LjLHT1.2 - a mycorrhiza-inducible plant amino acid transporter from Lotus japonicus

LjLHT1.2—a mycorrhiza-inducible plant amino acid transporter from *Lotus japonicus*

Mike Guether, Veronica Volpe, Raffaella Balestrini, Natalia Requena, Daniel Wipf, Paola Bonfante

Abstract

In mycorrhizal associations, the fungal partner assists its plant host by providing nitrogen (N) in addition to phosphate. Arbuscular mycorrhizal (AM) fungi have access to inorganic and organic forms of N and translocate them, via arginine, from the extra- to the intraradical mycelium, where N is transferred to the plant as inorganic N compounds such as ammonium. However, several putative amino acid transporters (AATs) with an altered expression in *Lotus japonicus* mycorrhizal roots were recorded in a previous microarray-based investigation, which led to the question of whether a transfer of organic N, mainly in the form of amino acids, could occur in AM roots. Here, we have characterized an AAT gene (*LjLHT1.2*) that encodes for lysine–histidine–transporter (LHT)-type amino acid transporter. We show that it is induced in mycorrhizas, but not in nodulated roots. By using in situ hybridization and laser microdissection technology, the corresponding transcripts have been demonstrated to be located above all in arbusculated cells but also in the non-colonized cells of the root cortex. The gene expression resulted to be differentially regulated by the availability of the N sources. Furthermore, functional experiments, via heterologous expression in yeast, have demonstrated that the protein was a high-affinity amino acid transporter. Taken together, the results show that LjLHT1.2 may allow the uptake of energy-rich N compounds, such as amino acids, towards the cortical cells. We suggest that LjLHT1.2 could be involved in complex mechanisms that guarantee the re-uptake and recycle of amino acids and which are particularly efficient in mycorrhizal roots.

Keywords

Amino acid transport*Lotus japonicus*Arbuscular mycorrhizaPlant nutritionNitrogen

Introduction

Mutualistic arbuscular mycorrhizal fungi (AMF) colonize the roots of the vast majority of land plants and improve the mineral nutrition of their hosts via an efficient uptake of mineral nutrients from the soil. The plant in return provides the fungus access to the photosynthetic C pool, creating an energetic flux, which is needed for the fungal nutrient uptake as well as for its proliferation and spore formation. The main sites for such a nutrient exchange are the arbusculated cells (Bonfante and Genre 2010). Arbuscules are highly branched characteristic fungal structures that develop inside a living cortex cell. Each fungal branch is surrounded by a proliferating plant plasma membrane that is called the periarbuscular membrane (PAM).

Since the largely acknowledged benefit of an AM plant is an improved inorganic phosphate (Pi) nutrition, a landmark in the current knowledge is that the transport of Pi from the fungus towards

the plant is facilitated by transporters located in the PAM (Bucher 2007; Javot et al. 2007). However, it has been shown in recent years that AMF play an additional role in soil nutrient cycling since they are able to acquire organic and inorganic N from the soil and, at least in part, transfer it to the plant via an active interface (Lanfranco et al. 2011) in the form of inorganic N compounds, such as ammonium. Mycorrhiza-specific ammonium transporters have been identified (Gomez et al. 2009; Guether et al. 2009b; Kobae et al. 2010) and like mycorrhiza-specific Pi-transporters are localized in the PAM, where they mediate the uptake of the ammonium released by the fungus. An optional transfer of organic N has been postulated to be mediated by LHT-type amino acid transporters (lysine–histidine–transporter) and to be active at the fungus–root interface (Chalot et al. 2006; Hirner et al. 2006), but this has never been experimentally demonstrated.

Several putative amino acid transporters (AATs), with an altered expression, were found in a previous microarray-based investigation of *Lotus japonicus* roots colonized by the AM fungus *Gigaspora margarita* (Guether et al. 2009a). In order to test the hypothesis that they may be involved in the transfer of fungal amino acids to the plant and to have a better understanding of the relationships between AMF and the N metabolism in a colonized root, we characterized an AAT that had resulted to be the strongest up-regulated one in the previous investigation on mycorrhizal roots of *L. japonicus* (Guether et al. 2009a). We show here that the transporter is specifically induced in mycorrhizas and is not altered in nodulated roots. We also demonstrate that its transcripts are located above all in arbusculated cells, and its expression is regulated by the availability of N sources. Lastly, uptake experiments have proved that the protein is a functional high-affinity AAT.

Materials and methods

Plant materials, growth conditions and inoculation methods

Gi. margarita Becker and Hall (strain deposited in the Bank of European Glomales as BEG 34) was used as the fungal inoculum. The *L. japonicus* mycorrhization method (Regel) K. Larsen (Gifu; wild type) is described in detail in Guether et al. (2009a). *Lotus* seedlings were grown, for the nodulation experiment, in a vermiculite and Long–Ashton nutrient solution (Broughton and Dilworth 1971) containing 10 μM or 4 mM KNO_3, respectively. Some of the N-starved plants were inoculated with *Mesorhizobium loti* strain NZP2235 (Ott et al. 2005). Bacterial cells were taken from a TY plate, washed and then suspended in 50 mL of water. Twenty-eight days after the inoculation with the fungus, the samples were cut from the mycorrhizal roots after observation under a stereomicroscope. Some segments were stained with 0.1% cotton blue in lactic acid and the infection was quantified (Trouvelot et al. 1986); other fragments were processed for further analysis. To investigate *LjLHT1.2* expression as N source dependent, novel sets of mycorrhizal and non-mycorrhizal *L. japonicus* plants were prepared. After 28 days, a solution of 20 mM nitrate or ammonium was applied for further 7 days.

RNA isolation, cDNA synthesis and real-time RT-PCR

The RNA isolation, cDNA synthesis and quantitative RT-PCR methods were described in detail in Guether et al. (2009a). Prior to quantitative RT-PCR, gene-specific primers were tested for *LjLHT1.2* on genomic DNA and cDNA for amplification. Since the RNA extracted from mycorrhizal roots contains plant and fungal RNAs, the specificity of the primer pair was also analysed by PCR amplification of the *Gi. margarita* genomic DNA. No amplification products were obtained on the fungal DNA. The oligonucleotide sequences for *LjUBQ10* (TC14054) and *LjEF1* (TC14038) are 5′-TTCACCTTGTGCTCCGTCTTC-3′ and 5′-AACAACAGCACACACAGACAATCC-3′ and 5′-TGTGAAGGATCTCAAGCGTG-3′ and 5′-

GTATGGCAATCAAGGACTGG-3′ respectively (Guether et al. 2009a). The oligonucleotide sequences for *LjLHT1.2* were as follows: forward primer, 5′-GATGTTCAGCTTATCCTGGATC-3′; reverse primer, 5′- CAGATAGCTCTTGAGAAGTAGG -3′.

5′/3′ RACE and sequence analysis

5′-RACE and 3′-RACE were performed with the aforementioned total RNA extracted from the mycorrhizal roots using a SMART RACE cDNA amplification kit (CLONTECH, Mountain View, CA, USA). The gene-specific primer sequences used are as follows: reverse primer, 5′-CATCTTCCCAACTTCAACGCCA -3′; forward primer, 5′-AGTCCACATCTTCCTGAACTCCCT-3′. PCR was performed according to the CLONTECH protocol using the Advantage 2 PCR enzyme system and 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 2 min and a final extension at 72°C for 10 min. The RACE products were subjected to electrophoresis, cloned in pCRII (TOPO cloning kit, Invitrogen, Carlsbad, CA, USA) and analysed by DNA sequencing.

The LjLHT1.2 secondary structure was predicted using the TMHMM programme.

Plasmid constructs

The coding region of *LjLHT1.2* (1,398 bp) was amplified from the aforementioned cDNA by PCR using Phusion DNA-Polymerase (Finnzymes, Espoo, Finland) and the following oligonucleotides: forward primer, 5′-CGTACATTAAACATGTCTACTGTT-3′; reverse primer, 5′-TTTAGTTTGAGGTCATCTCGA-3′. The PCR product was cloned into the yeast expression vector pDR196 (Wipf et al. 2003) and 199 (Schaaf 2004) and the full length was sequenced as previously described (Guether et al. 2009b). The GenBank accession numbers for the coding sequences of the control AATs are At5g40780 for AtLHT1 and X95736 for AtAAP6.

Heterologous expression in yeast and uptake measurements

The plasmids containing the AAT coding sequences were heat shock-transformed (Gietz and Woods 2002) into the yeast mutant strain 22Δ8AA (Fischer et al. 2002), which is defective in multiple amino acid uptake systems. The N-deficient growth medium, lacking uracil, was YNB without amino acids and ammonium sulfate (ForMedium™, Norfolk, UK) and was supplemented with 3% Glc and 10 mM NH_4Cl as the only N source. The growth of the yeast was not visibly affected by the expression of the different constructs under non-selective conditions. The following AATs were used as controls for the heterologous yeast complementation experiment: AtLHT1 and AtAAP6 (Hirner et al. 2006).

Complementation growth tests were performed on arginine, aspartate, glutamate, (0.5 mM), citrulline, GABA and proline (1 mM each) as the only N sources. The uptake measurements and K_M determination with [^{14}C]Pro were conducted as previously described (Cappellazzo et al. 2008). Samples were removed for linear uptake rates after 30, 60, 120 and 180 s. The measurements represent the means of three parallel experiments.

In situ hybridization

Sample fixation and embedding

Mycorrhizal and non-mycorrhizal roots were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS): 130 mM NaCl; 7 mM Na_2HPO_4; 3 mM NaH_2PO_4, pH 7.4, overnight at 4°C. The

samples were fixed under a vacuum, for the first 15 to 30 min, to facilitate infiltration with the fixative. Thereafter, the fixative solution was removed by washing in a saline solution (150 mM NaCl) for 15 min at room temperature. The tissue was dehydrated in successive steps of 30 to 60 min in solutions (in 150 mM NaCl) of 30%, 50%, 70%, 80%, 95% and 100% ethanol (twice) and 100% Neoclear (twice). Finally, the samples were embedded in paraffin wax (Paraplast plus; Sigma-Aldrich, St Louis, MO, USA) at 60°C. Sections of 7 to 8 µm were then transferred to slides, treated with 100 µg/mL poly-l-Lys (Sigma) and dried on a warm plate at 40°C overnight.

Preparation of the riboprobes

A 298-bp fragment of the LjLHT1.2 cDNA was amplified and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). The gene-specific primer sequences are as follows: reverse primer, 5′-CCCAGAGAAGCCATCTAAAGG -3′; forward primer, 5′- CCACAAATCGTAGCAGACCG -3′.

DIG-labelled RNA probes were synthesized starting with 1 mg of linearized template (Langdale 1993). DIG-labelled riboprobes (antisense and sense probes) were produced with DIG-UTP by in vitro transcription using the pGEM-T Sp6 and T7 promoters according to the manufacturer's protocol (RNA labelling kit; Roche, Basel, Switzerland).

In situ hybridization and detection

The sections were treated as follows: deparaffinized in Neoclear, rehydrated through an ethanol series, treated with 0.2 M HCl for 20 min, washed in sterile water for 5 min, incubated in 2X SSC for 10 min, washed in sterile water for 5 min, incubated with proteinase K (1 µg/mL in 100 mM Tris–HCl, pH 8.0, 50 mM EDTA; Roche) at 37°C for 30 min, washed briefly in PBS and then treated with 0.2% Gly in PBS for 5 min. After two rinses in PBS, the slides were incubated in 4% paraformaldehyde in PBS for 20 min, washed in PBS (2 × 5 min) and then dehydrated in an ethanol series from 30% to 100%. Hybridizations were carried out overnight at 55°C with denatured DIG-labelled RNA probes in 50% formamide, 6X SSC, 3% SDS, 100 µg/mL tRNA, 100 µg/mL poly A. The slides were then washed twice in 1X SSC, 0.1% SDS at room temperature and rinsed with 0.2X SSC, 0.1% SDS at 55°C (twice for 10 min). After rinsing with 2X SSC for 5 min at room temperature, the non-specifically bound DIG-labelled probe was removed by incubating it in 10 mg/mL RNase A in 2X SSC at 37°C for 30 min. The slides were then rinsed twice in 2X SSC before proceeding to the next stage. The hybridized probe was detected using an alkaline phosphatase antibody conjugate (Roche). After rinsing in TBS (100 mM Tris–HCl, pH 7.5, 400 mM NaCl) for 5 min, the slides were treated with a 0.5% blocking reagent in TBS for 1 h, incubated for 2 h with the anti-DIG alkaline phosphatase conjugate diluted 1:500 in 0.5% BSA Fraction V in TBS and then washed in TBS (thrice for 5 min). Colour development was carried out as previously reported (Torres et al. 1995). The colour reaction was stopped by washing in distilled water, and the sections were then dehydrated through an ethanol series, deparaffinized in Neoclear and mounted in Neomount (Merck, Darmstadt, Germany).

Laser MicroDissection (LMD), RNA extraction and RT-PCR

Mycorrhizal and non-mycorrhizal root segments were fixed in freshly prepared Methacarn (absolute methanol/chloroform/glacial acetic acid [6:3:1]) at 4°C overnight for paraffin embedding (Balestrini et al. 2007). A Leica AS laser microdissection system (Leica Microsystems, Bensheim, Germany) was used to isolate cells from the prepared tissue sections as described in Guether et al. (2009a). After collection, the RNA extraction buffer from the PicoPure kit (Arcturus Engineering, Mountain View, CA, USA) was added and samples were incubated at 42°C for 30 min, centrifuged at 800 *g*

for 2 min and stored at −80°C. Then, about 1,500 cells were pooled for each cell type population in a single tube with a final volume of 50 µL for the subsequent RNA extraction steps.

The RNA extractions were performed according to a slightly modified PicoPure kit protocol (Arcturus Engineering), as described by Balestrini et al. (2007). RNA quantification was obtained using the NanoDrop 1000 spectrophotometer. A one-step RT-PCR kit (Qiagen, Valencia, CA, USA) was used for the RT-PCR experiments, which were conducted on the RNA extracted from the several samples. Reactions were carried out, as described in detail in Guether et al. (2009a), with the *LjLHT1.2* oligonucleotides used for amplification of the in situ hybridization riboprobe and the housekeeping gene *LjEF1* in order to validate the presence of plant RNA in all the microdissected samples (Guether et al. 2009a). The RT-PCR experiments were conducted on at least three independent biological and technical replicates.

Results

Gene isolation and phylogenetic analysis of *LjLHT1.2*

The 1,692-bp full-length cDNA of the *LjLHT1.2* gene (GenBank accession no. JF705953), obtained using a 5′ and 3′ RACE, encodes a 466-amino-acid-long peptide with a relative molecular mass of 51.8 kDa. Using a transmembrane prediction programme (http://www.cbs.dtu.dk/services/TMHMM/), the LjLHT1.2 protein is likely to have nine transmembrane domains with a cytosolic N-terminus and an extracellular C-terminus (Fig. 1b). The phylogenetic analysis (Fig. 2) showed a high similarity to the LHT type of amino acid transporter, a subfamily of the ATF superfamily (Wipf et al. 2002). The highest homology within this group was found to be with the AtLHT1 (Chen and Bush 1997) transporter of *Arabidopsis thaliana*, with an overall identity of 80%. Interestingly, AtLHT1 is known to be involved in general amino acid uptake in roots. From the genomic sequence database of *L. japonicus* (Sato et al. 2008), it results that the genomic DNA of the *LjLHT1.2* gene contains eight exons and seven introns and it is located on chromosome 1 (Fig. 1a). An analysis of the putative promoter sequence upstream of the 5′ UTR revealed the partial sequence of another putative *LHT* gene (Fig. 1a). Translated into protein, this partial coding sequence shows an even higher identity, 84% (Fig. 2), to AtLHT1 and was therefore named LjLHT1.1.

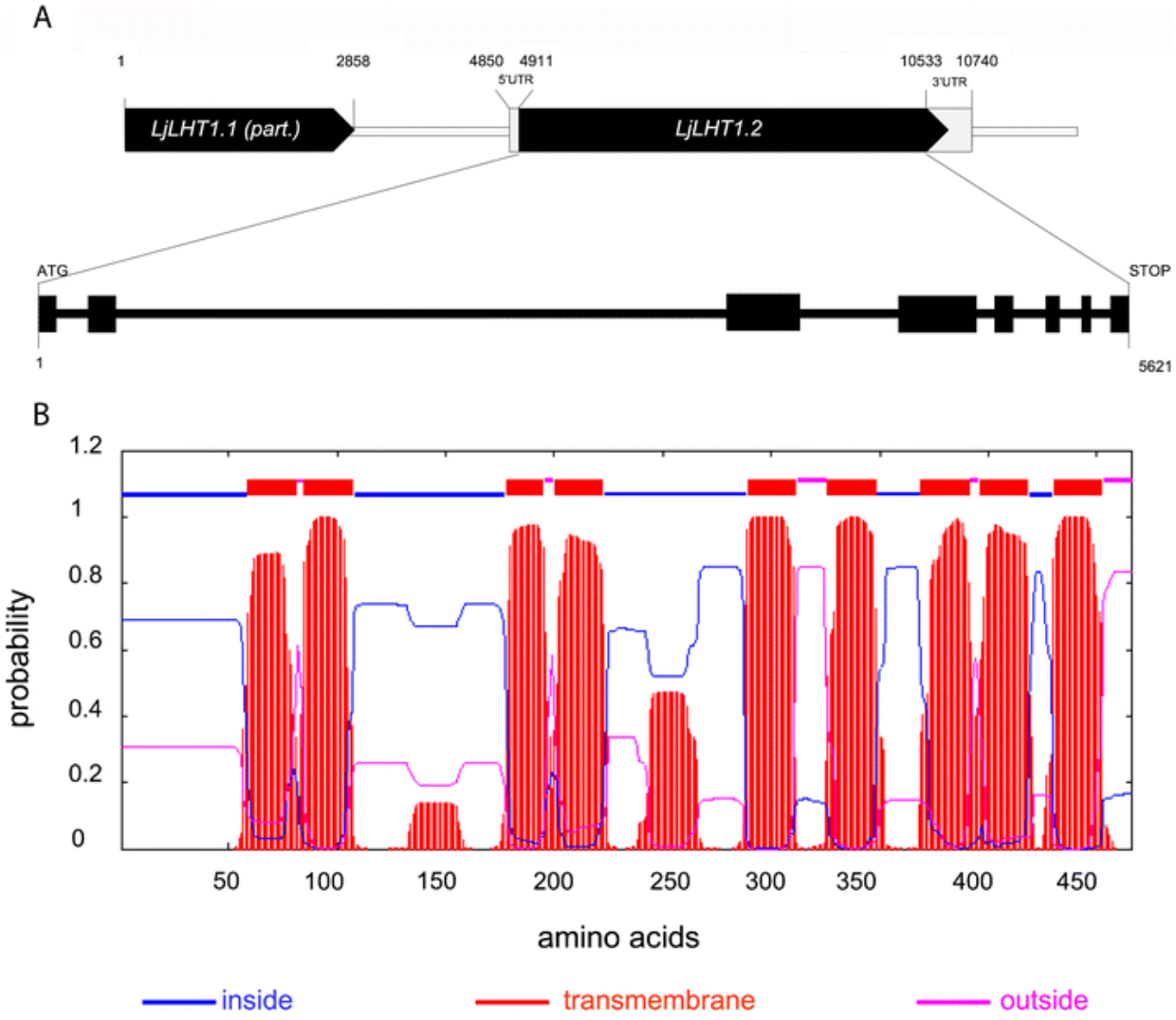

Fig. 1 In silico analysis of *LjLHT1.2*. **a** Genomic orientation of *LjLHT1.1* and *LjLHT1.2* on contig chr.1.CM0064 from *L. japonicus* genome assembly build 1.0 (Sato et al. 2008) and intron–exon structure of the *LjLHT1.2* gene; *black blocks* = exons. **b** Prediction of transmembrane domains of LjLHT1.2 using the TMHMM programme (http://www.cbs.dtu.dk/services/TMHM)

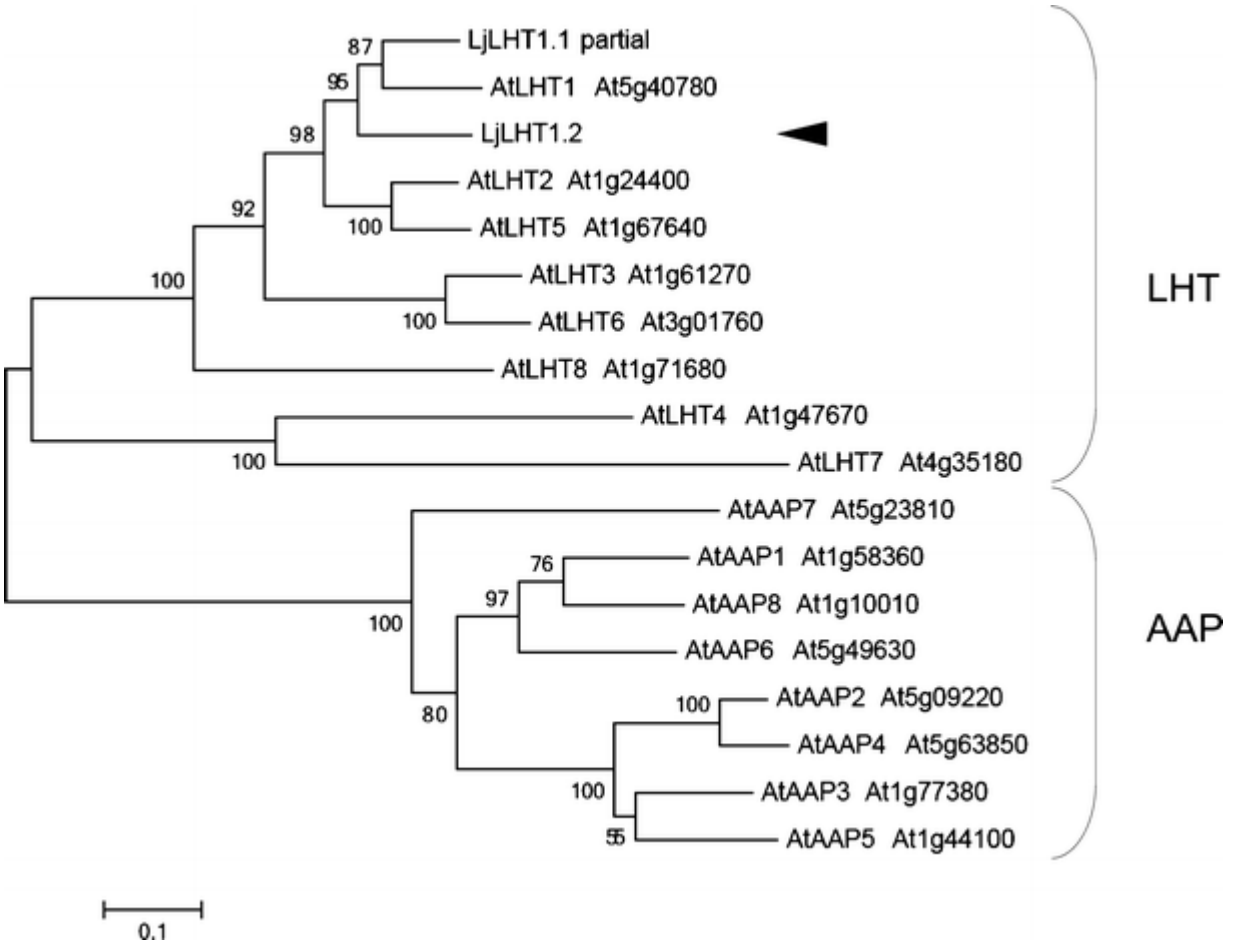

Fig. 2 An un-rooted phylogenetic tree for the amino acid sequences of plant LHT- and AAP-type amino acid transporters that was generated by Mega 4.0 software using ClustalW for the alignment and the neighbour-joining method for the construction of the phylogeny (Tamura et al. 2007). Bootstrap tests were done using 1,000 replicates. The branch lengths are proportional to the phylogenetic distances. *At A. thaliana*, *Lj L. japonicus*. Gene accession numbers are provided behind gene name. The *black arrow* marks the LjLHT1.2 protein sequence

AM symbiosis and N-source-dependent *LjLHT1.2* expression

The *LjLHT1.2* gene resulted to be one of the most up-regulated genes in a previous microarray study (Guether et al. 2009a) with 360-fold regulation upon mycorrhization. To test whether this regulation was mycorrhiza-specific or dependent upon the improved N nutritional status of the plant, we carried out a qRT-PCR analysis on transcripts from mycorrhizal and nodulated roots as well as from the respective controls treated with high and low N and P availability. To perform these experiments, mycorrhizal and control roots were sampled at 28 days and the extension of the colonization process was quantified as corresponding to 61% of the whole root length. Control and nodulated roots were sampled at 35 dpi. There were 32 to 36 nodules counted in each root system from the inoculated plant.

Mycorrhizal roots were confirmed to show the highest expression level (350-fold compared to the non-mycorrhizal P-starved roots). In the nodulated roots, the expression of the *LjLHT1.2* gene was similar to that of the respective control, with high N availability (Fig. 3). The non-nodulated control roots (under N starvation and P availability) showed a threefold up-regulation and the non-mycorrhizal control roots (under P starvation and N availability) a 0.4-fold down-regulation of its *LjLHT1.2* gene activity in comparison to the respective control (P and N availability) (Fig. 3).

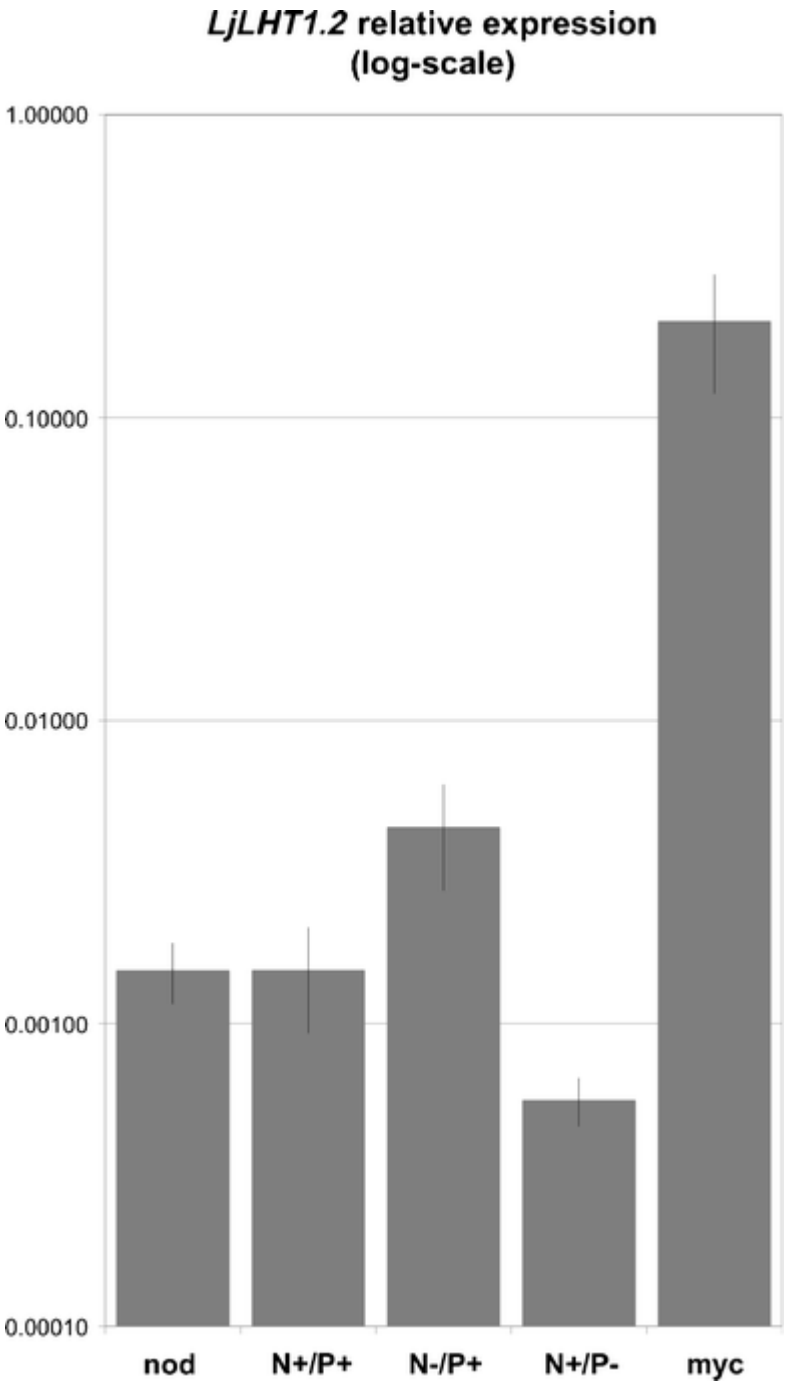

Fig. 3 Relative expression of *LjLHT1.2* assessed by qRT-PCR in *L. japonicus* roots after 28 days of mycorrhization and 35 days after nodulation. The c_t values of the samples were normalized against the c_t values of the housekeeping gene UBQ10. Data for each condition are presented as mean±standard deviation and were obtained from three biological and three technical replicates. −N = 10 µM KNO_3; +N = 4 mM KNO_3; −P = 20 µM PO_4^{3-}; +P = 500 µM PO_4^{3-}

These experiments demonstrate that the gene expression level is mycorrhiza dependent and that N and P are involved in its regulation as crucial nutrients, even though to different degrees.

Since the expression of the orthologous *AtLHT1* transporter in *A. thaliana* roots is induced by nitrate and repressed by ammonium (Hirner et al. 2006), a qRT-PCR expression analysis was performed on cDNA from mycorrhized and control roots treated with nitrate or ammonium. To rule out growth and developmental dependent effects, all of the plants were grown on a medium containing 20 mM nitrate as the N source for

4 weeks; half of the plants was then watered daily with the same medium for 1 week, whereas the other half was watered with 20 mM ammonium as the N source. The N source had no significant effect on the *LjLHT1.2* expression in *Lotus* mycorrhized roots (Fig. 4). However, in the non-mycorrhized control roots, the *LjLHT1.2* expression was about fourfold higher when watered with nitrate than in the ammonium-treated control roots. This regulation follows the trend described for the AtLHT1 transporter, even though the quantitative data differ due to the diverse treatment conditions (Hirner et al. 2006).

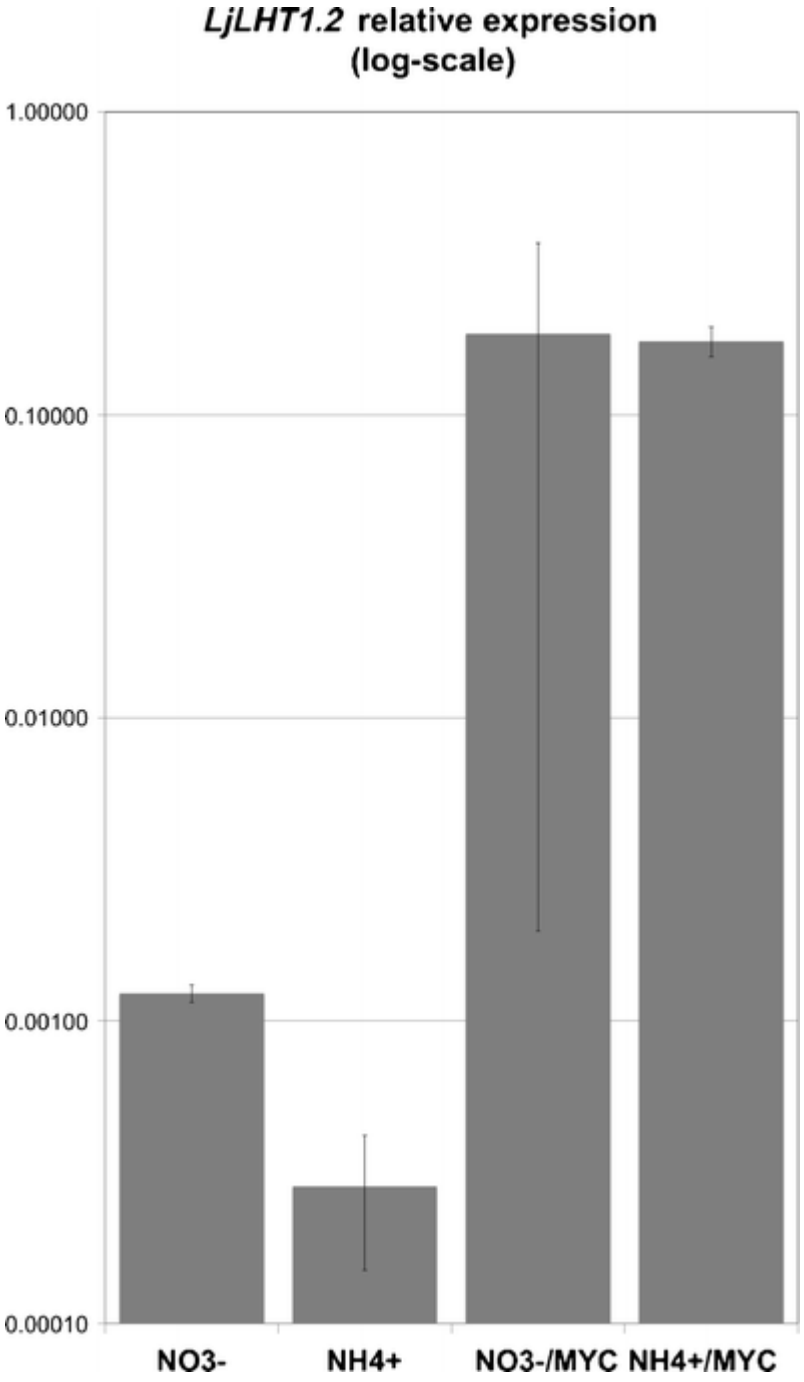

Fig. 4 Relative expression of *LjLHT1.2* assessed by qRT-PCR in *L. japonicus* roots after 28 days of mycorrhization and the application of 20 mM nitrate or ammonium as N source. The c_t values of the samples were normalized against the c_t values of the housekeeping gene ubiquitin. Data for each condition are presented as mean±standard deviation and were obtained from three biological and three technical replicates

Taken as a whole, the experiments demonstrate that *LjLHT1.2* expression is affected to a great extent by mycorrhization and moderately sensitive to N and P starvation and to the N source, but not affected by nodulation.

Cell-specific expression of *LjLHT1.2*

In situ hybridization experiments were performed to show the distribution of the *LjLHT1.2* transcripts in mycorrhizal roots. A higher level of chromogenic signal, which mirrors the presence of the *LjLHT1.2* transcripts, was observed above all in arbusculated cells (as indicated by the black arrows in Fig. 5a, b) as well as in the vascular tissue. A weak signal was also sometimes present in the non-colonized cortical cells. No staining was detected in the cortical layer of the non-mycorrhizal roots, while a strong signal was still evident in the vascular tissue (Fig. 5c). Control experiments conducted with the sense probe did not show any signal (Fig. 5d).

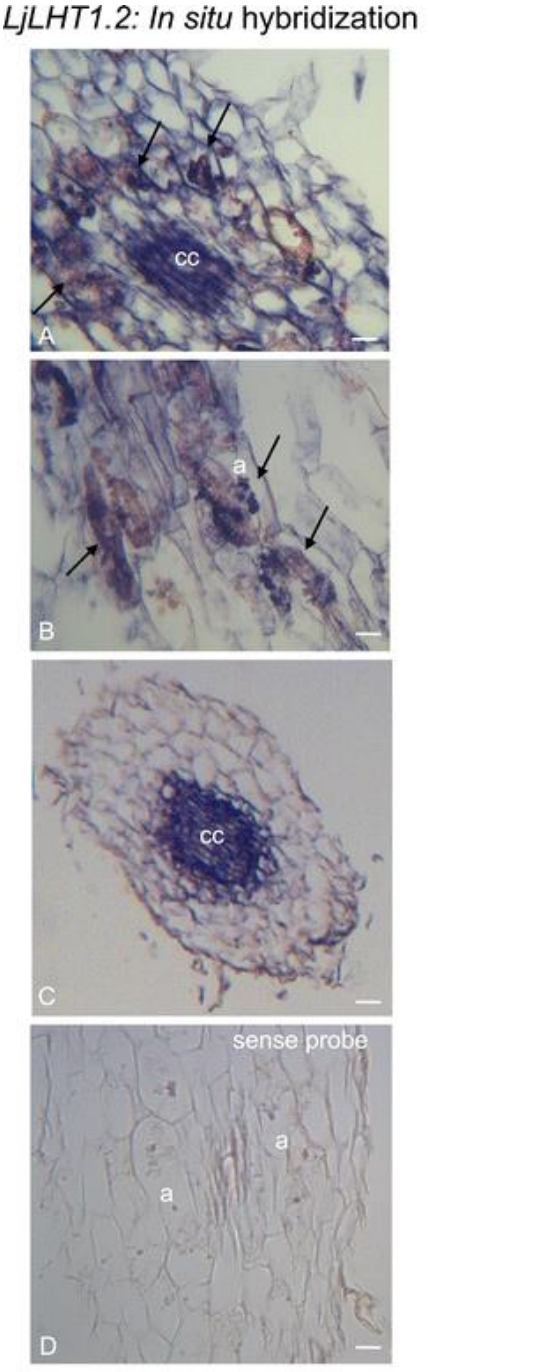

Fig. 5 **a**–**d** In situ hybridization. **a**, **b** Section of mycorrhizal roots with a higher level of chromogenic signal in arbusculated cell (*a*) and in central cylinder (*cc*); **c** section of non-mycorrhizal roots with an evident signal only in vascular tissue (*cc*); **d** sense probe shows a basic signal; **e** RT-PCR analysis of *LjLHT1.2* in LMD samples: amplified fragments were detected in arbusculated cells (*ARB*) and in non-colonized cortical cells from mycorrhiza roots (*MNM*); in cortical cells from non-mycorrhizal roots (*C*) the level of *LjLHT1.2* was undetected

The distribution of the *LjLHT1.2* transcripts was confirmed using a laser microdissection or LMD approach (Fig. 5e). To this aim, three cell types were collected: arbusculated cells (ARB), non-colonized cortical cells from mycorrhizal roots (MNM) and cortical cells from non-mycorrhizal roots (C). The extracted RNA was subjected to RT-PCR one-step analysis. Amplified fragments of the expected size were always detected in all of the samples using specific primers for the housekeeping gene *LjEF1* (data not shown). The amplification with specific primers for *LjLHT1.2* shows that a fragment of the expected size was present in all the RNA samples after 40 cycles, except for the C cell-type population where *LjLHT1.2* transcripts were not always detectable (Fig. 5e), which suggests a low level of expression in this cell type. In all of the biological replicates, amplified fragments were instead observed in ARB and MNM cell-type populations, which shows that *LjLHT1.2* is consistently expressed in cortical cells from mycorrhizal roots. LMD samples were validated by analysing the expression of the mycorrhiza-specific plant phosphate transporter *LjPT4* (data not shown) which is currently considered a positive control for arbusculated cells (Guether et al. 2009a). The two approaches have convincingly demonstrated that *LjLHT1.2* is prevalently expressed in mycorrhizal roots and have pointed to the arbusculated cells as the preferential site of the transcript presence.

Heterologous complementation of a yeast mutant defective in various amino acid uptake systems

To demonstrate that the *LjLHT1.2* gene encodes a functional amino acid transporter, a heterologous complementation experiment was performed with the yeast strain 22Δ8AA. Due to mutations in multiple amino acid transport systems, this yeast strain is incapable of growing properly on a minimal medium with certain amino acids (arginine, aspartate, citrulline, γ-aminobutyrate (GABA), glutamate, proline) as a single N source (Fischer et al. 2002). The yeast was transformed with the shuttle vectors pDR196/199, which express the genes of interest under the control of the constitutive PMA1 promoter (Schaaf 2004; Wipf et al. 2003). The already characterized amino acid transporters AtAAP6 (Okumoto et al. 2002) and AtLHT1 (Hirner et al. 2006) from *Arabidopsis* were included as positive controls. The empty vectors served as the respective negative controls. The 22Δ8AA strain carrying these constructs was spotted on plates with a YNB minimal medium and the respective amino acids (0.5–1 mM).

The yeast transformed with the *LjLHT1.2* construct was able to grow on all of the provided amino acids, whereas the negative controls showed almost no growth (Fig. 6a). The ability of the yeast expressing the *LjLHT1.2* to use the non-proteinogenic amino acid citrulline and the biogenic amine GABA indicates a similar substrate preference for LjLHT1.2 as that described for AtLHT1 (Hirner et al. 2006). However, the yeast expressing *AtAAP6* was not able to use these two N compounds efficiently. These results reinforce the hypothesis of phylogenetic clustering of the *Lotus* gene in the LHT amino acid transporter sub-family. However, the use of GABA as N source was remarkably lower for LjLHT1.2 than for AtLHT1, which pointed to a significant difference between the two transporters.

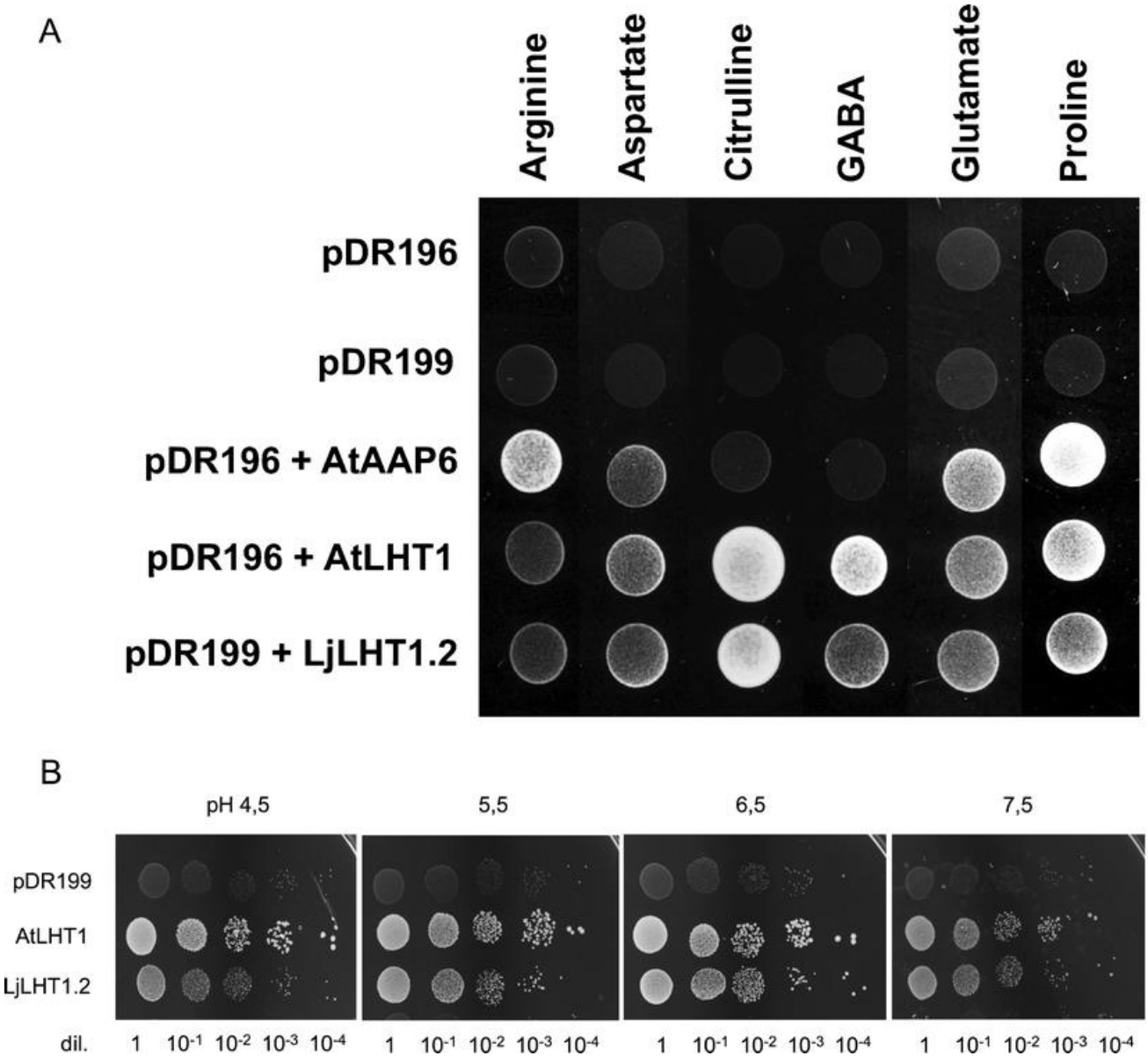

Fig. 6 **a** Growth of the amino acid uptake-deficient strain 22Δ88AA (Fischer et al. 2002) transformed with *AtLHT1*, *AtAAP6*, *LjLHT1.2* and the respective control plasmids pDR196 and pDR199 on different amino acids and amines as the only N sources (1 mM citrulline, 1 mM GABA, 1 mM proline, 0.5 mM arginine, 0.5 mM glutamate, 0.5 mM aspartate). OD of the undiluted yeast suspensions = 1. **b** pH-dependent growth test of the *AtLHT1* and *LjLHT1.2* transformed yeasts on 1 mM proline as a single N source. Serial dilutions (from *right* to *left*) $\mathrm{OD} = 1 - 1 \times 10 - 4$

The results prove that LjLHT1.2 is a high-affinity AAT with a K_M of 12.8 µM for proline. Since previous studies demonstrated that membrane transporters located at the plant–fungus interface often exhibit a dependency on an acidic pH (Guether et al. 2009b; Harrison et al. 2002), the transformed yeasts were further subjected to a growth test on media with different pH values. Such a strict acidic pH dependency, however, was not shown for LjLHT1.2 (Fig. 6b). The LjLHT1.2-expressing yeast showed a slightly reduced growth on plates with pH 4.5 and 7.5, suggesting an optimum pH for the transport between 5.5 and 6.5 as previously reported for other AATs and confirmed by the positive control AtLHT1 (Fig. 6b).

The ability of LjLHT1.2 to increase the permeability of the yeast membrane for amino acids was further investigated at different substrate concentrations. Due to the good growth shown by the *LjLHT1.2*-

expressing yeast on proline and since AtLHT1 has been reported to have a very low K_M for proline, ^{14}C-labelled proline was chosen as a substrate for the uptake experiments. The *LjLHT1.2*-expressing yeast even exhibited transport activity at very low proline concentrations (Fig. 7).

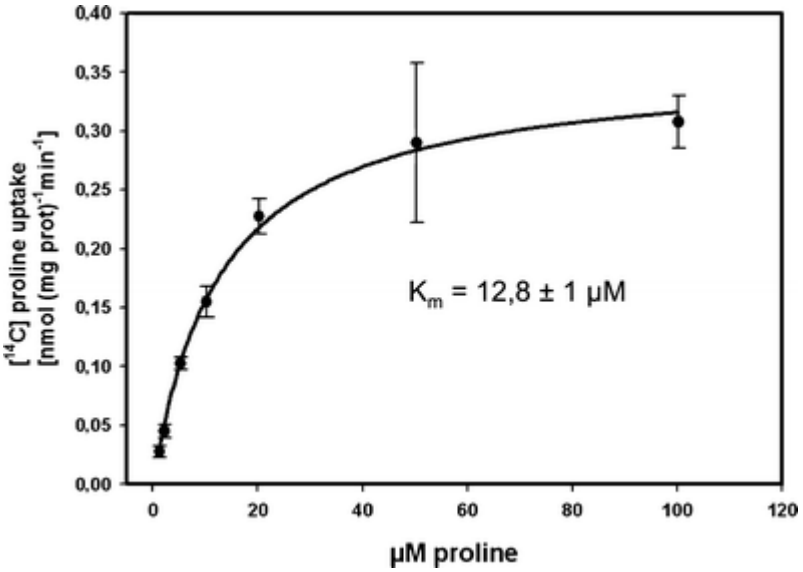

Fig. 7 Concentration-dependent kinetics of [^{14}C]Pro uptake by yeast strain 22Δ88AA (Fischer et al. 2002) transformed with the pDR199 *LjLHT1.2*. Yeast strain 22Δ88AA transformed with insert-free pDR199 were used to measure background transport activity, and the results are reported as net transport

Discussion

As one of the elements necessary for life, N is a limiting factor for plant growth and development. It is usually taken up by roots directly from the soil, where it is available in different organic and inorganic forms. Between the latter, nitrate and ammonium represent the preferential N sources. However, in cold-climate ecosystems, a reduced rate of N mineralization may lead to an increased uptake of amino acids, peptides and other organic N compounds by plant roots. In addition to P, AM fungi improve N availability for their host plants (Lanfranco et al. 2011), thanks to an uptake of inorganic and organic N sources (Govindarajulu et al. 2005; Hodge et al. 2001). Many findings have indicated ammonia as the preferential N compound transferred from AMF to the host (Govindarajulu et al. 2005; Guether et al. 2009b; Kobae et al. 2010), which raises the question on whether other N transporters are activated by the mycorrhizal status (Guether et al. 2009a). Here, we characterize the *Lotus* gene *LjLHT1.2* as a high-affinity transporter for amino acids and demonstrate that its expression is induced by mycorrhization and, to a lesser extent, by N starvation and nitrate. The results demonstrate that *LjLHT1.2* activation is the result of a novel N status, upon AM colonization, and open speculations on LjLHT1.2's role in N use efficiency of the mycorrhizal plant.

The *LjLHT1.2* gene belongs to a widespread family of plant amino acid transporters

Plant amino acid transporters have been classified in two superfamilies: the amino acid transporter family (ATF) and the amino acid polyamine choline family (APC) (Wipf et al. 2002). The ATF superfamily comprises several subfamilies and includes the LHT type (Wipf et al. 2002). Little is known so far about this kind of transporter; most of the current knowledge originates from *A. thaliana*, whose genome harbours eight LHT members, including *AtLHT1*, which is homologous to the *LjLHT1.2* sequence characterized in this work. Phylogenetic analysis has shown that the *LjLHT1.2* sequence clusters with another identified sequence (*LjLHT1.1*) and codes for a putative

LHT transporter (Fig. 2). Since *Arabidopsis*, a non-host plant for AM fungi, possesses only one *LHT1* gene, it is tempting to speculate that a duplication event in the plant ancestors has led to a mycorrhiza-specific function for one of the two homologous genes (*LjLHT1.2*). Alternatively, due to the ancient symbiotic status of most land plants (Bonfante and Genre 2008), non-host plants might have lost the symbiosis-specific homologous genes. However, such a hypothesis is difficult to test due to the lack of full-length coding sequences of all LHT-type family members of *Lotus* as well as of basal and other model plants.

LjLHT1.2 reveals broad-substrate specificity

The AtLHT1 sequence which was originally described as a transporter for the cationic amino acids lysine and histidine (LHT) (Chen and Bush 1997) is now considered as a transporter of a broad range of amino acids (Hirner et al. 2006). Based on the protein sequence similarity, LjLHT1.2 was expected to exhibit transport characteristics and substrate specificities similar to the AtLHT1 ones. Functional complementation experiments, using a yeast mutant defective in amino acid uptake, confirmed the hypothesis and showed that LjLHT1.2, like AtLHT1, mediates amino acid uptake into the cell and shows a broad specificity for proteinogenic and non-proteinogenic amino acids. The high affinity of LjLHT for proline, as reflected by the very low K_M (12.8 μM), is in agreement with the AtLHT1 function. On the other hand, the differences in the transport of the non-proteinogenic amino acid GABA by AtLHT1 and LjLHT1.2 would seem to point to a specialized function for the single transporters.

LjLHT1.2 expression depends on the nutritional status of the plant and on the external N source

Apart from the energy-rich C skeleton, amino acids contain a considerable amount of N. Plants growing under limited N conditions are likely to use systems to reuptake and recycle N from the amino acids and proteins that are lost from the cell during developmental processes. *Arabidopsis AtLHT1* has been convincingly demonstrated to be involved in the uptake of amino acids via the rhizodermis of roots and the mesophyll of leaves (Hirner et al. 2006). It was also suggested a role in amino acid retrieval from the apoplast as well as in amino acid cycling. Experiments performed in N-starved plants have demonstrated that *L. japonicus* shows a drastic reduced growth compared to nodulated and high-N-treated plants (Mike Guether, unpublished results). In addition, *LjLHT1.2* expression resulted to be up-regulated under N starvation, suggesting an enhanced uptake capacity for amino acids and a potential increased recycling capacity for organic N. This over-expression was strictly N dependent since the roots of the P-starved non-mycorrhizal control plants, which had access to a sufficient amount of N, showed a 0.4-fold down-regulation of gene activity. In addition, and again like *Arabidopsis*, *LjLHT1.2* expression depended on the N source as it was activated by nitrate, but not by ammonium. Taken as a whole, the results clearly indicate the importance of the amino acid LHT transporter for plants growing under N starvation or where reduced forms of N are limiting. Furthermore, these results also suggest that the roots may perceive the quantity and the nature of N that is supplied and regulate *LjLHT1.2* expression according to these environmental parameters.

LjLHT1.2 is preferentially expressed in mycorrhizal roots

A particular characteristic of the LjLHT1.2 amino acid transporter is its highly increased expression in mycorrhizal roots but not in nodulated roots. This indicates that the over-expression in mycorrhizal roots is not correlated to a general change in N status following the symbiosis but is specific of an AM interaction. On the basis of sequence similarities with *Arabidopsis AtLHT1* as well as the functional complementation of the yeast mutant, we have concluded that *LjLHT1.2* gene encodes a putative amino acid high-affinity transporter.

Transcript localization and laser microdissection experiments in *L. japonicus* mycorrhizal roots have shown that *LjLHT1.2* is expressed above all in the cortical arbuscule-containing cells and in the neighbouring non-colonized cells. Due to the complex morphology of arbusculated cells (Bonfante and Genre 2010), we can speculate that *LjLHT1.2* is located at both the peripheral membrane and the periarbuscular membrane that surrounds each fungal branch. In both locations, *LjLHT1.2* may allow amino acids to be retrieved directly from the apoplasm or from the interfacial material, which is a specialized form of apoplast (Balestrini and Bonfante 2005).

At the interface, LjLHT1.2 could be involved in the active transfer of amino acids from the fungus to the plant. However, this could not be the exclusive function as LjLHT1.2 does not show a close dependency on an acidic pH. Such a dependency has been reported for the mycorrhiza-specific phosphate and ammonium transporters located at the plant–fungus interface (Guether et al. 2009b; Harrison et al. 2002). Amino acids can also be originated from protein degradation processes in the interface around the senescing arbuscules. These fungal structures have, in fact, a relatively short life cycle (Alexander et al. 1989), which suggests a high turnover rate, involving protein recycling. Several transcriptome studies have already reported that proteases are transcriptionally activated during mycorrhization (Guether et al. 2009a; Hohnjec et al. 2005; Küster et al. 2004; Liu et al. 2003) and, in particular, a special family, the subtilases, seems to play an important role in arbuscule development (Takeda et al. 2007, 2009). A transfer of organic N from the fungus towards the plant is probably not quantitatively important since radiotracer studies have not revealed it (Govindarajulu et al. 2005; Jin et al. 2005). It is already known that amino acids are long-distance transport forms of N which are distributed in both symplastic and apoplastic ways (Ludewig and Koch 2008). In situ hybridization experiments, *LjLHT1.2* transcripts were detected in the vascular tissues of both mycorrhizal and non-mycorrhizal roots. This observation could nicely point to the involvement of the amino acid permease in the long-distance amino acid transport as a constitutive mechanism that is enhanced under the mycorrhizal condition. Although we have not at the moment any experimental evidence on its subcellular localization, we speculate that in addition to a vascular tissue location LjLHT1.2 transporter could be also localized in the peripheral plasma membrane of arbusculated and non-colonized neighbouring cells, with a possible role in the re-distribution of amino acids in mycorrhizal roots.

Taken as a whole, our findings add a novel element to the general claim that AM symbiosis causes changes in N root metabolism. Such modifications seem to be even more important than expected so far (Lanfranco et al. 2011) due to their impact on the whole nutritional network. Complex mechanisms which guarantee the re-uptake and recycling of energy-rich organic N compounds, such as amino acids, seem to be activated and require new functional actors, including specialized amino acid transporters. The characterization of the *LjLHT1.2* gene and of its transcripts represents therefore the starting point to elucidate the effective role of these amino acid transporters during AM symbiosis.

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