LETTERS

Nitrogen transfer in the arbuscular mycorrhizal symbiosis

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Most land plants are symbiotic with arbuscular mycorrhizal fungi (AMF), which take up mineral nutrients from the soil and exchange them with plants for photosynthetically fixed carbon. This exchange is a significant factor in global nutrient cycles[1] as well as in the ecology[2], evolution[3] and physiology[4] of plants. Despite its importance as a nutrient, very little is known about how AMF take up nitrogen and transfer it to their host plants[5]. Here we report the results of stable isotope labelling experiments showing that inorganic nitrogen taken up by the fungus outside the roots is incorporated into amino acids, translocated from the extraradical to the intraradical mycelium as arginine, but transferred to the plant without carbon. Consistent with this mechanism, the genes of primary nitrogen assimilation are preferentially expressed in the extraradical tissues, whereas genes associated with arginine breakdown are more highly expressed in the intraradical mycelium. Strong changes in the expression of these genes in response to nitrogen availability and form also support the operation of this novel metabolic pathway in the arbuscular mycorrhizal symbiosis.

The uptake of mineral nutrients from the soil by plants is greatly aided by mutualistic associations with mycorrhizal fungi, which grow into and extend out of the plant roots. Of these symbioses the arbuscular mycorrhizal one is the oldest, most anatomically intimate and ecologically widespread[6]. As well as benefiting plants by aiding phosphorus uptake from the soil[7], AMF can take up and transfer significant amounts of nitrogen to their host plants[5]. The availability of nitrogen frequently limits plant growth, and depending on soil conditions nitrogen transfer by mycorrhizal fungi can represent a significant route of uptake by the plant[5,8]. AMF have been strongly implicated in the transfer of nitrogen from one plant to another[5], can increase the utilization of different forms of nitrogen by plants[9] and have been shown to take up nitrogen directly and transfer it to host roots[5,10,11]. However, despite the identification in AMF of enzymes and genes of primary nitrogen assimilation and catabolism (nitrate reductase, glutamine synthetase and glutamate dehydrogenase[12–14]), we know very little about how nitrogen is transferred from fungus to plant. In particular, we do not know the form in which nitrogen is translocated within the fungus from the hyphae in the soil (extraradical mycelium) to the fungal structures within roots (intraradical mycelium), or the form in which nitrogen is transferred across the mycorrhizal interface to the plant. This ignorance limits our understanding both of underground nitrogen movement globally and of nutrient exchange in what is arguably the world's most important symbiosis.

In order to follow the uptake, assimilation and transfer of nitrogen in the arbuscular mycorrhizal symbiosis, we supplied isotopically labelled substrates to *in vitro* arbuscular mycorrhizal cultures of carrot (*Daucus carota* L.) roots colonized by *Glomus intraradices*. When grown in divided Petri plates[15], this model mycorrhiza excludes other microorganisms and prevents diffusion of non-volatile solutes between the compartments. Thus, nutrient transfer between the compartment in which the colonized roots grow and the compartment in which the extraradical mycelium (ERM) proliferates takes place only via uptake, metabolism and transport by the fungus[16–20]. This model system develops normally with respect to fungal morphology and life cycle[15], and functions in phosphorus uptake and transfer[20,21]; it has also yielded considerable insight into carbon handling[16–19,22–23], and has been shown to take up and metabolize nitrogen[10,14].

The free amino acids of the ERM became highly labelled after this tissue was exposed to either $^{15}NO_3^-$ (Fig. 1a) or $^{15}NH_4^+$ (not shown) added to the fungal compartment. This observation is consistent

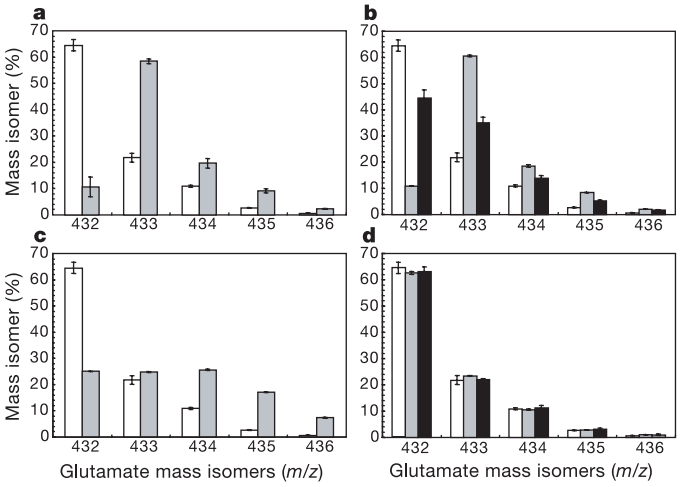

Figure 1 | Labelling of amino acids after supplying labelled nitrate or acetate to the extraradical mycelium. a–d, Mass isomer distributions of glutamate from free amino acids of the ERM (**a**, **c**), and free and protein amino acids of mycorrhizal roots (**b**, **d**) after supplying the ERM with ^{15}N-labelled NO_3^- (**a**, **b**) or ^{13}C-labelled acetate (**c**, **d**) for 6 weeks. White bars represent the mass isomer distribution from an unlabelled glutamate standard, where most of the molecules (after derivatization) have a mass of 432 atomic mass units (a.m.u.), and the higher mass molecules have heavier isotope atoms at natural abundance levels. Grey bars represent glutamate from extracted free amino acids, and black bars represent glutamate from hydrolysed protein. In **b** and **c**, but not **d**, free glutamine and glutamine from protein have more molecules of higher mass than the unlabelled glutamine sample due to the metabolic incorporation of ^{13}C and ^{15}N atoms. Results are shown for glutamine as an abundant amino acid, but labelling patterns in other amino acids support the same conclusions. Error bars represent s.e.m.; $n = 3$.

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with the known uptake and assimilation of nitrogen into amino acids by the ERM[10–11,14]. ^{15}N labelling in the free amino acid pool from colonized roots was also very high (Fig. 1b), demonstrating nitrogen translocation to the colonized roots. Furthermore, the amino acids obtained by hydrolysis of total protein extracted from colonized roots were also significantly labelled (Fig. 1b). This finding is consistent with the transfer of nitrogen from the ERM to the host[5]. The protein extracted from mycorrhizal roots is expected to consist mostly of host protein, because the fungus and its protein represent a tiny proportion of the totals for these colonized roots (D.D.D. and H.B., unpublished results) as in all other arbuscular mycorrhizae examined[6]. The average ^{15}N level in the nitrogen of root protein amino acids was 29.0 ± 1.0%, which shows that close to one-third of the total nitrogen in the roots was provided by the fungus from inorganic nitrogen taken up by the fungal ERM in the fungal compartment. This is a high value when one considers that most of the root biomass was made before the labelling period. ^{15}N labelling of the free amino acids of roots after supplying $^{15}NO_3^-$ or $^{15}NH_4^+$ to the fungal compartment was very high, even when the nitrogen levels supplied in the root compartment were threefold higher than in this study (Y. S.-H., P.E.P. and D. Rolin, unpublished results), which shows that the uptake of nitrogen by the ERM and translocation to the mycorrhizal roots occurs whether the host roots are nitrogen limited or not.

In order to track the synthesis and fate of the carbon skeletons of amino acids made in the ERM, $^{13}C_2$ acetate was provided to the ERM compartment (sugars are not taken up by the ERM[22]). Free amino acids in the ERM became highly labelled (Fig. 1c), with 34–76% of the molecules of the three abundant amino acids arginine, glutamate and aspartate having one or more ^{13}C atoms. This finding indicates that the carbon skeletons of these amino acids are synthesized in the ERM, consistent with previous observations that acetate can enter central metabolism in the ERM via acetyl CoA and the glyoxylate cycle[17]. Free arginine extracted from the colonized roots after the ERM was exposed to ^{13}C acetate was labelled between 6% and 18% (data not shown). This indicates that one or more amino acids are being translocated from the ERM to the intraradical mycelium (IRM). The alternative explanation that ^{13}C from acetate is transferred to the IRM as storage lipid[18] or carbohydrate and then made into amino acids is unlikely, because fungal compounds such as storage lipids and trehalose within the mycorrhizal roots did not become labelled in such experiments[23].

If nitrogen is transferred to the host root in the form of one or more free amino acids, then one would expect to see ^{13}C labelling in amino acids of isolated root proteins after ^{13}C acetate supply to the ERM. However, protein from the colonized roots did not become detectably labelled with ^{13}C under these conditions (Fig. 1d). The absence of ^{13}C labelling in plant protein under conditions when large amounts of nitrogen are being transferred from fungus to plant indicates that nitrogen is transferred in inorganic form. To test the possibility that carbon is transferred with the nitrogen but that they are metabolically separated before nitrogen is incorporated into protein (as hypothesized for the ectomycorrhizal symbiosis[24]), we analysed plant storage lipid and sucrose after ^{13}C acetate was provided to the ERM, and found that neither of these metabolic pools became labelled[23]. This indicates that carbon is not incorporated into other host carbon pools. In addition, we found that when ^{14}C-labelled amino acids (alanine, arginine, glutamate, glutamine, or ornithine) were provided to uncolonized roots, between 10% and 20% of the amino acids that were taken up in one week were recovered in the soluble protein extracted from the roots.

The absence of ^{13}C labelling in mycorrhizal root proteins indicates that the labelled free amino acids found in mycorrhizal root tissues after provisioning the ERM with ^{13}C acetate are in the IRM, and that they are broken down to release inorganic nitrogen inside the fungus before transfer to the host. Analysis of the levels of free amino acids by high-performance liquid chromatography revealed that arginine is by far the most abundant fungal amino acid, (between 50 and 200 mM depending on developmental stage), representing >90% of the total free amino acids in the ERM. Arginine levels are also substantially higher in colonized compared with uncolonized roots (54.2 ± 19.3% versus 10.9 ± 4.8% of free amino acids). This is consistent with a previous observation[11], and, together with the finding that arginine in the ERM is rapidly turned over (I. Jakobsen, C. Trujillo, P. Ambus, N. Requena and H. Egsgaard, unpublished data), suggests that arginine may be transported from the ERM to the IRM.

To test whether arginine is translocated from the ERM to the IRM, $^{13}C_{U6}$ arginine was supplied to the ERM. After 6 weeks, 34% of the free arginine in the ERM and 33% of the free arginine in the colonized roots showed $^{13}C_{U6}$ labelling (Fig. 2). The mass spectra show that the free arginine molecules in the colonized roots were either completely unlabelled (natural abundance mass isomer distribution) or labelled in all six carbon positions, thus indicating that arginine is transported intact from ERM to IRM. The absence of detectable ^{13}C arginine labelling in proteins from the colonized roots in these experiments, despite the appearance of labelled free amino acids from colonized root samples, contrasts with the finding that

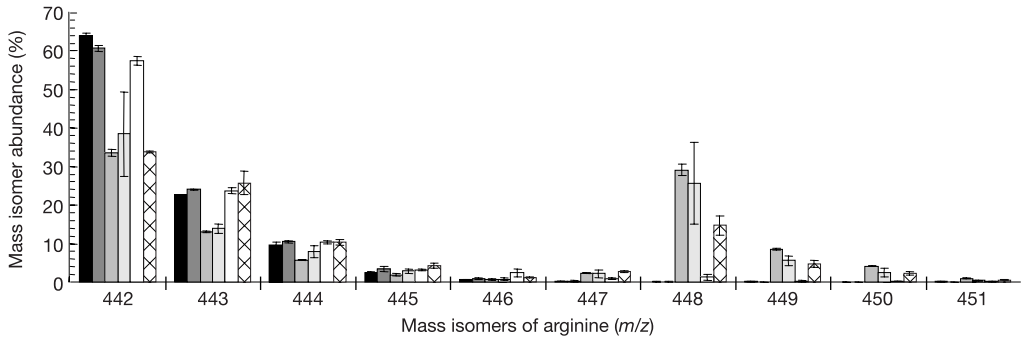

Figure 2 | Labelled arginine after addition of 2 mM $^{13}C_{U6}$ arginine to the ERM compartment for 6 weeks. Mass isomer distributions were measured by mass spectrometry after extraction of free amino acids or hydrolysis of extracted soluble protein followed by derivatization (see Methods). Black bars, unlabelled arginine standard showing the natural abundance mass isomer distribution; dark grey bars, arginine extracted from unlabelled mycorrhizal root tissue; medium grey bars, arginine extracted from ERM after labelling; light grey bars, free arginine from mycorrhizal roots after labelling; white bars, arginine from soluble protein of mycorrhizal roots after labelling; hatched bars, arginine from soluble protein of uncolonized roots after they were exposed to $^{13}C_{U6}$ arginine (positive control, showing that if arginine is made available to the root tissue, it is detectable in root protein). Error bars represent s.e.m.; $n = 3$.

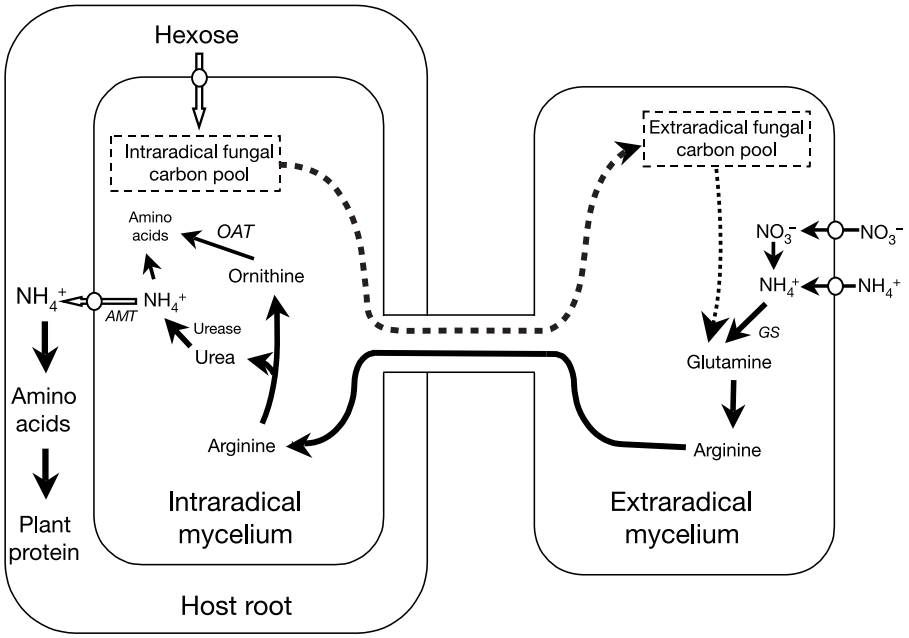

Figure 3 | Model of nitrogen movement in the arbuscular mycorrhizal symbiosis. Inorganic nitrogen is taken up by the fungal ERM and assimilated via nitrate reductase and the GS–GOGAT cycle. It is then converted into arginine, which is translocated along the coenocytic fungal hyphae from the ERM into the IRM. Arginine is broken down in the IRM, releasing urea and ornithine, which are further broken down by the actions of urease and ornithine aminotransferase (OAT). Ammonia released from arginine breakdown passes to the host via ammonia channels (AMT). Amino acids from ornithine breakdown and/or NH_4^+ assimilation in the IRM may be catabolized within the IRM or translocated to the ERM.

roots supplied directly with labelled arginine incorporate significant levels into protein (see Fig. 2 and ^{14}C results described above). Supplying ^{15}N arginine to the ERM resulted in ^{15}N labelling of all the free amino acids in the root tissue, including those present at high levels in uncolonized roots and at low levels in the ERM (mass spectra not shown). Thus, the nitrogen but not the carbon of arginine is transferred from fungus to host across the host–fungus interface.

On the basis of these labelling patterns, we conclude that arginine synthesized in the ERM is translocated by the fungus from the ERM to the IRM, but that it is not transferred to the host; rather it is broken down in the IRM and nitrogen is transferred to the host as ammonium. This proposed mechanism, illustrated in Fig. 3, is consistent with both the suggestion that arginine may be involved in nitrogen transfer[11] and a hypothetical scheme we have previously presented[25]. There is also evidence that arginine can bind to polyphosphate[26], which is the form of phosphorus thought to be translocated by the fungus[6], suggesting a possible link between nitrogen and phosphorus movement.

The proposed mechanism (Fig. 3) predicts that enzyme activity required for nitrogen assimilation should be induced in the ERM and suppressed in the IRM when inorganic nitrogen is available to the ERM. We tested this prediction by measuring messenger RNA levels for key enzymes in ERM and IRM tissues by quantitative real-time polymerase chain reaction (PCR). Figure 4a shows that glutamine synthase expression follows this prediction, particularly when NO_3^- is added to the ERM compartment, supporting previous findings that nitrogen assimilation occurs in the ERM via the glutamine synthetase–glutamate synthase (GS–GOGAT) pathway[11]. Figure 4a also shows that expression of a putative NAD-dependent glutamate dehydrogenase (GDH) gene is downregulated in ERM tissue supplied with either NO_3^- or NH_4^+ in the ERM compartment, consistent with this enzyme having a catabolic role[14,27,28].

The proposed mechanism also requires that enzymes required for the breakdown of arginine are more active in the IRM than in the ERM in order to release ammonia for transfer to the host roots. Figure 4b shows that *G. intraradices* genes with high similarity to known ornithine aminotransferase, urease accessory protein and an ammonium transporter are indeed preferentially expressed in the IRM, consistent with the predictions of the model. This was true regardless of whether NO_3^- or NH_4^+ was added to the ERM compartment, although the effect was greater for NH_4^+ treatments. The suggestion that NO_3^- is directly transferred from the ERM to the IRM and then to the plant[29] is not supported by the strong induction of glutamine synthase transcripts in the ERM, observed when NO_3^- is provided to the ERM; similarly the high levels of labelling in ERM amino acids after $^{15}NO_3^-$ is supplied to the fungal compartment points to the assimilation of NO_3^- into amino acids in the ERM.

Our results show the operation of a metabolic route in which nitrogen is moved by the AMF from the soil to its host. This pathway

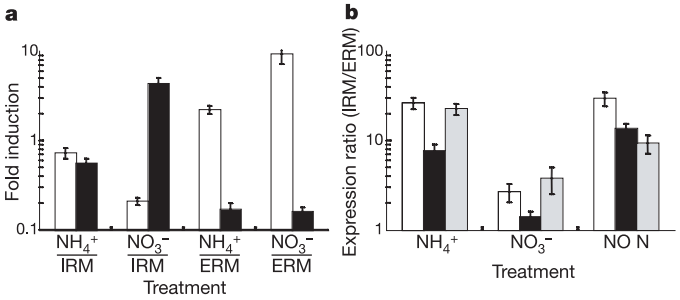

Figure 4 | Expression of primary nitrogen metabolic genes. Expression of putative nitrogen metabolic genes was measured in IRM and ERM by quantitative real-time PCR after 8 weeks of growth. Ribosomal protein S4 gene expression was used as the reference. **a**, The response of fungal gene expression to nitrogen availability. Levels of glutamine synthetase (white bars) and NAD-dependent glutamate dehydrogenase (black bars) mRNA are expressed as fold change relative to when no nitrogen was supplied to the fungal compartment. **b**, The ratio of intraradical to extraradical mycelial expression of urease accessory protein (white bars), ornithine amino transferase (black bars) and ammonium transporter (grey bars). Relative expression values represent the ratios of normalized IRM to ERM mRNA levels. Error bars represent s.e.m.; $n = 3$.

consists of metabolic processes known to operate in fungi (the assimilation of inorganic nitrogen) together with a new variant of the urea cycle in which the anabolic and catabolic parts are separated by the long-distance translocation of arginine. The assimilation of nitrogen into arginine allows it to be moved in a concentrated form (four nitrogens per molecule) that is also non-toxic. Its potential to bind to polyphosphate might allow phosphorus translocation to also carry nitrogen. The use of the catabolic arm of the urea cycle allows the transfer of nitrogen to the host plant with minimal loss of carbon by the fungus. The existence of this pathway and the high flux of nitrogen through it indicate that the arbuscular mycorrhizal symbiosis can effectively transfer large amounts of nitrogen from the soil to plant roots. This means that the symbiosis may have a much more significant role in the global nitrogen cycle than has been widely believed[6].

METHODS

Tissue culture. Root-inducing T-DNA-transformed carrot (*D. carota* clone DC2) roots colonized by *G. intraradices* (DAOM 197198) were grown[20] in bi-compartmented Petri plates[21] as previously described[28]. For labelling experiments, $Ca(NO_3)_2{\cdot}_4H2O$ was replaced with 180 mg l^{-1} $CaCl_2{\cdot}2H_2O$ in the medium of both compartments. KNO_3 was increased to 100 mg l^{-1} in the root compartment, whereas in the fungal compartment the only nitrogen source was $^{15}NO_3$ (4 mM), $^{15}NH_4^+$(4 mM), arginine-guanido-$^{15}N_2$·HCl (^{15}N, 98%) (2 mM), or $^{13}C_{U6}$ (98%) arginine (2 mM). For ^{13}C labelling of the ERM, $^{13}C_2$ acetate was added to the fungal compartment. Filter-sterilized solutions of the labelled compounds (pH 6.0) were added to autoclaved medium gelled with 4 g l^{-1} phytalgel (Sigma). Tissue from two plates was combined per sample and three replicates were analysed per treatment. ERM and mycorrhizal roots were harvested after 6 weeks of labelling.

To test the possibility that nitrogen moved across the barrier, we conducted experiments in which ^{13}C-labelled glucose was supplied to the fungal or colonized root compartment. This substrate is highly mobile in the medium and is taken up and incorporated into sucrose by the roots but is not taken up by the extraradical fungal mycelium[22]. When supplied to the root compartment, labelling in host and fungal metabolites was measurable within 36 h by gas chromatography-mass spectrometry (GC-MS). When supplied to the fungal compartment, no labelling was detected in host or fungal metabolites at any time up to 12 weeks of incubation. Thus, there is no significant diffusion across the barrier connecting the two compartments. Experiments with ^{14}C acetate also showed that there was no significant movement of substrates between the compartments by diffusion.

For gene expression experiments, split plates with mycorrhizal roots growing on both sides were incubated at 24 °C for 5 weeks, after which the roots and solidified minimal medium[28] from one compartment of each plate were transferred intact to a new split plate. Seventeen millilitres of fresh liquid M medium was then added to the empty half of each plate.

The liquid M medium contained 3.2 mM NH_4Cl (with no nitrate) or 3.2 mM NO_3^- (as KNO_3 and $Ca(NO_3)_2{\cdot}4H_2O$), or no nitrogen (KCl and $CaSO_4{\cdot}2H_2O$ replacing KNO_3 and $Ca(NO_3)_2{\cdot}4H_2O$, respectively). Only fungal hyphae grew over the barrier. ERM and mycorrhizal root samples were harvested 3 weeks after transfer.

Isolation of soluble and protein amino acids. Samples were stored at −80 °C, ground with acid-washed sand in liquid nitrogen, and extracted twice with NH_4HCO_3 buffer (pH 8 with 0.2% NaN_3). After centrifugation the supernatant was lyophilized, re-suspended in NH_4HCO_3 buffer and dialysed twice against 40 ml of NH_4HCO_3 buffer at 4 °C for 24 h using a dialysis membrane with a molecular mass cutoff of 2,000 Da (spectra/Por7 cellulose ester). Dialysed samples were pooled, lyophilized, stored at −20 °C, then 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) were added and the samples were incubated for 12 h at 30 °C with constant shaking; fresh enzymes were added at 6 h. Samples were centrifuged for 10 min at 10,000*g* at 4 °C, and the supernatants were lyophilized and re-suspended in 2 ml H_2O, then lyophilized again and re-suspended in 1 ml H_2O. The solution was loaded onto a cation exchange column (1 ml of DOWEX 50 *4-200, hydrogen form) and eluted with three 1-ml aliquots of 1 N NH_4OH. The eluate was lyophilized, re-suspended in 1 ml of water, acidified with 500 μl 1 N HCl, vortexed and then lyophilized. Free amino acids were purified as above either from the pooled dialysates (about 80 ml)—which were lyophilized, twice re-suspended in 2 ml water and lyophilized—or extracted MeOH as previously described[21].

Amino acid analysis. Free amino acid abundances were determined using a Waters Pico-Tag amino acid analyser using the Pico-Tag method. The threshold for detection of amino acids in standard solutions was 30 pM of each amino acid per assay, corresponding to <10 nmol g^{-1} dry weight of tissue.

Derivatization of amino acids. Amino acid samples were dissolved in 20–50 μl of dry dimethyl formamide at room temperature. The dimethyl formamide was evaporated under N_2 gas, 75 μl of *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide containing 1% *N*-methyl-*N*-(*t*-butyldimethylchlorosilane was added and the sample was heated for 30–50 min at 106 °C.

GC-MS analysis of amino acid labelling. MS analyses were performed with a Trace 2000 gas chromatograph (Thermo Electron) equipped with a splitless injector, open-tubular column of 0.25-μm-thick BP-15 film (0.18 mm internal diameter, 30 m long, Agilent) interfaced to a Thermo Finnigan quadrupole mass detector (Thermo Electron). Temperatures were: injector, 290 °C; column, 50 °C for 1 min after injection then increasing to 250 °C at 10 °C min^{-1}, then to 280 °C at 25 °C min^{-1}, and 280 °C for 8 min; detector, 350 °C. Carrier gas velocity was 1 ml min^{-1}. Peak identities were confirmed by GC-MS of authentic samples. The mass isomer distribution for each derivatized amino acid was measured from the intensity of ions having masses of 57 atomic mass units below the molecular ion, except for arginine whose m-188 ion was used. ^{15}N and ^{13}C labelling were measured from the intensities of signals with increased mass. Correction for background contributions to higher mass ions was made from the known N, C and Si isotopic natural abundances and confirmed with unlabelled standards and extracts.

The reliability of measuring fractional isotopic labelling was determined with samples of known labelling levels (natural abundance samples and glycine containing 0, 10%, 25%, 50%, 75% or 100% ^{15}N). Agreement between measured and predicted mass isomer distributions was always >95% and >99% for >75% of the repetitions.

Real-time RT–PCR. Total RNA was extracted from ERM tissues (Plant RNeasy kit, Qiagen) and from mycorrhizal roots (RNAqueous TM-Midi kit, Ambion) frozen in liquid nitrogen. RNAs were treated with DNase I (Ambion) and quantified by fluorescence (Ribogreen assay, Molecular Probes). Reverse transcription and PCR were performed with 1 ng and 2.5 ng of total RNA from ERM and IRM tissues, respectively (1-step master mix, Qiagen). Primers and probes (IDT, Synthegen) were as follows: glutamine synthetase forward 5′-CCT CAAGGTCCCTATTATTGTTCTG-3′, reverse 5′-ACGATAATGAGCTTCCA CAACGT-3′, dual-labelled fluorogenic probes 5′-CGACCAAAAGCAACA TTCGCACCA-3′; GDH forward 5′-CCACTTATTGCATTTACGTCAAAGA-3′, reverse 5′-CCCAGTCATCTCAGCAAGAGAA-3′, dual-labelled fluorogenic probes 5′-CTTCTTCGCCATCCAATGGCACG-3′; S4 ribosomal protein forward 5′-TCTTGTGAAGGTTGATGGCAAA-3′, reverse 5′-CGCCATTTCTTTC GATCGA-3′, dual-labelled fluorogenic probes 5′-TTCGAACCGATTCAACA TACCCTGCC-3′; UAP forward 5′-TTCCGCAGTCGTTGAATTGA-3′, reverse 5′-CCTTCGACAATGCTTAAAAATTATCA-3′, dual-labelled fluorogenic probes 5′-CACCAGACGCCTTCCAAGCACTCAAT-3′; OAT forward 5′-AGGGCTCAAGGTGCGTATGT-3′, reverse 5′-ACTGCCGAATAGGCACAC AAA-3′, dual-labelled fluorogenic probes 5′-TCCATATATTTGTTGCC TTCTGGGTCCCA-3′. Gene-specific PCR products were monitored in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using TaqMan probes. The *G. intraradices* S4 ribosomal protein was used for normalization. Expression values for IRM samples were compared with the expression in ERM tissues. The comparative ($\Delta\Delta C_T$) method was used to measure changes in gene expression[30]. Errors are expressed as standard error of the mean (s.e.m.) of three independent biological triplicates determined as the mean of technical duplicates.

Received 6 October 2004; accepted 4 April 2005.

1. Read, D. J. & Perez-Moreno, J. Mycorrhizas and nutrient cycling in ecosystems — a journey towards relevance? *New Phytol.* **157,** 475–492 (2003).
2. Allen, M. F., Swenson, W., Querejeta, J. I., Egerton-Warburton, L. M. & Treseder, K. K. Ecology of Mycorrhizae: A conceptual framework for complex interactions among plants and fungi. *Annu. Rev. Phytopathol.* **41,** 271–303 (2003).
3. Redecker, D., Kodner, R. & Graham, L. E. Glomalean fungi from the Ordovician. *Science* **289,** 1920–1921 (2000).
4. Marschner, H. *Mineral Nutrition of Higher Plants* 2nd edn (Academic, London, 1995).
5. He, X.-H., Critchley, C. & Bledsoe, C. Nitrogen transfer within and between plants through common mycorrhizal networks (CMNs). *Crit. Rev. Plant Sci.* **22,** 531–567 (2003).
6. Smith, S. E. & Read, D. J. *Mycorrhizal Symbiosis* 2nd edn (Academic, San Diego, California, 1997).
7. Harrison, M. J. & Van Buuren, M. L. A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. *Nature* **378,** 626–629 (1995).

8. Ames, R. N., Reid, C. P. P., Porter, L. K. & Cambardella, C. Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by *Glomus mosseae*, a vesicular-arbuscular mycorrhizal fungus. *New Phytol.* **95,** 381–396 (1983).
9. Hodge, A., Campbell, C. D. & Fitter, A. H. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature* **413,** 297–299 (2001).
10. Bago, B., Vierheilig, H., Piché, Y. & Azcon-Aguilar, C. Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. *New Phytol.* **133,** 273–280 (1996).
11. Johansen, A., Finlay, R. D. & Olsson, P. A. Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. *New Phytol.* **133,** 705–712 (1996).
12. Kaldorf, M., Zimmer, W. & Bothe, H. Genetic-evidence for the occurrence of assimilatory nitrate reductase in arbuscular mycorrhizal and other fungi. *Mycorrhiza* **5,** 23–28 (1994).
13. Breuninger, M., Trujillo, C. G., Serrano, E., Fischer, R. & Requena, N. Different nitrogen sources modulate activity but not expression of glutamine synthetase in arbuscular mycorrhizal fungi. *Fungal Genet. Biol.* **41,** 542–552 (2004).
14. Toussaint, J. P., St-Arnaud, M. & Charest, C. Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith and Ri T-DNA roots of *Daucus carota* L. in an *in vitro* compartmented system. *Can. J. Microbiol.* **50,** 251–260 (2004).
15. St-Arnaud, M., Hamel, C., Vimard, B., Caron, M. & Fortin, J. A. Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an *in vitro* system in the absence of host roots. *Mycol. Res.* **100,** 328–332 (1996).
16. Bago, B., Pfeffer, P. E. & Shachar-Hill, Y. Carbon metabolism and transport in arbuscular mycorrhizas. *Plant Physiol.* **124,** 949–957 (2000).
17. Lammers, P. J. *et al.* The glyoxylate cycle in an arbuscular mycorrhizal fungus: gene expression and carbon flow. *Plant Physiol.* **127,** 1287–1298 (2001).
18. Bago, B. *et al.* Translocation and utilization of fungal lipid in the arbuscular mycorrhizal symbiosis. *Plant Physiol.* **128,** 108–124 (2002).
19. Bago, B. *et al.* Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. *Plant Physiol.* **131,** 1496–1507 (2003).
20. Bücking, H. & Shachar-Hill, Y. Phosphate uptake, transport and transfer by the AM fungus *Glomus intraradices* is stimulated by increased carbohydrate availability. *New Phytol.* **165,** 899–912 (2005).
21. Nielsen, J. S., Joner, E. J., Declerck, S., Olsson, S. & Jakobsen, I. Phospho-imaging as a tool for visualization and noninvasive measurement of P transport dynamics in arbuscular mycorrhizas. *New Phytol.* **154,** 809–819 (2002).
22. Pfeffer, P. E., Douds, D. D., Bécard, G. & Shachar-Hill, Y. Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. *Plant Physiol.* **120,** 587–598 (1999).
23. Pfeffer, P. E., Douds, D. D., Bücking, H., Schwartz, D. P. & Shachar-Hill, Y. The fungus does not transfer carbon to or between roots in an arbuscular mycorrhizal symbiosis. *New Phytol.* **163,** 617–627 (2004).
24. Martin, F., Stewart, G. R., Genetet, I. & Le Tacon, F. Assimilation of $^{15}NH_4^+$ by beech (*Fagus sylvatica* L.) ectomycorrhizas. *New Phytol.* **102,** 85–94 (1986).
25. Bago, B., Pfeffer, P. E. & Shachar-Hill, Y. Could the urea cycle be translocating nitrogen in the arbuscular mycorrhizal symbiosis? *New Phytol.* **149,** 4–8 (2001).
26. Martin, F. ^{15}N-NMR studies of nitrogen assimilation and amino acid biosynthesis in the ectomycorrhizal fungus *Cenococcum graniforme*. *FEBS Lett.* **182,** 350–354 (1985).
27. Cliquet, J. B. & Stewart, G. R. Ammonia assimilation in *Zea-mays* L. infected with a vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatum*. *Plant Physiol.* **101,** 865–871 (1993).
28. Vallorani, L. *et al.* Biochemical and molecular characterization of NADP glutamate dehydrogenase from the ectomycorrhizal fungus *Tuber borchii*. *New Phytol.* **154,** 779–790 (2002).
29. Hildebrandt, U., Schmelzer, E. & Bothe, H. Expression of nitrate transporter genes in tomato colonized by an arbuscular mycorrhizal fungus. *Physiol. Plant.* **115,** 125–136 (2002).
30. Winer, J., Jung, C. K. S., Shackel, I. & Williams, P. M. Development and validation of real-time quantitative reverse-transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocetes *in vitro*. *Anal. Biochem.* **270,** 41–49 (1999).

Acknowledgements We thank A. Abdul-Wakeel and D. Schwartz for their technical assistance, and acknowledge the financial support of this project from USDA-NRICGP.

Author Contributions Experimental work was carried out by J.A., J.W.A., H.B., D.D.D., M.G., H.J. and PEP; experimental design was by H.B., M.G., P.J.L., P.E.P. and Y.S.-H.; data analysis was carried out by M.G., H.J., P.J.L., P.E.P. and Y.S.-H.; and project planning was by Y.S.-H.

Author Information Accession numbers (GenBank) for gene sequences used to design the TaqMan assays are as follows: GS (DQ063587), GDH (AY745984), UAP (CV186300), OAT (BI452207) and S4 RP (BI452093). Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.J.L. (plammers@nmsu.edu) or P.E.P. (ppfeffer@arserrc.gov).