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Research

The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis

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Summary

• Nitrogen (N) is known to be transferred from fungus to plant in the arbuscular mycorrhizal (AM) symbiosis, yet its metabolism, storage and transport are poorly understood.
• *In vitro* mycorrhizas of *Glomus intraradices* and Ri T-DNA-transformed carrot roots were grown in two-compartment Petri dishes. ^{15}N- and/or ^{13}C-labeled substrates were supplied to either the fungal compartment or to separate dishes containing uncolonized roots. The levels and labeling of free amino acids (AAs) in the extraradical mycelium (ERM) in mycorrhizal roots and in uncolonized roots were measured by gas chromatography/mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC).
• Arginine (Arg) was the predominant free AA in the ERM, and almost all Arg molecules became labeled within 3 wk of supplying $^{15}NH_4^+$ to the fungal compartment. Labeling in Arg represented > 90% of the total ^{15}N in the free AAs of the ERM. [Guanido-2-^{15}N]Arg taken up by the ERM and transported to the intraradical mycelium (IRM) gave rise to ^{15}N-labeled AAs. [U-^{13}C]Arg added to the fungal compartment did not produce any ^{13}C labeling of other AAs in the mycorrhizal root.
• Arg is the major form of N synthesized and stored in the ERM and transported to the IRM. However, NH_4^+ is the most likely form of N transferred to host cells following its generation from Arg breakdown.

Key words: ^{13}C labeling, ^{15}N labeling, arbuscular mycorrhiza (AM), arginine (arg), *Glomus intraradices*, *in vitro* mycorrhizal culture, mass spectrometry, urea cycle.

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Introduction

Arbuscular mycorrhizal (AM) fungi form a mutualistic symbiosis with the roots of most land plants. They confer a nutritional benefit to their hosts by taking up phosphorus (P) and other macronutrients, trace elements and water from the soil and transferring them to their host plants. Most attention has been focused on P acquistion (Smith *et al.*, 1994; Harrison & Van Buuren, 1995; Smith & Read, 1997; Rausch *et al.*, 2001; Ezawa *et al.*, 2002). However nitrogen (N) is also an important element whose uptake from nutrient poor soils to roots can be improved by AM fungal colonization (Ames *et al.*, 1983; Jennings, 1995; Johansen *et al.*, 1996; Smith & Read, 1997).

The extraradical hyphae of (AM) fungi are able to take up and assimilate ammonium (NH_4^+) (Ames *et al.*, 1983; George *et al.*, 1992; Johansen *et al.*, 1992, 1993, 1996; Frey & Schüpp, 1993), nitrate (NO_3^-) (Tobar *et al.*, 1994; Bago *et al.*, 1996; Johansen *et al.*, 1996) and amino acids (AAs; Hawkins *et al.* 2000; Hodge *et al.*, 2001) from their surroundings and

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translocate N from diverse sources to the plant (Hawkins *et al.*, 2000; Azcón *et al.*, 2001; Vazquez *et al.*, 2001). AM fungi can apparently also transfer N from one plant to another (Bethlenfalvay *et al.*, 1991; He *et al.*, 2003; Cheng & Baumgartner, 2004) and can increase the availability of different forms of N to plants (Hodge *et al.*, 2001). However, when and where fungal transport of N plays an important part in plant nutrition remains unclear (Smith & Read, 1997; He *et al.*, 2003).

Assimilation of NH_4^+ is a principal means of N absorption both in ectomycorrhizal (Martin *et al.*, 1986; Finlay *et al.*, 1989; Chalot *et al.*, 1991; Martin & Botton, 1993) and AM fungal systems (Bago *et al.*, 1996; Hawkins *et al.*, 2000; Toussaint *et al.*, 2004). N uptake and incorporation into AAs via the glutamine synthetase, glutamate synthase (GS/GOGAT) cycle has been found in ectomycorrhizal fungi (Martin, 1985; Vèzina *et al.*, 1989; Chalot *et al.*, 1994). Smith *et al.* (1985) also described the activity of the GS/GOGAT enzymes in AM fungi. Also in an AM fungus, Govindarajulu *et al.* (2005) found support for N assimilation in the extraradical mycelium (ERM) via the GS/GOGAT pathway by measuring mRNA levels for key enzymes in the ERM and intraradical mycelium (IRM) tissues with quantitative real-time polymerase chain reaction. They also demonstrated the expression of a putative nicotinamide adenine dinucleotide (NAD)-dependent glutamate dehydrogenase (GDH) gene, which is down-regulated in the ERM tissue supplied with either NO_3^- or NH_4^+ in the ERM compartment, consistent with this enzyme having a catabolic role (Vallorani *et al.*, 2002). The report by Breuninger *et al.* (2004) that GS was constitutively expressed during all stages of the fungal life, whereas exposure to NH_4^+ produced a general increase in GS activity when compared with hyphae grown in NO_3^- as a sole N source, is also consistent with assimilation of inorganic N through the GS/GOGAT pathway in the ERM. Kaldorf *et al.* (1998) revealed that nitrate reductase is differentially expressed in arbuscular mycorrhizas of maize and concluded that NO_3^- may be transferred from fungus to plant.

The mechanisms involved in the fungal delivery of N are a matter of considerable interest because, depending on N availability and mobility and given the near-ubiquity of the AM symbiosis, these processes may represent a significant nutritional benefit to the plant (Ames *et al.*, 1983; Johansen *et al.*, 1996; Smith & Read, 1997). We have previously postulated a mechanism for N handling in the AM symbiosis which involves transfer of N from fungus to plant without carbon (Bago *et al.*, 2001). We recently provided evidence in support of the transfer of N without carbon (C) (Govindarajulu *et al.*, 2005), and here we test this potentially important mechanism, particularly the role of arginine (Arg) in the transport of N along AM hyphae.

We utilized a sterile, divided, Petri dish system (St-Arnaud *et al.*, 1996) that separates a portion of the extraradical mycelium (ERM) of *Glomus intraradices*-colonized RiT-DNA carrot roots from the mycorrhiza by using a barrier that does not allow soluble nutrients to move from one compartment to the other (Pfeffer *et al.*, 1999; Pfeffer *et al.*, 2004). By allowing the ERM to grow over the barrier while the roots remain in the initial compartment, we were able to study the uptake, metabolism and transport of ^{15}N-labeled substrates added to the fungal compartment. This *in vitro* model mycorrhizal system eliminates the complications arising from the uptake and metabolism of N by other microorganisms and from the diffusion of N to the host roots. Our observations show that Arg is a major form of N stored and transported in the mycelium of the AM fungus, and they are consistent with the proposed model in which NH_4^+ is the form of N transferred to the host.

Materials and Methods

In vitro culture of mycorrhizas and ^{15}N labeling

Ri T-DNA-transformed carrot roots (*Daucus carota* L.) [roots created by the incorporation of Ri T-DNA (Rhizogenes DNA plasmid) from the soil-borne bacterium *Agrobacterium* rhizogenes into the plant's genome], were grown at 24°C in modified M medium (Bécard & Fortin, 1988) gelled with 4 g l^{-1} phytagell (Sigma, St Louis, MO, USA) using the split-plate method of St-Arnaud *et al.* (1996). The medium of the root compartment was modified to limit the N concentration as follows: $Ca(NO_3)\cdot 4H_2O$ was replaced with $CaCl_2\cdot 2H_2O$ (180 mg l^{-1}) and KNO_3 was increased to 100 mg l^{-1}. This yielded a NO_3^- concentration of 1 mM as compared with 3.2 mM in M medium, which limits total root growth. This medium was also used for culturing uncolonized roots. The medium in the fungal compartment was modified M medium with no N added except for the labeled substrate: [^{15}N, 98%]NH_4Cl (4 mM), [guanido-2-^{15}N, 98%]Arg (2 mM) or [U-^{13}C, 98%]Arg (2 mM). Solutions of labeled compounds were adjusted to pH 6.0, filter sterilized through a 0.2-micron filter and added to cooled (~50°C) autoclaved M medium with no N, which was then poured into the fungal compartment. The uncolonized roots were cultured in the solid medium with the same concentration of labeled substrates as added to the ERM. The original growth of roots before subculturing onto plates was performed in liquid medium.

The level of colonization was 14–20%, based on the microscopic examination of cleared and stained roots as described earlier (Pfeffer *et al.*, 1999). Estimates made through microscopic examination of the amount of fungal material in the fungal compartment were at least 20 times larger than those outside the roots in the root compartment. Estimates of the fungal material inside the roots indicated that the fungus comprised < 2% of the mycorrhizal root mass (Pfeffer *et al.*, 1999).

To test the possibility that N could diffuse across the barrier between compartments, we grew uncolonized roots in one compartment and labeled the compartment into which the fungal ERM grows into after crossing the barrier with 4 mM

$^{15}NH_4Cl$. Extraction of the root AAs from the uncolonized root in the root compartment 2 wk later showed no detectable ^{15}N labeling.

Extraction and isolation of free amino acids for GC-MS and HPLC analysis

After approx. 3 wk of growth, the developing ERM grew across the compartment divider into the fungal compartment. The ERM extended into the fungal compartment and grew for 1, 3 or 6 wk, and at those times the tissues from the root and fungal compartments were collected by dissolving the media in 10 mM sodium citrate buffer pH 6.0 (Doner & Bécard, 1991). Tissues from four dishes were combined for each sample before analysis, and there were three replicate samples per experiment.

The tissues were recovered on a 40-μm sieve, rinsed with deionized water and lyophilized. The lyophilized mycorrhizal roots and ERM were each ground in a mortar and pestle with a pinch of acid washed sand and extracted with a mixture of methanol : chloroform : water (12 : 5 : 3, v/v/v), which gave a 30–35% higher recovery of AAs than extraction with NH_4^+ buffer. Methylene chloride and water were added to the extraction solution to facilitate the separation of chloroform and the methanol–water phases. The methanol–water phase containing the AAs was collected and evaporated in a rotary evaporator at 50°C, and the residues containing the AAs were dissolved in 2 ml of 0.01 M HCl and loaded onto a cation exchange column (0.3 ml of DOWEX 50 X8-200 – hydrogen form; Sigma-Aldrich, St Louis, MO, USA), which was previously washed with 2 M NH_4OH, deionized H_2O and 2 M HCl, and followed by deionized H_2O until the effluent was neutral. The neutral compounds, principally carbohydrates washed off the column with 5 ml of water and the free AAs (except Cys and Met, whose recoveries were low), were eluted with 2 ml of 1 M NH_4OH (Bengtsson & Odham, 1979). This eluent was collected and lyophilized for analysis.

Isolation of soluble protein and enzymatic hydrolysis of extracted protein

Tissues were stored at −80°C and then ground with acid-washed sand. The soluble protein was extracted twice with cold NH_4HCO_3 buffer (pH 8 with 0.2% NaN_3). After centrifugation, the supernatants containing soluble protein were lyophilized and resuspended in NH_4HCO_3 buffer (pH 8 with 0.2% NaN_3).

The extracts were dialyzed twice against 40 ml of NH_4HCO_3 buffer (pH 8 with 0.2% NaN_3) at 4°C for 24 h using a dialysis membrane with a molecular weight cut-off of 2000 (Spectra/Por 7 cellulose ester; Spectrum Medical Industries, Los Angeles, CA, USA). The dialysates were pooled, lyophilized, stored at −20°C, then lyophilized and resuspended in 600 μl of 20 mM NH_4HCO_3 buffer. Freshly dissolved proteases (2 μl aminopeptidase M, 2 μl of pronase E and 2 μl of carboxypeptidase Y) (Sigma, St Louis, MO, USA) were added and the samples were incubated for 6 h at 37°C with constant shaking. Fresh enzymes then were added and the incubation continued for a further 6 h. Samples were centrifuged for 10 min at 10 000 ***g*** at 4°C, and the supernatants were lyophilized and resuspended in 2 ml of water, then lyophilized again and resuspended in 1 ml of 0.1 M HCl. The AAs were isolated from this solution as described above for extracted free AAs.

GC-MS analysis of labeling in amino acids

AAs were derivatized as follows: 10–20 μl of dry N,N-dimethylformamide (DMF) was added to the sample, depending on the original tissue mass, then 30–50 μl of N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) was added to the sample, which was then heated for 30 min at 110°C.

Gas chromatography/mass spectrometry (GC-MS) analysis of labeling in AAs was performed as described by Mawhinney *et al.* (1986) by injecting the silylated extracts into a Finnigan Trace MS 2000 (Thermo Electron, Madison, WI, USA) equipped with a splitless injector (at 250°C), fused silica capillary column (RTX-5MS, 0.25 mm thick, 0.25 mm internal diameter, 30 m long; Restek Inc. Flemington, NJ, USA) interfaced to a Thermo Finnigan quadrupole mass detector (Thermo Electron, Madison, WI, USA). Helium was used as a carrier gas at a flow rate of 1 ml min^{-1}. The oven temperature was 110°C for 2 min after injection, rising to 260°C at 10°C min^{-1}, and remaining at 260°C for 5 min. Electron impact ionization was at an electron energy of 70 eV, and the detector mass range was scanned between mass-to-charge ratios *m/z* of 150 and 600 with a total scan time of 0.5 s. Identities of AAs were confirmed by comparison with mass spectra of authentic standards. Except for Arg, AAs labeling was determined by measuring the M-57 ions (ions having a mass of 57 less than the intact molecule) of the bis-(dimethylsilyl) trifluoroacetamide (MTBSTFA) derivatives, which result from the loss of a t-butyl group. The Arg ion measured had a *m/z* of 442, which is the M-188 (molecular ion minus 188) fragment arising from the loss of one guanido nitrogen together with a tBDMS and DMS group from the tetra-substituted tert-butyldimethylsilyl(tBDMS)-derivatized Arg (Fig. 1). When using [guanido-2-^{15}N] Arg, we observe an ion at a *m/z* of 443 (M-189, molecular ion minus 189). This isotopomer corresponds to the derivatized [guanido-2-^{15}N]Arg because the ion loses one of the equivalent guanido nitrogens by fragmentation at 70 eV (Fig. 1). Unlabeled, derivatized Arg yields an ion at a *m/z* of 442, which corresponds to a molecular fragment containing three ^{14}N atoms. Thus the maximum number of ^{15}N atoms detected is three, resulting in a mass isomer distributions of M, M+1, M+2 and M+3. These were used to calculate the isotopic enrichment in each AA after correction for natural abundance isotopic contents by comparison with the mass isomer distributions measured for unlabeled standards.

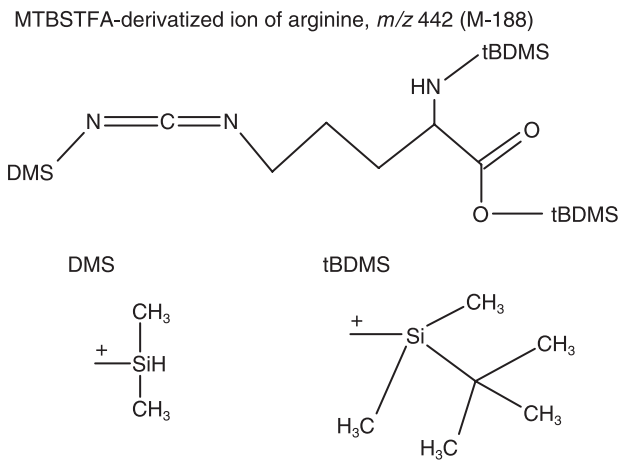

Fig. 1 Molecular structure of ions of bis-(trimethylsilyl) trifluoroacetamide (MTBSTFA)-derivatized Arg. This figure gives the structure of the ion remaining after a 70-eV impact on the MTBSTFA derivative of Arg. It demonstrates that the ion being detected has lost one of the quanido Ns and a t-butyl group. This fragmentation is different from that of the other amino acids (AAs) that only lose a t-butyl group (M-57) on impact in their fully derivatized form.

HPLC analysis of amino acid concentrations

The amount of each AA was measured by high-performance liquid chromatography (HPLC) of the phenylisothiocyanate derivatives as described by Endres & Mercier (2003). The extracted AAs were dissolved in 0.1 M HCl and vacuum dried on a Pico-Tag (Millipore Corp, Waters Chromatography Division, Milford, MA, USA) work station, then dried again by adding ethanol : water : triethylamine which then was evaporated by vacuum drying. Then 20 ml of ethanol : water : triethylamine : phenylisothiocyanate (7 : 1 : 1 : 1) was added to derivatize the AAs at 23°C for 20 min. The samples were then dried under vacuum and redisolved in 100 µl of Pico-Tag sample diluent. Then 20 µl of each sample was loaded onto a reverse-phase C18 column (3.9 mm ID X 150 mm long) using a Waters 510 autosampler using an eluent gradient consisting of 38 ml Pico-Tag Eluent A and Eluent B (Waters Corporation, Milford, MA, USA) as mobile phase. The flow rate was 1.0 ml min^{-1}, with the proportion of Eluent B rising from 0–100%. The elution was monitored at 254 nm with a Waters 486 tunable absorbance detector. Prior to chromatography, all solutions were degassed under vacuum for 1 min. The concentrations of the AAs were calculated by comparing the integrated peak area with those of standard AAs at known concentrations using Waters MILLENIUM software (Waters Chromatography Division).

NMR analysis of the ^{15}N content in mycorrhizal and uncolonized root tissues

Mycorrhizal root tissue was digested in H_2SO_4 followed by H_2O_2, as described by Wall & Gehrke (1975). Volatile NH_3 was distilled from the solution following the addition of 10 ml 2.0 M NaOH, and absorbed into 20 ml 0.1 M H_2SO_4. This solution was evaporated down in a forced air drying oven at 80°C. The resulting $(NH_4)_2SO_4$ was dissolved in 99% chlorosulfonic acid/H_2O containing 10% perdeutero-dimethyl-sulfoxide ($DMSOd_6$) and adjusted to pH 1.0 (Preece & Cerdant, 1993). The ^1H spectrum was obtained with 2048 transients at 400 MHz with a 30° pulse width, spectral width of 6000 Hz, pulse delay of 5 s and acquisition time of 2 s at 25°C. The triplet resonance of the ^1H–^{14}N and doublet resonance of the ^1H–^{15}N protons were observed centered at 3.72 ppm relative to the H_2O resonance at 4.67 ppm with ^1H–^{15}N couplings of 53 and 74 Hz, respectively. The integrated area of the ^1H–^{15}N doublet resonances divided by the sum of the doublet and triplet resonances yielded the percentage ^{15}N of the total N in the tissue.

Results

The concentrations and ^{15}N enrichments of free amino acids after $^{15}NH_4Cl$ labeling

The levels of the free AAs in the ERM and in the mycorrhizal roots were measured after exposure to 4 mM NH_4Cl. Arg is by far the most abundant AA in the extract of ERM with asparagine, glutamine and glutamate being next in abundance (in the range of 10–25 nmol mg^{-1} d. wt; Fig. 2). The levels of Arg dropped between 1 and 3 wk and then showed little change between 3 and 6 wk of development of the ERM; the levels of most free AAs did not change substantially during this period. Nevertheless, the level of Arg in the ERM remained high (50 nmol mg^{-1} d. wt) even after 6 wk, which is consistent with the fact that mature spores also were found to contain substantial levels of Arg (25 nmol mg^{-1} d. wt) (data not shown). The ERM contained a higher concentration of Arg than the mycorrhizal root and uncolonized root tissues (Fig. 2a). The levels of Arg in the mycorrhizal roots increased from 2.5 nmol mg^{-1} d. wt to 10 nmol mg^{-1} d. wt between 1 and 3 wk but dropped back to 5 nmol mg^{-1} d. wt after 6 wk of culture. Uncolonized roots contained predominantly asparagine (Asn) and glutamine (Gln) after 1 wk of labeling, whereas the concentration of Arg was relatively low (Fig. 2c). After 6 wk Gln was the most abundant AA. Arg levels also rose somewhat and Asn levels decreased.

Exposure of the ERM in the fungal compartment to 4 mM $^{15}NH_4Cl$ resulted in ^{15}N labeling of all free AAs in the ERM and colonized roots (Fig. 3a,b). After 1 wk of labeling, glutamate, glutamine and asparagine were 85% labeled by ^{15}N, and Arg was > 99% enriched with ^{15}N (Fig. 3a). In the mycorrhizal roots, Arg had the highest enrichment after 1 wk of $^{15}NH_4$ labeling, but after 6 wk of $^{15}NH_4^+$ labeling (Fig. 3b), most of the other AAs were highly labeled in the ERM and in mycorrhizal roots. Nonmycorrhizal roots exposed directly to the same ^{15}N treatment contained AAs that were somewhat less labeled than those from labeled ERM tissue. Labeling of

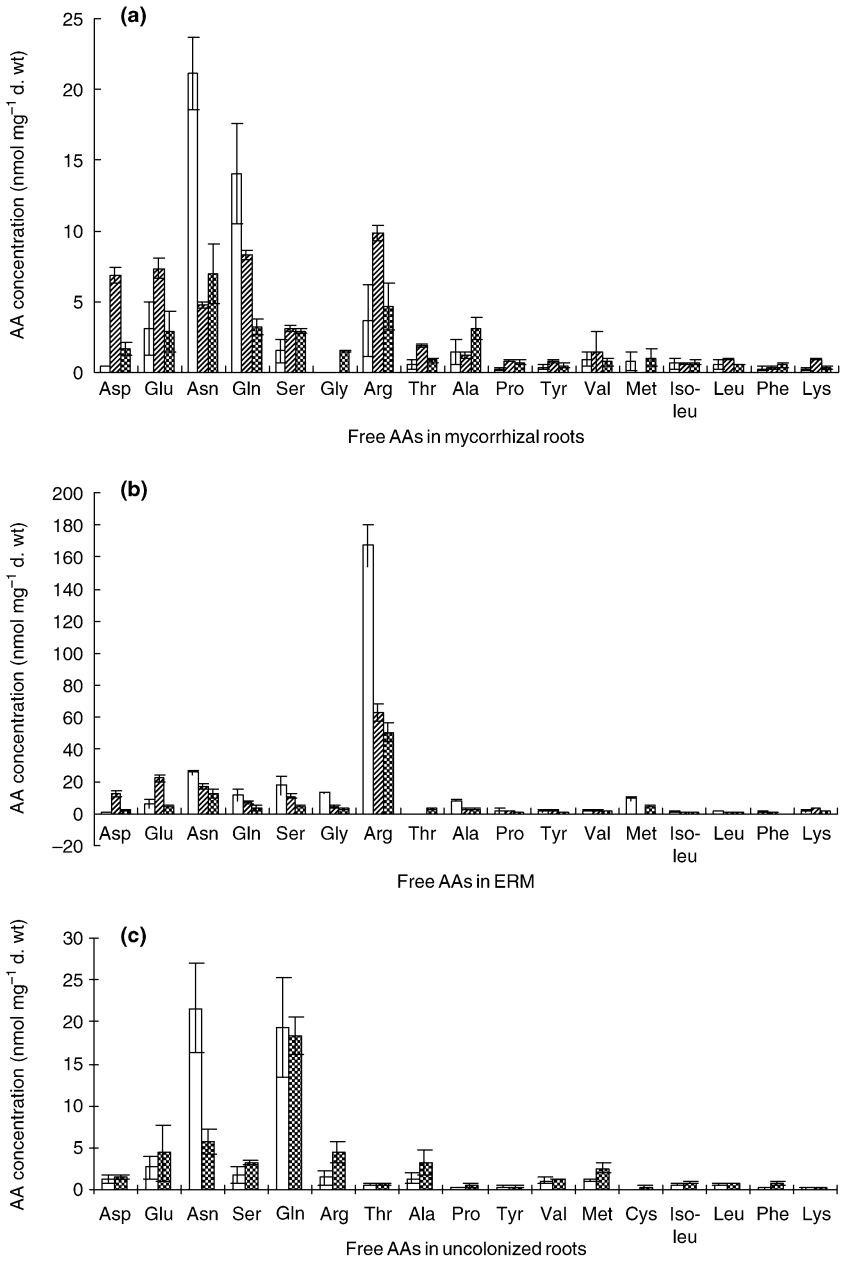

Fig. 2 The concentration of free amino acids (AAs) in mycorrhizal root tissue (a) and extraradical mycelium (ERM) (b) after culturing for 1 wk (empty bars), 3 wk (hatched bars) and 6 wk (checkered bars) with $^{15}NH_4Cl$ in the fungal side after the ERM had grown into the fungal compartment in the two-compartment Petri dishes. (c) Free AAs from uncolonized roots which were cultured in nitrate-limiting M medium supplied with $^{15}NH_4Cl$ in the root compartment for 1 wk (empty bars) and 6 wk (checkered bars). Some bars are not visible because the level of these AAs was very low or not determined. Values are expressed as means of three replicate experiments ± SD.

the free AAs in uncolonized roots exposed to ^{15}N for 1 wk was slightly higher than that of mycorrhizal roots, although after 6 wk the AAs of the mycorrhizal tissue reached essentially the same ^{15}N content as the uncolonized root.

Mass spectrometry revealed that Arg from the ERM tissue became highly labeled within a day after introducing $^{15}NH_4Cl$ into the fungal compartment and remained highly labeled thereafter (Fig. 4a,b). The predominant ion was the one having a *m/z* of 445 (three ^{15}N atoms per molecule, with the fourth lost during mass spectrometry; see the Materials and Methods section and Fig. 1). In fact, ^{15}N-labeled Arg (*m/z* of 445) accumulates to a significant level in the ERM within 8 h of supplying $^{15}NH_4^+$ to the fungal compartment (data not shown). The high levels and high fractional enrichment of Arg means that this AA accounted for about 90% of the ^{15}N in the free AA pool of the ERM. In mycorrhizal roots, at 1 and 3 d, 35 and 21%, respectively, of ^{15}N enriched Arg (*m/z* of 443) was observed; after 1 and 6 wk of labeling it increased to a *m/z* of 445. Thus, within 3 d, slightly more partially labeled [$^{15}N_2$]Arg molecules had been transported to the root tissue, whereas 50% of Arg within the ERM was already fully labeled (Fig. 4a). After 1 wk of labeling, fully labeled Arg (*m/z* of 445) dominated both mycorrhizal root and ERM with 50 and 70% isotopic percentage, respectively (Fig. 4b).

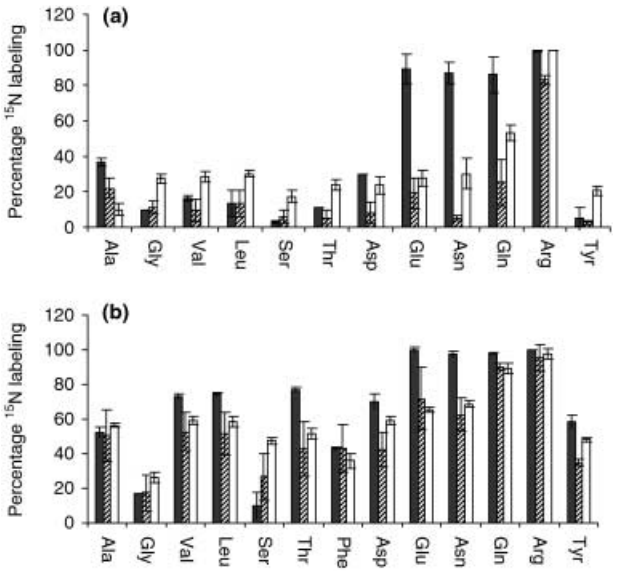

Fig. 3 ^{15}N labeling of the free amino acids (AAs) in extraradical mycelium (ERM) (black bars), mycorrhizal compartment tissue (hatched bars) and uncolonized roots (empty bars), following 1 wk (a), 6 wk (b) of $^{15}NH_4Cl$ labeling in the fungal compartment after the ERM grew into the fungal compartment in the two-compartment Petri dishes. Values are expressed as means of three replicate experiments ± SD.

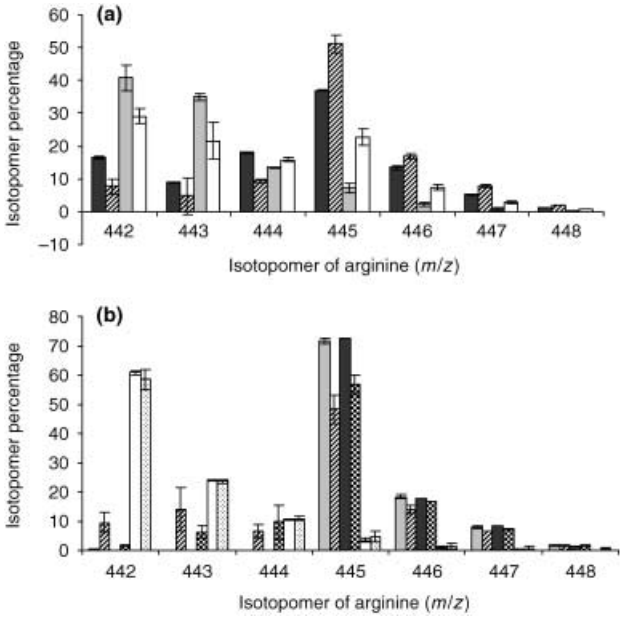

Fig. 4 Histogram of Arg percentage isotopomer composition in the mycorrhizal and fungal compartment tissues after labeling with $^{15}NH_4Cl$ in the fungal compartment for different times after the extraradical mycelium (ERM) grew into the fungal compartment in the two-compartment Petri dishes. (a) Arg in the ERM after 1 d (black bars) and 3 d (hatched bars) of culture; Arg in root compartment tissue after 1 d (gray bars) and 3 d (empty bars) of culture. (b) Arg in the ERM after 1 wk (gray bar) and 6 wk (black bar) of culture; Arg in the root compartment tissue after 1 wk (hatched bar) and 6 wk (checkered bars) of culture. Arg in the unlabeled root compartment tissue (empty bars) and unlabeled ERM (dotted bar). Values are expressed as means of three replicate experiments ± SD.

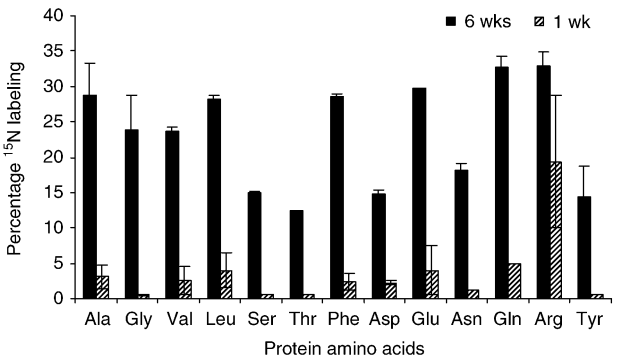

Fig. 5 ^{15}N Labeling of the protein amino acids (AAs) in mycorrhizal roots after $^{15}NH_4^+$ was supplied for 1 wk (hatched bars) and 6 wk (black bars) in the fungal side after the extraradical mycelium (ERM) grew into the fungal compartment in the two-compartment Petri dishes. Values are expressed as means of three replicate experiments ± SD.

To determine the extent of N transfer from the fungus to the host roots, the labeling of proteins extracted from the mycorrhizal roots also was measured after labeling the ERM with $^{15}NH_4^+$ for 1 and 6 wk (Fig. 5). Gel electrophoresis, total protein measurements and antibody based comparisons between colonized and uncolonized roots showed that the large majority of the protein in colonized roots was of plant origin (data not shown). ^{15}N labeling of the protein AAs of mycorrhizal roots increased with culture time as N was transferred to the host root following translocation to the IRM. Following Kjeldahl digestion of the mycorrhizal root tissue (6 wk of labeling), we analyzed the enrichment in total N using proton nuclear magnetic resonance (^{1}H NMR) (Preece & Cerdant, 1993). This gave a value of 50.4 ± 7.4% for the ^{15}N content (see the Materials and Methods section for details). Considering that most of the root biomass was formed before ^{15}N labeling, this is a remarkably high value and indicates that most of the N taken up by the roots during the labeling period came from the fungus.

Uptake and translocation of Arg by extraradical hyphae

In addition to being a major sink for N in the fungus, Arg is also a candidate for being the major form of N translocated within the fungus from ERM to the mycorrhizal roots. We supplied labeled [guanido-2-^{15}N]Arg to the ERM to track the movement of Arg within the fungus. In these experiments, the Arg content in all tissues was lower than when $^{15}NH_4Cl$ was the substrate in the fungal compartment. When [guanido-2-^{15}N]Arg was supplied to the fungal compartment for 6 wk, highly labeled [guanido-2-^{15}N]Arg whose MTBSTFA derivative ion with a *m/z* of 443 (M-189; Figs 6, 7) was observed in the mycorrhizal compartment tissue. Additionally, ^{15}N enrichment of Arg (*m/z* of 444, 445) in the ERM increased after 6 wk of labeling compared with standard [guanido-2-^{15}N]Arg and unlabeled ERM tissue (Fig. 7). Uncolonized roots exposed to

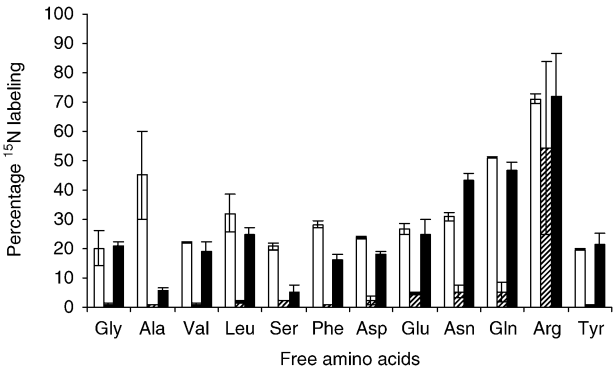

Fig. 6 ^{15}N labeling of the free amino acids (AAs) in uncolonized roots (empty bars), extraradical mycelium (ERM) (black bars) and mycorrhizal compartment tissue (hatched bars) from [guanido-2-^{15}N]Arg labeling in the fungal compartment for 6 wk after the ERM grew into the fungal compartment in the two-compartment Petri dishes. Values are expressed as means of three replicate experiments ± SD.

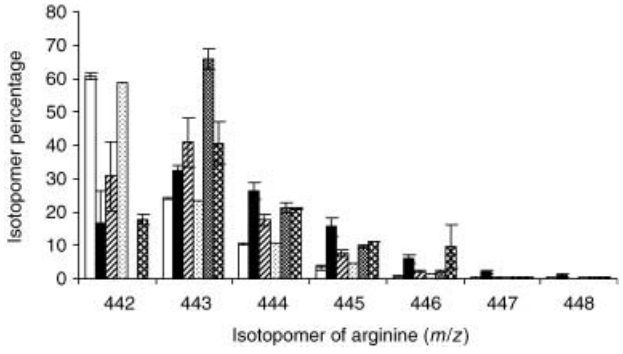

Fig. 7 Histogram of Arg isotopomers percentage composition in the mycorrhizal compartment tissue and extraradical mycelium (ERM) following the labeling with [guanido-2-^{15}N]Arg in the fungal side for 6 wk after the ERM grew into the fungal compartment in the two-compartment Petri dishes. Unlabeled mycorrhizal compartment tissue (empty bars); unlabeled ERM (dotted bars); labeled mycorrhizal compartment tissue (hatched bar); labeled ERM (black bars); standard [guanido-2-^{15}N]Arg (checkered bars); labeled uncolonized root (diamond bars). Values are expressed as means of three replicate experiments ± SD.

[guanido-2-^{15}N]Arg broke it down and incorporated its N into other free AAs in uncolonized roots (Fig. 6); also traces of intact [guanido-2-^{15}N]Arg were observed in protein AAs of uncolonized roots (data not shown). When uncolonized roots were treated with [U-^{13}C]Arg for 6 wk, we observed that intact [U-^{13}C]Arg isotopomers were found in the free AAs and protein AAs, representing 36 and 13% of the Arg, respectively. Of the other free AAs, only Glu and Gln were enriched in ^{13}C (approx. 5.0%) (data not shown). When doubly labeled [U-^{13}C/U-^{15}N]Arg was used as a substrate for uncolonized roots for 6 wk, all free AAs of the uncolonized roots were labeled by ^{15}N only (Govindarajulu *et al.*, 2005). Intact [U-^{13}C/U-^{15}N]Arg isotopomers were also observed in free AAs and protein AAs of uncolonized roots: 42 and 5%, respectively. When

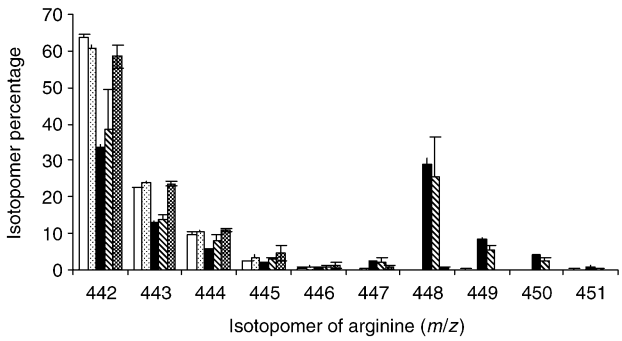

Fig. 8 A histogram representing isotopomer distributions for derivatized Arg from the [U-^{13}C]Arg labeling in the fungal compartment for 6 wk after the extraradical mycelium (ERM) grew into the fungal compartment in the two-compartment Petri dishes. Standard unlabeled Arg (open bars); unlabeled mycorrhizal compartment tissue (dotted bars); labeled ERM (black bars); labeled mycorrhizal compartment tissue (hatched bars); unlabeled ERM (checkered bars). Values are expressed as means of three replicate experiments ± SD.

[U-^{13}C]Arg (MTBSTFA derivative with a *m/z* of 448) was used as the labeled substrate in the fungal compartment, it was observed intact in the mycorrhizal root tissue (*m/z* of 448) as well as the ERM (Fig. 8). However, no C labeling was observed in root proteins. A similar result was observed when [U-^{13}C/U-^{15}N]Arg was used as a substrate in the fungal compartment (data not shown), with no C atoms in the other free AAs of mycorrhizal roots becoming labeled with ^{13}C, although N atoms in all AAs became labeled with ^{15}N. When [U-^{13}C]Arg was introduced into the mycorrhizal root compartment, derivatized Arg whose MS ion had a mass of 448 ([U-^{13}C]Arg) was found in the ERM (data not shown).

Discussion

The ^{15}N labeling data address the route of N assimilation in the ERM. Glu, Gln, Asn and Arg were the first AAs to be highly enriched with ^{15}N (Fig. 3a). This is consistent with the assimilation of N being via NO_3^- and nitrite reductases (for NO_3^-) and then via glutamine synthetase (GS) and glutamate synthase (GOGAT) asparagine synthase (AS) and arginino-succinate lyase. The fungal ERM takes up N quickly and incorporates most of it into Arg which contained over 90% of the total ^{15}N in the free AAs. Johansen *et al.* (1996) have previously observed, without reporting absolute levels, that Arg is the dominant free AA in extraradical mycelium of *Glomus claroideum*; however, they failed to observe it in *G. intraradices* by GC/MS owing to problems of derivatization and decomposition of the silated product (see the Materials and Methods section). Arg was previously found in the ERM and its synthesis was demonstrated in germinating spores with ^{13}C-NMR (Bago *et al.*, 1999), leading to the proposal that it is transported from the ERM to the IRM (Johansen *et al.*, 1996; Bago *et al.*, 2001). This speculative proposal highlighted how

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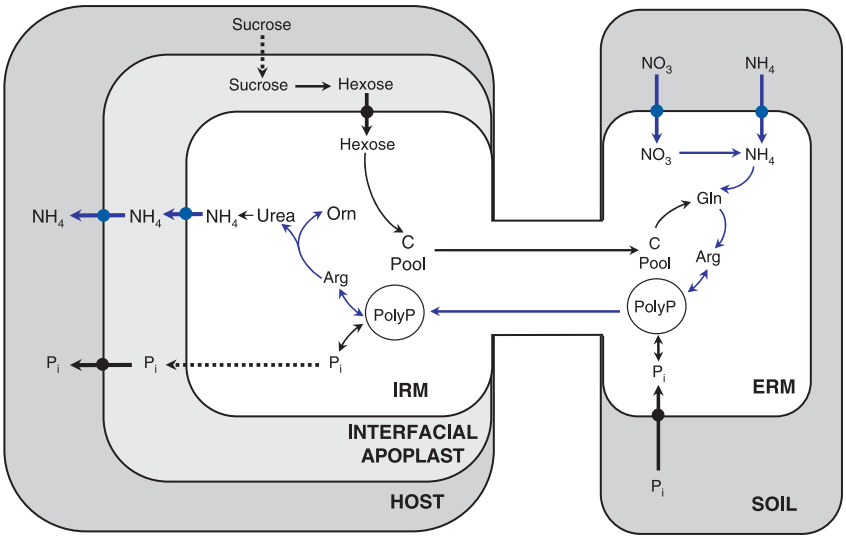

Fig. 9 The model of N transport and metabolism in the symbiotic system between transformed carrot root and arbuscular mycorrhizal fungi. Inorganic N is taken up by the fungal extraradical mycelium and assimilated via nitrate reductase and the GS/GOGAT cycle. It is then incorporated into Arg, which is translocated along the coenocytic fungal hyphae from the extraradical mycelium (ERM) into the intraradical mycelium (IRM). Arg is then broken down in the IRM, releasing urea and ornithine, which are further broken down by the actions of urease and ornithine aminotransferase (OAT). NH_4 released from Arg breakdown passes to the host via ammonium transporters (AMT). P_i, orthophosphate; PolyP, polyphosphate.

N movement in the AM symbiotic system might work; however, no evidence was presented to support it until recently (Govindarajulu *et al.*, 2005).

Excess NH_4^+ is toxic unless it is rapidly assimilated into nontoxic organic compounds (Temple *et al.*, 1998). It seems likely that synthesis of Arg in the ERM of AM fungi, as observed for Gln in ectomycorrhizas, prevents an excessive accumulation of NH_4^+ in fungal hyphae when the external N is plentiful and uptake is rapid. In addition, Arg may be associated with polyphosphate (PolyP) in the ERM of AM fungi, as proposed by Martin & Botton (1993) in ectomycorrhizas. Similarly, coordination of the regulation of P and N accumulation and transport may occur in the ERM of AM fungi (Fig. 9), however, confirmation of this must await further investigation.

The ERM of AM fungi can utilize exogenously supplied [^{15}N]Arg. When Arg was available to the ERM in the medium, it was metabolized within 6 wk, as evidenced by N enrichment of other AAs within the ERM, as shown in Fig. 6. However, Arg was not easily metabolized by the hyphae because the ^{15}N enrichment of the free AAs with the exception of Arg was relatively low compared with that after $^{15}NH_4^+$ labeling. Also, only Arg found in the root compartment tissue was over 50% enriched by ^{15}N when labeled by $^{15}NH_4^+$ or [guanido-2-^{15}N]Arg, indicating that it was transported from the ERM. This was not true for any other AAs found in the mycorrhizal root tissue (Fig. 3a,b). In addition, because the concentrations of all the other AAs were low in the ERM relative to Arg, it is unlikely that they could play a role in N storage within the hyphae.

Results of [U-^{13}C]Arg labeling in the fungal compartment confirmed that the Arg that is taken up or synthesized in the ERM is transported to the IRM. This is also the finding with the [U-^{13}C/U-^{15}N]Arg labeling experiments (Govindarajulu *et al.*, 2005). However, the C atoms of [U-^{13}C]Arg or [U-^{13}C/U-^{15}N]Arg were not incorporated into the free AAs in mycorrhizal roots even though traces of ^{15}N enrichment were observed in the AAs, and traces of intact [U-^{13}C]Arg or [U-^{13}C/U-^{15}N]Arg were found in protein extracted from the mycorrhizal roots (this work; Govindarajulu *et al.*, 2005). Subsequently, we confirmed that the intact, labeled Arg observed in the protein came from contamination with free Arg in mycorrhizal roots because the protein dialysis process does not remove it completely. Both [U-^{13}C]Arg and [U-^{13}C/U-^{15}N]Arg were found intact in extracts of free AAs and protein AAs when added to uncolonized roots, indicating that if Arg were transferred by the fungus to the host it would have been detected (this work; Govindarajulu *et al.*, 2005). Additionally, when uncolonized roots were labeled with [U-^{13}C]Arg for 6 wk, low levels of ^{13}C enrichment were observed in free Gln and Glu. Introduction of [U-^{13}C/U-^{15}N]Arg to cultured uncolonized roots produced free AAs (especially Glu and Gln) enriched in ^{15}N and ^{13}C. Thus if roots are exposed to Arg, it is taken up and utilized.

Arg is the major form in which N is transported from the ERM to IRM in the mycorrhizal symbiosis, Furthermore, we determined by ^{1}H NMR that about 50% of the total N found in mycorrizal roots was delivered from the hyphae under these experimental conditions. These results indicate that the amounts of N transported via hyphae can constitute a large contribution to the N nutrition of the plant, as opposed to earlier suggestions to the contrary (Hawkins *et al.*, 2000). Although these data demonstrate the capacity of the fungal partner to deliver a large proportion of the N taken up by roots, further study is needed using whole plants to determine the contribution that the fungus makes to N uptake under different conditions.

In a model of N uptake, transport and transfer by AM fungi (Fig. 9), we suggest that: (i) AM fungi take up inorganic N (NH_4^+, NO_3^-); (ii) absorbed N is mostly incorporated and

stored in Arg; (iii) AM fungi assimilate the N through GS/GOGAT, asparagine synthase and the urea cycle; (iv) stored Arg can be co-transported with PolyP intact to the IRM from the ERM of AM fungi, and Arg is also bi-directionally transported within the ERM; and (v) N released from transported Arg is transferred to the host as NH_4^+ and can be incorporated into other free AAs in mycorrhizal roots, while C not transferred to the host is recycled back to the ERM.

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