdialysis and probes inserted into the root zone of pots used in the labelling experiment. Samples from induced diffusive flux collected by the dialysis probes were taken at 30 min intervals. Arrow indicates time of addition of labelled L-glutamine to pots. (Symbols indicate means \pm SE, $N = 8$).

introduced as a tool for evaluating results from dual labelling experiments when variations in ^{15}N and ^{13}C contents between replicates precluded use of means (Näsholm *et al.*, 1998) and was always deemed to produce conservative estimates of the fraction of ^{15}N from derived from intact uptake of organic N. The current experiment compared quantitative LC-MS and IRMS analyses to assess the degree to which the linear regression analysis underestimates intact organic N uptake. The current study aimed to study organic N uptake with as low perturbation as possible of added, dual-labelled tracer and thus was not designed for studying metabolism of absorbed compounds. The clear increase in the m/z 324.135 ion

Table 2. Carbon and nitrogen concentrations, C/N ratios and natural abundance of ^{15}N in *A. thaliana* *LHT1* knock-out plants (*LHT1-KO*), wild type plants and plants overexpressing the *LHT1* transporter (*35S-LHT1*) grown on agricultural soil. Plants were grown in a climate chamber for 31 days in pots filled with agricultural soil and shoots harvested and, C and N contents and natural abundance of ^{15}N ($\delta^{15}\text{N}$, ‰) determined. Mean values \pm SE, $N = 25$. Different letters indicate statistical differences between genotypes at $P \leq 0.05$

	<i>LHT1-KO</i>	Wild type	<i>35S-LHT1</i>
N concentration (% DW)	4.06 ± 0.1^a	3.9 ± 0.1^a	3.5 ± 0.1^b
C concentration (% DW)	40.5 ± 0.3^a	40.6 ± 0.2^a	41.4 ± 0.2^b
C/N ratio	10.2 ± 0.3^a	10.5 ± 0.2^a	12.3 ± 0.5^b
$\delta^{15}\text{N}$ (‰)	11.6 ± 0.2^a	12.2 ± 0.2^a	18.3 ± 1.3^b

(corresponding to $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine) for wild type and *35S-LHT1* plants at both the 15 and 60 min harvests (Fig. 3 & Table 1) suggests that these two genotypes efficiently acquired intact L-glutamine from soil. Only small changes in the Gln +1 and the +2 isotopologues were recorded and only at the 60 min harvest (Table 1). These isotopologues would probably correspond to glutamine isotopically enriched at the amide and amino positions, and the label may originate from internal metabolism of absorbed $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine or from absorbed, ^{15}N -labelled, inorganic N. The lack of significant differences between genotypes in the Gln +1 and +2 ions may suggest the latter.

The majority of studies that have targeted the question of competition for N between plants and microbes conclude that the latter outcompetes the former for organic N (Kuzakov & Xu, 2013). Our study did not attempt to directly measure competition between the two. Instead, we asked if a non-mycorrhizal weed is a weaker competitor against soil microbes for organic N than for mineral N. Figure 5a shows that wild type plants acquired similar ^{15}N labelling from the two N sources, indicating similar N uptake from L-glutamine or ammonium and hence similar competitive ability of plants against microbes for these two N sources. Our results, thus, do not support the notion that plants are weaker competitors for organic N than for inorganic N against soil microbes.

To minimize the issue of dramatic increases in concentrations of a single amino acid following addition to the soil, we used a 10-fold lower concentration of labelled substance compared to earlier studies (100 μM ; Jones *et al.*, 2005). Monitoring of soil solution using microdialysis revealed a transient increase in induced diffusive glutamine fluxes from $1.7 \text{ nmol m}^{-2} \text{ s}^{-1}$ before tracer addition to $4.1 \text{ nmol m}^{-2} \text{ s}^{-1}$ during the period 0–30 min following tracer addition and then returning to background concentrations during the period 30–60 min after tracer addition (Fig. 7). These values correspond to a calculated maximum increase in soil solution concentration from ca. 1.0 to $2.4 \mu\text{M}$. Moreover, the rapid return of soil solution glutamine fluxes corroborates uptake of the tracer by plants and microbes was swift. A concomitant increase in induced fluxes of NO_3^- (Fig. 7) may suggest rapid mineralization of added L-glutamine and/or may have resulted from increased availability of soil NO_3^- due to addition of the solution. Detection of ^{15}N -labelled N in *LHT1-KO* plants would suggest the former alternative,

that is, rapid mineralization of L-glutamine (Fig. 5). The significant differences between genotypes in contents of ^{15}N -labelled N, and the even larger differences in contents of ^{13}C -labelled C (Figs 1 & 5), together with the clear differences in contents of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine (Fig. 3 & Fig. S2), show the importance of direct uptake of intact L-glutamine molecules. At any rate, the limited increase in soil solution fluxes following injection of labelled L-glutamine suggests that the experiment did assess plant acquisition of organic N under realistic conditions.

Shoot contents of isotopes were generally much lower than for roots and differences between genotypes small (Fig. S3). No difference in ^{15}N -labelled N between genotypes was recorded when L-glutamine was the tracer, while for plants exposed to labelled NH_4^+ , a slightly lower value was found for LHT1-KO plants compared to 35S-LHT1 plants and with wild type plants taking the intermediary position. Similarly, a content of ^{13}C -labelled C of shoots of plants exposed to labelled L-glutamine was significantly higher for 35S-LHT1 plants than for LHT1-KO plants and again wild type plants taking the intermediary position (Fig. S3). The low labelling of shoots limits the scope of detecting differences between genotypes in our experiment, suggesting that longer incubation times may be needed to enable for labelled compounds to reach shoots. However, and as stated above, due to rapid internal metabolism, longer incubation times also constrain the possibilities of inferring in what form (organic or inorganic) N was acquired.

Arguments have been raised that the dual labelling approach cannot be used to infer uptake of intact amino acids from soil because simultaneous occurrence of ^{13}C and ^{15}N in plant tissues after exposure to labelled amino acids may result from root absorption of products of amino acid decomposition in soil (i.e. $^{15}\text{NH}_4^+$ and $^{13}\text{HCO}_3^-$ or ^{13}C -organic acids; Rasmussen *et al.*, 2010). The current study used genotypes of *A. thaliana* differing in amino acid uptake capacities to test this possibility. A significant absorption of products of amino acid breakdown in soil would have resulted in small or insignificant differences between LHT1-KO plants and the two other genotypes. Further, we would have expected to find a significant relationship between ^{13}C -labelled C and ^{15}N -labelled N also in LHT1-KO plants following exposure to labelled L-glutamine. Figures 1–6 show clearly that LHT1-KO plants differ from the two other genotypes by acquiring significantly lower amounts of ^{15}N and ^{13}C from labelled L-glutamine and in contrast to the other genotypes by not showing any relationship between the two isotopes. LC–MS analysis of root extracts further strengthens this conclusion by displaying very low contents of $\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2$ L-glutamine in LHT1-KO plants but high contents in wild type and 35S-LHT1 plants (Fig. 3). Under the conditions of this experiment with soil-grown, non-mycorrhizal *A. thaliana* plants, we argue that the dual labelling approach is informative for assessing plant organic N acquisition.

The dual labelling approach estimates the capacities of plants to acquire amino acids from soil, although it cannot be used to assess the overall importance of such compounds for the N economy of plants and alternative methods enabling such estimates have not been presented (Näsholm *et al.*,

2009). Indirect support for plant use of organic N may, however, come from organic N sources having distinct effects on plant C/N ratios (Franklin *et al.*, 2016) and plant natural ^{15}N abundance (Ostle *et al.*, 1999). We analysed these parameters in plants of the three different genotypes grown in agricultural and found that the 35S-LHT1 plants displayed significantly higher C concentrations, C/N ratios and natural ^{15}N abundance than either LHT1-KO plants or wild type plants, while differences between wild type and LHT1-KO plants were non-significant (Table 2). The two labelling studies show clear differences between all three genotypes, while measurements of N-isotopes and C/N ratios only found a difference between the 35S-LHT1 genotype and the two other genotypes. At present, we cannot explain these somewhat contrasting results. Moreover, as stated above, the C/N and plant natural ^{15}N abundance differences between genotypes do not provide conclusive evidence for plant organic N acquisition. Nevertheless, differences between genotypes in these aspects are in line with the genotypic differences in uptake of labelled L-glutamine from soil (Figs 1–6), and with substantial soil fluxes of amino acids (Fig. 7).

CONCLUSIONS

Plant organic N nutrition has been intensively debated the last decade, and claims that it is of general relevance to plants have been criticized. The dual (^{13}C , ^{15}N) labelling approach, constituting a cornerstone of this research field, showing that plants could acquire organic N under realistic field conditions, has been deemed inconclusive in several studies. Results presented here, pertaining to a non-mycorrhizal weed growing in N-rich agricultural soil, suggest that uptake of amino acids may be of general relevance for plants. The use of *A. thaliana* mutants differing in expression of a key root amino acid transporter gene offered a new way of testing the reliability of the dual labelling approach as well as the importance of active and regulated root uptake mechanisms for plant organic N nutrition. These tests substantiate claims that plants access organic N, in the form of amino acids, from soil and extend earlier studies by showing the relevance of this process for a non-mycorrhizal, fast growing weed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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