Germinating spores of *Glomus intraradices* can use internal and exogenous nitrogen sources for *de novo* biosynthesis of amino acids

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Summary

• Here, nitrogen (N) uptake and metabolism, and related gene expression, were analyzed in germinating spores of *Glomus intraradices* to examine the mechanisms and the regulation of N handling during presymbiotic growth.

• The uptake and incorporation of organic and inorganic N sources into free amino acids were analyzed using stable and radioactive isotope labeling followed by high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC-MS) and liquid scintillation counting and the fungal gene expression was measured by quantitative polymerase chain reaction (Q-PCR).

• Quiescent spores store Asp, Ala and Arg and can use these internal N resources during germination. Although not required for presymbiotic growth, exogenous N can also be utilized for the *de novo* biosynthesis of amino acids. Ammonium and urea are more rapidly assimilated than nitrate and amino acids. Root exudates do not stimulate the uptake and utilization of exogenous ammonium, but the expression of genes encoding a putative glutamate dehydrogenase (GDH), a urease accessory protein (UAP) and an ornithine aminotransferase (OAT) were stimulated by root exudates. The transcript levels of an ammonium transporter (AMT) and a glutamine synthetase (GS) were not affected.

• Germinating spores can make effective use of different N sources and the ability to synthesize amino acids does not limit presymbiotic growth of arbuscular mycorrhizal (AM) spores.

Introduction

The majority of land plant species (80%) and families (92%) form mycorrhizal interactions and the arbuscular mycorrhiza is the predominant and ancestral type of these mutualistic plant–fungal interactions (Wang & Qiu, 2006). The symbiosis can enhance the uptake of phosphorus (P), zinc (Zn), copper (Cu), nickel (Ni) and nitrogen (N; Johansen *et al.*, 1992; Smith & Read, 2008). Nitrogen is the most abundant of the mineral elements in plant tissues and its availability often limits plant growth (Botton & Chalot, 1995). An increase in anthropogenic N depositions, however, will probably increase the production and tissue N concentration of plants, but will also have very serious environmental effects on microbial soil communities and activities (Näsholm, 1998).

It has been shown that arbuscular mycorrhizal (AM) fungal hyphae take up different forms of N from the soil and translocate it to the plant (Hawkins *et al.*, 2000; Azcon *et al.*, 2001; Hodge *et al.*, 2001; Vazquez *et al.*, 2001; Govindarajulu *et al.*, 2005; Leigh *et al.*, 2009). While there is evidence that AM fungi contribute to plant nutrition by N transfer under natural conditions, this has been difficult to assess directly. This is partly attributable to the high mobility of N in soils and to interactions with P nutrition (for review see He *et al.*, 2003),

so that the importance of AM fungi for N nutrition remains uncertain (Reynolds *et al.*, 2005). Studies in compartmented growth systems have shown, however, that N uptake by the extraradical mycelium (ERM) could account for > 30% of total plant N uptake (Frey & Schüepp, 1993).

Before a symbiosis with a host plant can be established, the AM fungus goes through a presymbiotic stage, consisting of spore germination, hyphal growth, host recognition and appressorium formation (Smith & Read, 2008). Spores of AM fungi possess the necessary genetic information and biosynthetic capacity to germinate under appropriate conditions (Barea, 1986). For those AM fungal species for which germination requirements have been studied, germination rates of over 90% have been obtained on water agar in the absence of any mineral or organic substrates or host roots (Azcòn-Aguilar *et al.*, 1986). Although not necessary for spore germination, root exudates can increase hyphal elongation and branching of AM fungi and thereby contribute to the mycorrhizal colonization of roots (Graham, 1982; Gianinazzi-Pearson *et al.*, 1989; Buee *et al.*, 2000; Nagahashi & Douds, 2000). Most studies on presymbiotic growth have focused on the morphological effects of root exudates on AM fungal spore germination and hyphal branching. Very little is known about the effects root exudates may have on nutrient uptake, metabolic fluxes, and gene expression in germinating spores, although it has been shown that respiration is increased in response to root exudates (Tamasloukht *et al.*, 2003; Bücking *et al.*, 2008).

Arbuscular mycorrhizal fungi are obligate symbionts that can only grow for a limited time without colonizing a susceptible host root (Bécard *et al.*, 2004). This has been suggested to be a consequence of one or more nutritional deficiencies during the presymbiotic phase, including the absence of *de novo* fatty acid synthesis (Bago *et al.*, 1999) or an inability to take up N (Bago *et al.*, 1996). Up to 95% of the total soil N is typically in organic form and consists mainly of peptides, proteins, purines, pyrimidines, and amino acids (Schulten & Schnitzer, 1998; Miller & Cramer, 2004). However, the extent to which germinating spores take up and metabolize different forms of N is not known. In order to investigate N uptake and metabolism in the presymbiotic phase of the life cycle, we measured the uptake of inorganic N and amino acids and ^{15}N labeling in free amino acids following exposure to ^{15}N-labeled inorganic and organic N sources. Furthermore, the effect of host root exudates on N uptake, metabolism and related gene expression was studied to obtain more information about the effects of host plants on presymbiotic growth of germinating spores.

Materials and Methods

Spore material

Axenic spores of *Glomus intraradices* Schenk & Smith (DAOM 181602) were purchased from Premier Tech Biotechnologies (Revière-du-Loup, Québec, Canada) in units of 10^6. The spores were kept refrigerated at 4°C. Before the start of the experiments, the spores were blended at high speed for 45 s in a commercial blender, filtered and suspended in sterile water. The spore suspension was continuously stirred and a homogenous volume of the spore suspension was added to Petri dishes for the experiments. During the experiments the spores were incubated at 30°C.

Root exudate preparation

Ri T-DNA transformed carrot (*Daucus carota* L.) roots were grown in Petri plates containing M medium (Bécard & Fortin, 1988) solidified with 0.2% (w/v) Phytagel (Sigma, St. Louis, MO, USA). After 4 wk of growth the roots were gently removed with forceps from the medium, transferred to a 4-l Erlenmeyer flask containing 1 l of liquid M medium with 3% sucrose, and grown for 4 wk at 25°C, while being continuously shaken. The roots were washed twice with autoclaved sterile deionized water, and were cultured under sterile conditions for another 1 wk on a shaker in 1 l of autoclaved deionized water at room temperature. The resulting crude root exudate solution was stored at −20°C for further use. Additionally, crude root exudates were partially purified, 200-fold concentrated, and dissolved in 70% methanol as previously described (Bücking *et al.*, 2008). The data for these partially purified root exudates were compared with a control to which 70% methanol was added (final methanol concentration in the medium was 1.7%).

Experimental design

Uptake of nitrogen N uptake and ^{15}N labeling experiments were conducted in modified liquid M medium (Bécard & Fortin, 1988) without sucrose and in which $Ca(NO_3)_2{\cdot}4H_2O$ was replaced with an equimolar concentration of $CaCl_2{\cdot}2H_2O$, so that the supplied N compound was the only N source (pH 6.0). To test whether germinating spores are able to use internal N sources for biosynthesis of amino acids, *c.* 25 000 spores per sample were incubated without an N source for 0, 7 or 14 d in sterile 12-well Petri plates containing 1 ml of M medium. In a second experiment the spores were incubated for 7 d in M medium containing 4 mM KNO_3 or NH_4Cl, or 2 mM urea, L-glutamine or L-arginine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA). The spores were collected by sieving and immediately frozen in liquid N, lyophilized, weighed and prepared for high-performance liquid chromatography (HPLC).

Labeling in free amino acids Approximately 20 000 spores per replicate were incubated in 50-mm Petri plates containing liquid M medium (the final volume was 10 ml) to which as filter sterilized solutions the following ^{15}N-labeled compounds were added: 4 mM $^{15}NH_4Cl$, $K^{15}NO_3$, $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$; or 2 mM [α-^{15}N]L-glutamine, [U-^{15}N]urea or

[guanido-$^{15}N_2$]L-arginine HCl (Cambridge Isotope Laboratories, Andover, MA, USA) (all 98% ^{15}N-enriched). After 7 d the spores were collected by centrifugation, washed with deionized water, centrifuged again and immediately frozen in liquid N, lyophilized, weighed and prepared for gas chromatography–mass spectrometry (GC-MS).

Uptake of amino acids The uptake of amino acids by germinating spores was studied by adding U-^{14}C-labeled amino acids (labeled alanine, arginine, glutamic acid, glutamine, glycine or ornithine; 50 μM; 0.1% ^{14}C-labeled) to 50-mm Petri dishes containing 8000 spores of *G. intraradices* in liquid M medium lacking an inorganic N source and sucrose. To examine the effect of root exudates on amino acid uptake, crude root exudate was added at a concentration of 7.7% (v:v) to half of the Petri dishes. After 1, 2, 4, or 8 d of incubation, aliquots of the medium were taken ($n = 8$) and the ^{14}C label remaining was determined by liquid scintillation counting (LSC; LS 6500; Beckman Coulter, Fullerton, CA, USA).

Effect of root exudates on NH_4^+ utilization To determine the effects of root exudates on N assimilation and metabolism, 7.7% crude or 2.5% partially purified root exudates (v:v) were added to Petri dishes containing M medium with 4 mM $^{15}NH_4Cl$ as the sole N source. Approximately 8000 spores were added to each Petri plate and incubated at 30°C for 24 h. To test whether potential effects of root exudates on N uptake are attributable to an increase in the nutrient-absorbing surface area produced by the presymbiotic growth response or to effects on the nutrient uptake efficiency per surface unit, a second set of samples was prepared and incubated first in M medium containing root exudates for 6 d before 4 mM $^{15}NH_4Cl$ was added for 24 h. The samples were centrifuged, washed in deionized water, centrifuged again, frozen in liquid N, lyophilized and weighed and then analyzed by GC-MS.

Gene expression studies Incubations for gene expression studies were conducted using liquid M medium without sucrose in 50-mm Petri dishes containing *c*. 8000 spores of *G. intraradices*. The spores were incubated with 7.7% crude root exudates or 2.5% partially purified root exudates. The plates were incubated for 2, 4, 8, 24, 48, or 96 h at 30°C and then spores were collected for quantitative RT-PCR.

HPLC analysis of amino acids concentrations

For the HPLC analysis the spores were first disintegrated in 0.1 ml of a 0.01 N HCl solution in 1.5-ml microcentrifuge tubes using a bead mill (Retsch MM301, Newtown, PA, USA) set to 30 Hz and two 3-mm stainless steel beads for 4 min. Complete disintegration was confirmed by examining small aliquots of five randomly chosen samples under a dissecting microscope. An additional 0.4 ml of 0.01 N HCl was then added followed by 10 min of vortexing. Then, 2.5 nM norleucine was added as an internal standard before extraction. Next, 0.5 ml of chloroform was added and the samples were vortexed for 5 min. After separation of the chloroform, aqueous solution, and cell debris by centrifugation, the aqueous phases were collected and lyophilized. For the analysis the dried samples were re-suspended in 20 μl of 0.02 N HCl and 60 μl of sodium borate buffer (pH 10.0), and derivatized with 20 μl of AccQ-Fluor™ reagent (6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate; Waters, Milford, MA, USA), which forms stable, fluorescent urea derivatives of primary and secondary amines (Cohen, 2000). Free amino acids were analyzed in 10- or 20-μl aliquots by HPLC-fluorescence using a Waters 2695 Alliance HPLC (Waters) equipped with a 37°C column oven, a Waters 2474 Fluorescence Detector, and EMPOWER software (Waters). A four buffer system was used for HPLC: sodium acetate buffer (Waters) adjusted to pH 5.7 and 6.8 with phosphoric acid; acetonitrile; and methanol:water (1 : 9, v:v). The concentrations of the amino acids were calculated by comparing the integrated peak area with those of standard amino acid samples with the same retention time and known concentration.

GC-MS analysis of free amino acids

For the GC-MS analysis the lyophilized spores and germ tubes were homogenized by grinding with pre-ground sand in a glass mortar. Next 2.5 ml of methanol/0.01 N HCl (70 : 30, v:v) was added and the sample ground again. After centrifugation, the supernatant was loaded on a cation exchange column filled with 0.5 ml of DOWEX 50X8 100–200 mesh (Sigma-Aldrich, St. Louis, MO, USA) (before use the DOWEX had been sequentially washed in 3 N NH_4OH, deionized water, 2 N HCl, and deionized water until the pH was neutral). Neutral compounds, especially carbohydrates, were washed off twice with 0.5 ml of deionized water. The free amino acids were eluted by adding twice 0.5 ml of 3 N NH_4OH (Bengtsson & Odham, 1979) and collected in a glass vial. The eluate was frozen, lyophilized, re-suspended in 0.3 ml of 0.01 N HCl, again frozen and lyophilized.

Amino acids were derivatized by first adding 10 μl of dry N, N-dimethylformamide (DMF), and then 30 μl of N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide (MTB-STFA). Samples were then heated on a hotplate for 45 min at 100°C. GC-MS analysis of labeling in amino acids was performed as described by Mawhinney *et al.* (1986) by injection of the silylated extracts into a Finnigan Trace MS 2000 (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a splitless injector (at 250°C), and a fused 0.25-μm-thick silica capillary column (0.25 mm i.d, 30 m long; RTX-5MS; Restek Inc., Flemington, NJ, USA), and interfaced to a Thermo Finnigan quadrupole mass detector (Thermo Electron, Madison, WI, USA). Helium was used as a carrier gas with a flow rate of 1 ml min^{-1}. The oven temperature was maintained at 110°C for 2 min after injection, then ramped up to 260°C at 10°C min^{-1}, and held there for 5 min. Electron impact

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Table 1 Forward and reverse primers and dual labeled fluorogenic probes used for the gene expression studies

Gene		Sequence
AMT	Forward	5′ GGTTGATATTGAGGCCGAAGAA 3′
	Reverse	5′ GGCAAGACGATCAACGTGATAA 3′
	Probe	5′ AGCCAATTCACCCAATTCAGTATCATCAATACCTT 3′
GS	Forward	5′ CCTCAAGGTCCCTATTATTGTTCTG 3′
	Reverse	5′ ACGATAATGAGCTTCCACAACGT 3′
	Probe	5′ CGACCAAAAGCAACATTCGCACCA 3′
GDH	Forward	5′ CCACTTATTG CATTTACGTCAAAGA 3′
	Reverse	5′ CCCAGTCATCTCAGCAAGAGAA 3′
	Probe	5′ CTTCTTCGCCATCCAATGGCACG 3′
OAT	Forward	5′ AGGGCTCAAGGTGCGTATGT 3′
	Reverse	5′ ACTGCCGAATAGGCACACAAA 3′
	Probe	5′ TCCATATATTTGTTGCCTTCTGGGTCCCA 3′
UAP	Forward	5′ TTCCGCAGTCGTTGAATTGA 3′
	Reverse primer	5′ CCTTCGACAATGCTTAAAAATTATCA 3′
	Probe	5′ CACCAGACGCCTTCCAAGCACTCAAT 3′
RP	Forward	5′ TCTTGTGAAGGTTGATGGCAAA 3′
	Reverse	5′ CGCCATTTCTTTCGATCGA 3′
	Probe	5′ TTCGAACCGATTCAACATACCCTGCC 3′
SRP	Forward	5′ CTTTATCTCAAGGCTCGAAACACA 3′
	reverse	5′ CAGGAGGAGCTTTCTTAATTGCAT 3′
	Probe	5′ TCGTCCGCCATGGTTGAAACGC 3′

AMT, ammonium transporter; GS, glutamine synthetase; GDH, glutamate dehydrogenase; OAT, ornithine aminotransferase; UAP, urease accessory protein; RP, S4 ribosomal protein; SRP, GATA-type transcriptional regulator.

ionization was at 70 eV, and the mass range was scanned from a mass-to-charge ratio of 150–600 with a scan rate of 2 Hz. The identities of amino acids were confirmed by comparison of mass fragments of authentic standards. Except for Arg, amino acid labeling was determined by evaluation of the M-57 (loss of a t-butyl group) fragments of the MTBSTFA derivatives. The measured fragment of Arg had an m/z ratio of 442 (M-188) because one guanido N was lost from the tetra-substituted tert-butyldimethylsilyl(tBDMS)-derivatized Arg (Jin *et al.*, 2005). The isotopic contents given by M, M + 1, M + 2, M + 3, and M + 4 were used to calculate the extent of isotopic enrichment in each amino acid by comparison with the isotopomer distribution of unlabeled amino acids. Isotopic enrichments were expressed as the percentage of ions detected that contained one or more ^{15}N atoms.

Measurements of gene expression by quantitative RT-PCR

RNeasy mini RNA isolation kits (Qiagen, Valencia, CA, USA) were used to extract RNA from samples containing 30 and 50 mg fresh weight of germinating spores. Samples were treated with DNase I (Ambion, Austin, TX, USA) and RNA was quantified using the RiboGreen® fluorescence assay (Molecular Probes/Invitrogen, Carlsbad, CA, USA) (Jun *et al.*, 2002). RNA yields were between 0.5 and 1 μg per sample. Reverse transcription and PCR were performed as follows: 60 min at 48°C for reverse transcription, 10 min at 95°C for denaturation, and 45 cycles of 15 s at 95°C and 1 min at 60°C. A no-RT control sample was used for every primer set which resulted in Ct values of > 45 to exclude the possibility of the presence of genomic DNA. Gene-specific PCR products were monitored in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan® probes. The primer and probe sequences (IDT, Coralville, IA, USA, or Synthegen, Houston, TX, USA) are given in Table 1. The effect of root exudates on the expression of the following putative genes was examined: ammonium transporter (AMT; EF379152), glutamate dehydrogenase (GDH; AY745984), glutamine synthetase (GS; DQ063587), ornithine-aminotransferase (OAT; BI452207), and urease accessory protein (UAP; CV186300). Sample extraction, design of primers and probes, and conditions during the quantitative PCR (Q-PCR) were chosen according to Udvardi (2008) to guarantee high and consistent PCR efficiency and reliable Q-PCR results.

The comparative ΔΔCT method was used to measure changes in the expression of the selected genes after treatment relative to the untreated controls (Pfaffl, 2001) using the following formula to calculate the relative gene expression: fold induction = $2^{-[\Delta\Delta CT]}$, where $\Delta\Delta CT = [Ct_{GI}$ (treated sample) $- Ct_{RG}$ (treated sample)] – [Ct_{GI} (control) – Ct_{RG} (control)]. GI is the gene of interest while RG is the reference gene and Ct is the number of PCR cycles required to reach the critical threshold signal. Each assay was validated by plotting ΔCT versus input [RNA] (slope of < 0.19) to demonstrate the

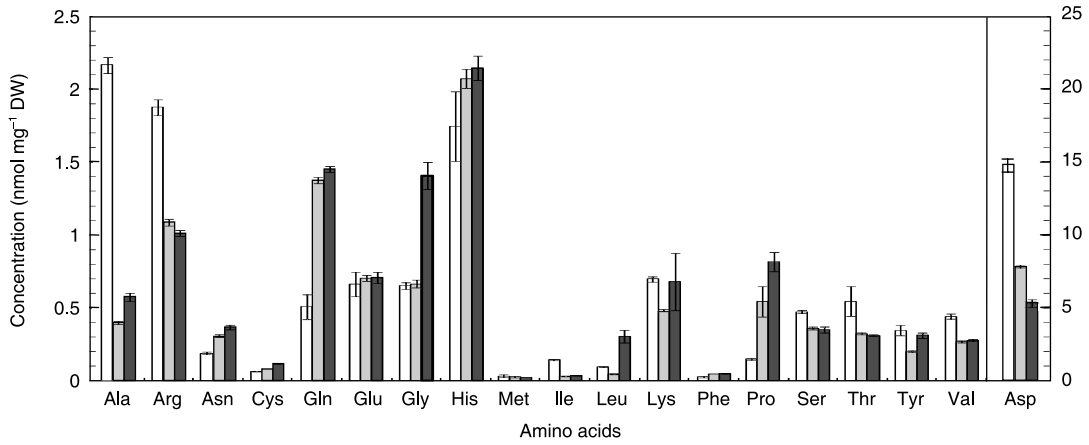

Fig. 1 The free amino acid composition in germinating spores of *Glomus intraradices* cultured in modified M medium without added nitrogen (N) after 0 d (white bars), 7 d (light gray bars) or 14 d (dark gray bars). The right *y*-axis shows the concentration of Asp, and the left *y*-axis the concentration of all other amino acids. Mean of $n = 4 \pm$ SEM.

near-identical efficiency of amplification for the gene of interest and the reference gene (Applied Biosystems, 2001). A putative S4 ribosomal protein (RP; BI452093) and a GATA-type transcriptional regulator (SRP; EU420034) were used as reference genes because of their high expression stability (Haas *et al.*, 1999; Vandesompele *et al.*, 2002). Analysis of the critical threshold levels for both genes confirmed that the transcript levels do not significantly change during spore germination or in response to crude or partially purified root exudates (see Supporting Information Table S1). SRP was used to normalize the data shown in Fig. 6 because RP transcript levels were found to vary in other samples (data not shown). The reported effects on gene expression, however, did not differ significantly between the two reference genes. Only twofold or greater changes in expression of all samples per treatment were considered to be biologically significant.

Statistical treatment

In the figures the means of three (gene expression studies), of four to five (uptake and ^{15}N experiments) or of eight (^{14}C uptake experiment) biological replicates and the standard errors (vertical bars) are shown. Results are only described if a statistically significant difference ($P \leq 0.05$) between the treatments by ANOVA with a repeated measures design and the Student–Newman–Keuls test was found. The data in Table 2 were also analysed using Mann and Whitney's *U*-test (Unistat Software, London, UK). Statistically significant differences between the treatments are indicated by letters.

Results

N uptake and metabolism by germinating spores

When spores were cultured in the absence of external N, the fungal tissue concentrations of the amino acids Asn, Cys, Gln, Gly, Leu, Phe and Pro increased two- to fivefold over 14 d, while the concentrations of Ala, Arg, Asp, Ile, Ser, Thr, and Val decreased over the time course (Fig. 1). When the changes in the concentrations of free amino acids are compared and the number of amino groups per molecule is considered (e.g. 4 N in Arg), the increase in some amino acids corresponds to approximately one-third of the N released by the catabolism of, for example, Ala, Arg or Asp. Asp was by far the most abundant amino acid in spores before germination (see different scale in Fig. 1), but Ala, Arg and His were also detected at relatively high concentrations. The concentrations of Gln, Gly and His, however, were significantly higher after 14 d than the concentrations of Ala and Arg. The concentrations of Glu, Met and Lys stayed constant over the time course.

Incubation with inorganic forms of N or urea resulted in dramatic increases in the free amino acid concentrations, with the largest increases being in Arg and Glu (Fig. 2a; the concentration of Cys, Phe, Met, Ile, and Leu also increased; results not shown). Supplying urea or NH_4^+ to germinating spores led to the highest concentrations of free amino acids. When urea was supplied, the concentration of Arg was 25-fold and that of Glu more than 100-fold higher than under control conditions. The concentration of the other amino acids was between 1.5 and 5 times higher than in the controls. Nitrate addition also led to an increase in the concentration of free amino acids, but the effect was generally smaller than after NH_4^+ or urea addition. However, NO_3^- induced the highest increases in Asp, Lys and Pro. Only the concentrations of Cys and Gln were not significantly affected by the N supply. The Gln concentration even remained constant when Gln was supplied as the sole N source (Fig. 2b). Compared with the inorganic N sources, only small increases in the free amino acid concentrations were observed after supply of Gln or Arg. Gln led only to small increases in the concentrations of Ala, Glu, Gly, and Ser. When Arg was supplied, only the concentration of Arg in germinating spores was significantly higher; the other amino acids were not affected.

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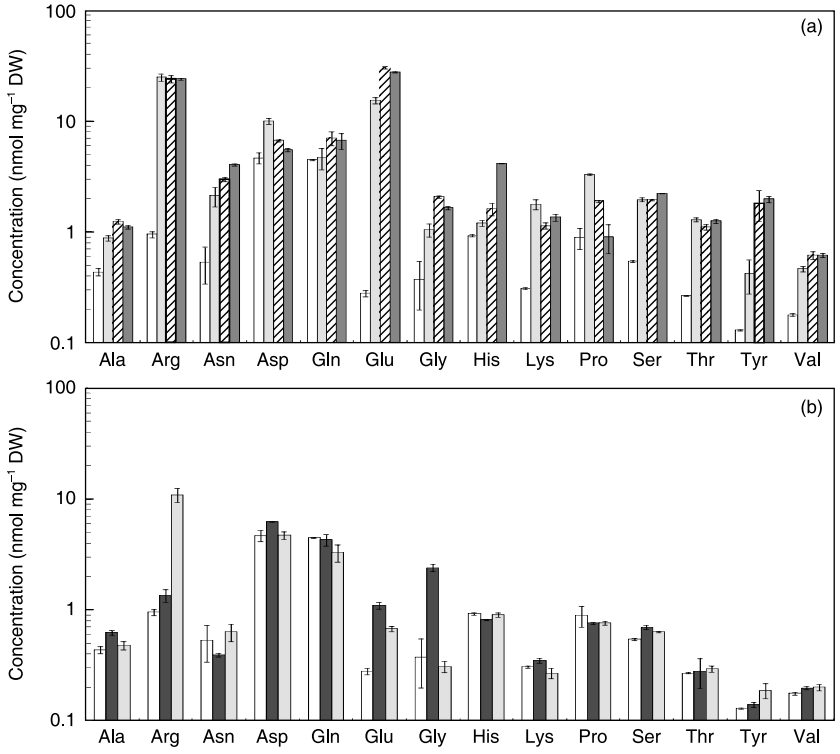

Fig. 2 Free amino acid contents of germinating spores of *Glomus intraradices* after supply of different nitrogen (N) sources for 7 d. (a) No nitrogen (white bars), 4 mM KNO_3 (light gray bars), 4 mM NH_4Cl (hatched bars) or 2 mM urea (mid-gray bars). (b) No nitrogen (white bars), 2 mM Arg (dark gray bars) or 2 mM Gln (light gray bars). Mean of $n = 4 \pm$ SEM.

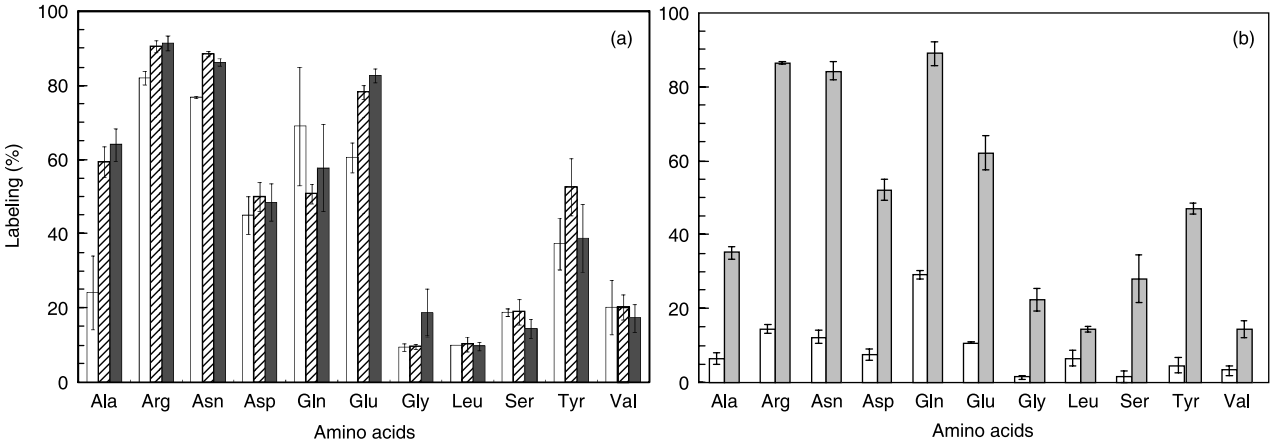

Fig. 3 ^{15}N labeling in free amino acids in germinating spores of *Glomus intraradices* after supply of different ^{15}N-enriched substrates for 7 d. (a) 4 mM KNO_3 (white bars), 4 mM NH_4Cl (hatched bars), or 2 mM urea (dark gray bars); (b) 4 mM $NH_4{}^{15}NO_3$ (white bars) or 4 mM $^{15}NH_4NO_3$ (gray bars). Mean of $n = 5 \pm$ SEM.

Labeling in free amino acids after incubation with ^{15}N-labeled substrates

Ala, Arg, Asn, Asp, Gln and Glu became highly labeled (50–90% contained one or more ^{15}N atoms) when germinating spores were supplied with different inorganic N sources (Fig. 3a). When ^{15}N-labeled NH_4^+ or urea was supplied as the sole N source for 7 d, the ^{15}N labeling in free amino acids was higher than after $^{15}NO_3^-$ supply (Fig. 3a). We also compared the ^{15}N incorporation from NO_3^- or NH_4^+ when the spores were exposed to both for 7 d. The incorporation of label into all amino acids and particularly into Ala, Arg, Asn, Gln, and Glu was substantially higher when the spores were exposed to $^{15}NH_4NO_3$ compared with $NH_4{}^{15}NO_3$ (Fig. 3b). When $^{15}NO_3^-$ was supplied as the sole N source for 7 d, *c.* 80% of the free Arg and Asn became labeled, whereas < 20% were labeled when $^{15}NO_3^-$ was supplied with NH_4^+. Similar to the results shown above (Fig. 2a,b), the labeling of free Gln was independent of the N source (Fig. 3a).

Compared with the inorganic N sources, a supply of Gln and Arg led only to a low ^{15}N enrichment in free amino acids (not shown). [^{15}N]Gln was taken up and mainly

Table 2 Labeling in free amino acids in germinating spores of *Glomus intraradices* after supply of root exudates and $^{15}NH_4Cl$ for 24 h (the spores of the 7 d treatment were labeled on day 6)

		Free amino acids						
Treatment	Days	Ala	Arg	Asn	Asp	Gln	Glu	Ser
Control W	1	41.8 ± 3.4 a	84.6 ± 0.6 a	76.1 ± 0.4 ac	55.7 ± 2.3 a	93.8 ± 1.6 a	77.5 ± 5.6 ac	14.0 ± 1.5 ab
Crude Ex	1	47.4 ± 1.7 a	83.8 ± 0.2 a	71.3 ± 0.2 ab	52.9 ± 1.7 a	92.1 ± 0.1a	64.7 ± 0.4 ab	19.3 ± 2.2 a
Control M	1	37.8 ± 2.9 a	83.9 ± 1.0 a	70.6 ± 1.0 ab	58.1 ± 1.9 a	77.5 ± 16.7 ab	68.8 ± 0.9 ab	13.3 ± 2.5 ab
Purified Ex	1	41.1 ± 2.2 a	83.4 ± 0.5 a	72.3 ± 0.3 ab	58.3 ± 0.6 a	95.3 ± 0.1a	70.1 ± 0.2 ab	14.4 ± 1.2 ab
Control W	7	47.5 ± 7.6 a	90.6 ± 3.0 a	80.9 ± 2.0 a	54.6 ± 5.3 ab	97.7 ± 1.2 a	85.0 ± 4.7 a	11.7 ± 4.9 ab
Crude Ex	7	40.9 ± 6.4 a	60.9 ± 5.8 b[2]	54.2 ± 10.6 ab	46.8 ± 3.3 ab	89.8 ± 3.7 a[2]	59.8 ± 13.0 ab[2]	11.8 ± 2.0 ab
Control M	7	9.9 ± 3.6 b	95.0[1]	48.2 ± 10.8 b	33.5 ± 20.1 bc	90.0[1]	52.3 ± 10.1 bc	7.2 ± 2.5 ab
Purified Ex	7	14.2 ± 4.4 b	44.5 ± 1.6 c	49.2 ± 0.4 bc	29.3 ± 3.6 c	65.4 ± 1.5 b	47.5 ± 2.1 b	7.3 ± 2.0 b

Values are mean of $n = 3–5 \pm$ SEM.
Control W, water control; Crude Ex, crude root exudates; Control M, methanol control; Purified Ex, partially purified root exudates. Different letters within a column indicate significant differences according to the Student–Newman–Keuls test ($P \leq 0.05$).
[1]Only one sample of the methanol control after 7 d contained detectable amounts of Arg and Gln.
[2]According to the nonparametric Mann–Whitney *U*-test significantly different from the water control at day 7.

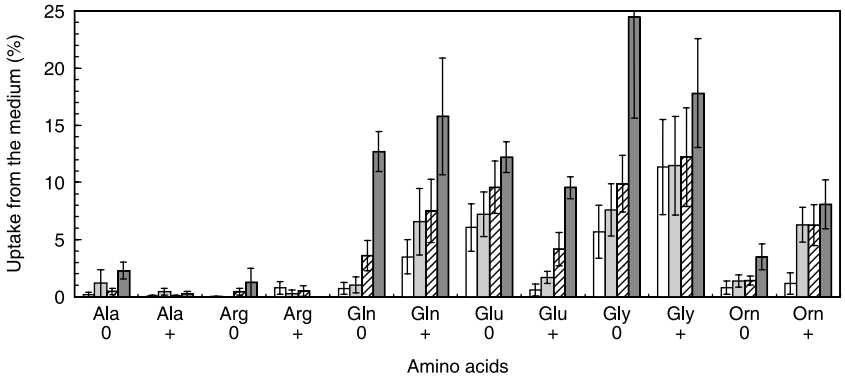

Fig. 4 Amino acid uptake (in %) by germinating spores of *Glomus intraradices* without (0) or with the addition of 7.7% crude root exudates (+). Shown is the uptake after 1 d (white bars), 2 d (light gray bars), 4 d (hatched bars) and 8 d (dark gray bars). Mean of $n = 8 \pm$ SEM.

incorporated into Glu (9.1 ± 1.2%), but the incorporation into other amino acids was < 4%. When [^{15}N]Arg was supplied, low labeling of Ala, Asn, Gly, Ser and Val (≤ 3%) was observed.

Effects of root exudates on the utilization of NH_4^+

The root exudates used in these experiments have been shown to stimulate presymbiotic growth, the uptake of carbon, respiration, and fungal gene expression under the conditions used here (Bücking *et al.*, 2008). To investigate whether potential effects of root exudates on N uptake are caused by an increase in the nutrient-absorbing surface area (as a result of the presymbiotic growth response) or by effects on nutrient uptake efficiency (nutrient absorption per surface unit), germinating spores were exposed for 24 h to root exudates and $^{15}NH_4^+$ or grown for 6 d with or without root exudates and then labeled for 24 h with $^{15}NH_4^+$. The uptake and incorporation of exogenous NH_4^+ into free amino acids were very fast and enrichments of > 80% for Arg and for Gln were found 24 h after supply of $^{15}NH_4^+$ (Table 2). The labeling in free amino acids was comparable to the results after 7 days shown in Fig. 3a. Crude and partially purified root exudates had no effect on the uptake and incorporation of $^{15}NH_4^+$ into amino acids after 24 h, but partially purified root exudates reduced the incorporation at the later time-point. The labeling in free amino acids when spores were first exposed to partially purified root exudates for 6 d and then supplied with $^{15}NH_4^+$ was as much as 70% lower than after the 1-d treatment. Also, the methanol control showed lower incorporation into Ala, Asn and Glu. The reduced incorporation into Arg, Gln, and Glu indicates that crude root exudates also have an effect (not all differences were significant at the 5% level with the Student–Newman–Keuls test, but significant according to the Mann–Whitney *U*-test).

Effects of root exudates on the uptake of amino acids

The effect of crude root exudates on the uptake of six amino acids during presymbiotic growth was measured using ^{14}C-labeled amino acids. In the absence of root exudates, the

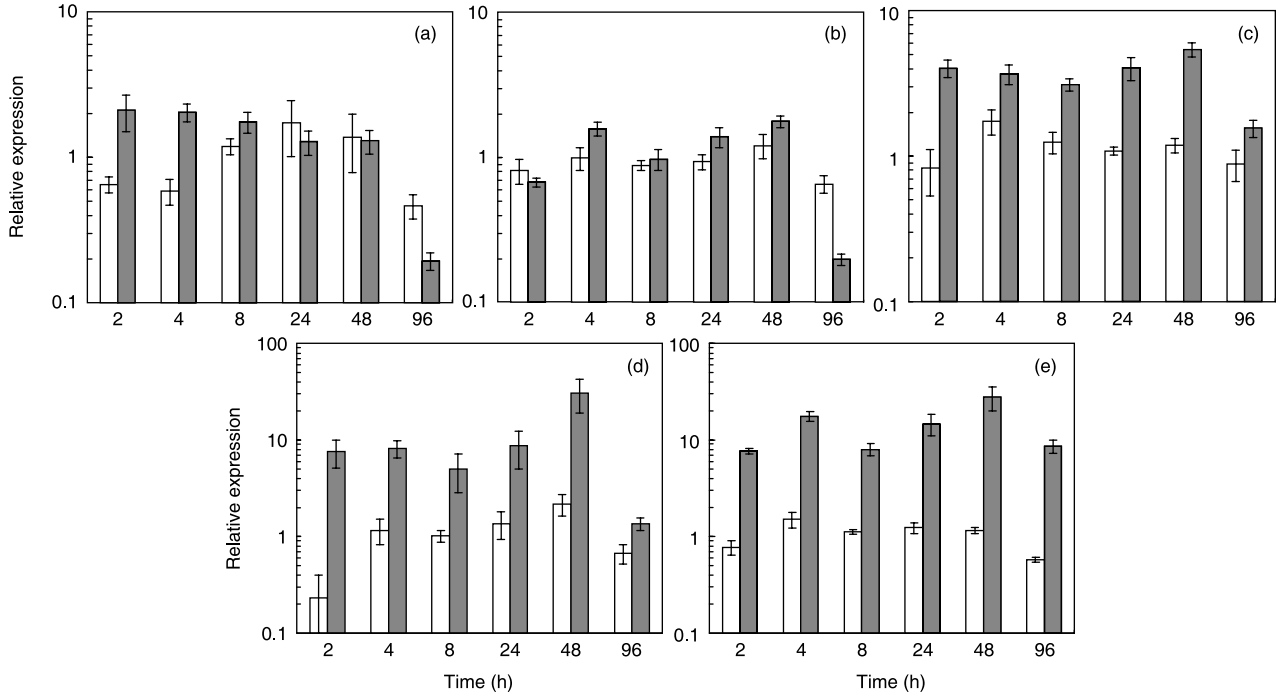

Fig. 5 Effect of root exudates on the expression of (a) a putative fungal ammonium transporter (AMT), (b) glutamine synthetase (GS), (c) glutamate dehydrogenase (GDH), (d) urease accessory protein (UAP), and (e) ornithine aminotransferase (OAT) relative to untreated controls using a GATA-type transcriptional regulator as reference protein. The addition of crude root exudates is shown as white bars, the addition of partially purified root exudates as gray bars. Mean of $n = 3 \pm$ SEM.

uptake of Gln, Glu and Gly was higher than that of Ala, Arg, and Orn (Fig. 4). The addition of crude root exudates tended to result in a slightly higher uptake of Gln, Gly, and Orn, but the effect was only significant for the first time-points (1 or 2 d). By contrast, the uptake of Glu at the earlier time-points was slightly lower than that in the controls when the spores were exposed to root exudates.

Effects of root exudates on gene expression in germinating spores

Changes in the expression of genes that were identified by sequence similarity as putative genes involved in the N metabolism of *G. intraradices* were measured in germinating spores using real-time quantitative PCR. The addition of crude root exudates did not have an effect on gene expression, but some of the genes responded strongly to the presence of partially purified root exudates in the medium. The relative expression of an AMT (Fig. 5a) and of GS (Fig. 5b) was not changed in the presence of either crude or partially purified root exudates. By contrast, the transcript levels of GDH (Fig. 5c), UAP (Fig. 5d), and OAT (Fig. 5e) increased significantly after the addition of partially purified root exudates to germinating spores. Increases in the transcript levels were already detectable 2 h after partially purified root exudates were added to the spores. The expression of GDH and UAP was three- to fivefold higher, and that of OAT approximately tenfold higher than in the controls. The expression of GDH and UAP, however, dropped significantly at the last time-point (4 d).

Discussion

Germinating spores use internal N sources during presymbiotic growth

The rise in the concentrations of some amino acids during presymbiotic growth in the absence of exogenous N sources indicates that germinating spores of *G. intraradices* can mobilize internal N reserves (Fig. 1). Fungal spores have been reported to store N as protein bodies (Petersen *et al.*, 1983; Cruz, 2004), and a decrease in the intensity of protein bands on gels supports the idea that proteins are utilized during germination (Avio & Giovannetti, 1998). The expression of GDH during presymbiotic growth (Fig. 5c) is also consistent with utilization of stored organic N.

The levels of Asp, Ala and Arg found in quiescent spores and their degradation during germination demonstrate that spores also store a significant amount of N in the form of amino acids and that their catabolic metabolism may also provide N and C skeletons for the biosynthesis of other amino acids and proteins during presymbiotic growth. Asp can serve as a precursor for the synthesis of Asn (the concentration of free Asn increased during the time course), or its amino group could be transferred by Asp aminotransferase (AAT) onto α-ketoglutarate to give

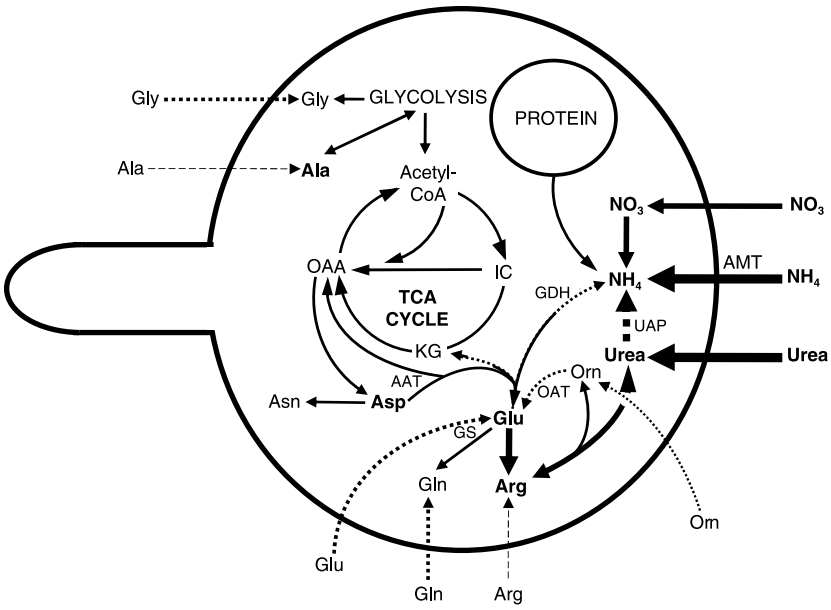

Fig. 6 Nitrogen metabolism in germinating spores of *Glomus intraradices*. The main metabolic pathways are shown as bold lines (e.g. uptake of NH_4), pathways of small importance as thin dashed lines (e.g. uptake of Arg) and pathways that are affected by root exudates as dotted lines (e.g. uptake of Orn). AMT, ammonium transporter; IC, isocitrate; KG, α-ketoglutarate; OAA, oxaloacetate; UAP, urease accessory protein; OAT, ornithine aminotransferase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; AAT, Asp aminotransferase.

Glu and oxaloacetate, which could anaplerotically replenish the TCA cycle (Fig. 6). A putative AAT has been identified in spores of *G. intraradices* (Lammers *et al.*, 2001) and ectomycorrhizal fungi show high activities of AAT (Botton & Chalot, 1995). The concentration of free Ala also dropped by > 70% after 7 d of germination without N supply. It has been shown that for ectomycorrhizal fungi Ala is a much better respiratory substrate than Glu or Gln and that the carbon skeletons derived from Ala are actively incorporated into TCA cycle intermediates through pyruvate formation and used for further amino acid biosynthesis (Chalot *et al.*, 1994). The storage of N in the form of Arg with its low C:N ratio (6C:4N) allows spores to store N with the minimum amount of C and it has been suggested that Arg could be associated with polyphosphates (Cramer *et al.*, 1980, Cramer & Davis, 1984). Spores of *G. intraradices* contain high amounts of polyphosphates (Maia & Kimbrough, 1998) and it has been reported that the activity of enzymes involved in polyphosphate breakdown is increased during spore germination of *Aspergillus* (Nishi, 1960). The expression of putative UAP and OAT genes in germinating spores and the increased expression when spores were exposed to root exudates (Fig. 5d,e) also indicate that the catabolic arm of the urea cycle is active during presymbiotic growth (Fig. 6). The urea cycle has been implicated in the translocation of inorganic N across the mycorrhizal interface, involving the synthesis of Arg in the extraradical mycelium (ERM) and its breakdown in the intraradical mycelium (Bago *et al.*, 2001; Govindarajulu *et al.*, 2005; Cruz *et al.*, 2007; Gomez *et al.*, 2009). Arg breakdown may thereby also make NH_4^+ available for the biosynthesis of amino acids and proteins during presymbiotic growth. Arg could serve in spores also as a precursor for the biosynthesis of putrescine via Arg decarboxylase (Sannazzaro *et al.*, 2004) during germination. Putrescine has been shown to be essential for hyphal growth and the colonization of the host plant (El Ghachtouli *et al.*, 1996).

Germinating spores can utilize exogenous inorganic N and synthesize amino acids *de novo*

Exposure to NH_4, NO_3, or urea resulted in large increases in free amino acid concentrations, most dramatically in Arg and Glu, but also in Asn (Fig. 2a). The high percentage of ^{15}N in free amino acids when labeled inorganic N sources or urea was supplied (Fig. 3a) also shows that germinating spores can make effective use of these forms of N for amino acid biosynthesis. Glu is a key intermediary in amino acid metabolism and Arg seems to play a role in the storage and transport of N (see the section 'Root exudates influence the expression of putative N metabolic genes') that is taken up during presymbiotic growth. In ^{13}C-labeling experiments Bago *et al.* (1999) also observed the synthesis of Arg and Glu during presymbiotic growth of this fungus. Beilby & Kidby (1980, 1982) found the highest label incorporation in Asn and Arg after supplying [1-^{14}C]acetate to germinating spores of *Glomus caledonius* in the presence of inorganic N. The labeling of Gly, Val and Cys was very low and Pro and His did not contain any radioactivity and their biosynthesis was accordingly thought to be a potential limitation of presymbiotic growth. This view was also supported by the finding of Hepper & Jakobsen (1983), who reported that the supply of sulfur-containing amino acids can increase the hyphal growth of germinating spores *in vitro*. By contrast, this study shows that the concentration of these amino acids increased when an inorganic N source was supplied and that the amino acids detected here by GC-MS incorporated exogenously supplied ^{15}N, showing that they can be synthesized *de novo*. Experiments with ^{35}S-labeled sulfate also showed that sulfur-containing

amino acids can be made during presymbiotic growth (J. W. Allen & Y. Shachar-Hill, unpublished). The low or lack of incorporation of ^{14}C into some amino acids in the work of Beilby & Kidby (1982) may have reflected the substrate (acetate, leucine and uracil) used rather than the absence of amino acid biosynthesis. Thus we suggest that the ability to synthesize amino acids does not limit presymbiotic growth and that, while AM fungi do not depend on exogenous N to germinate or to develop, they are able to take advantage of available inorganic N sources to synthesize amino acids.

In contrast to all other amino acids, the concentration of free Gln remained constant even when Gln was supplied as the sole N source (Fig. 2a,b). A possible explanation for this observation is the fact that in fungi Gln has been described as the key effector for N metabolite repression (Marzluf, 1997), and that the free Gln concentrations in spores are closely regulated. This global regulatory mechanism plays a key role in the transcriptional and post-translational regulation of, for example, N assimilation (Premakumar *et al.*, 1979) and ensures the preferential utilization of simple N sources over more complex N molecules (Margelis *et al.*, 2001).

When germinating spores of *G. intraradices* were supplied with $^{15}NH_4$, the labeling in free amino acids was higher than after they were supplied with $^{15}NO_3$ (Fig. 3a), and the presence of NH_4^+ in the medium when $NH_4{}^{15}NO_3$ was supplied repressed the incorporation of label from $^{15}NO_3$ into free amino acids (Fig. 3b). The preferential utilization of NH_4^+ over NO_3^- by spores is consistent with findings for NH_4^+ and NO_3^- uptake by the ERM of this fungus (Johansen *et al.*, 1996; Toussaint *et al.*, 2004). This can be explained by the fact that NO_3^- is more oxidized, so that the assimilation of N from NH_4^+ is energetically more efficient than from NO_3^- (Johansen *et al.*, 1996; Villegas *et al.*, 1996).

Germinating spores are able to utilize organic N sources

Glu, Asp, Ala and Gly are amongst the most prevalent amino acids in the soil, with concentrations between 1 and 10 $\mu g\ g^{-1}$ dry soil (Abuarghub & Read, 1988), and the results show that germinating spores are able to take up amino acids, such as Gln, Glu, Gly and Orn, during presymbiotic growth. The uptake of Ala and Arg (Fig. 4), however, and the incorporation of ^{15}N from Arg into other free amino acids (not shown) were extremely low. By contrast, the ERM of this fungus has been shown to be able to take up Arg and to transfer it to the intraradical mycelium (IRM) within the mycorrhizal root (Jin *et al.*, 2005). This is consistent with the findings of Cappellazzo *et al.* (2008), who characterized an amino acid permease from *Glomus mosseae* (GmosAAP1) with a preferential affinity for all nonpolar and hydrophobic amino acids that is expressed in the ERM but not in sporocarps. A partial sequence with a high similarity to GmosAAP1 has also been identified in *G. intraradices* (Cappellazzo *et al.*, 2008). The observation that germinating spores were able to take up Gln (Fig. 4), but that the incorporation of ^{15}N into other amino acids from Gln was low, could be attributable to the different concentration range used for the experiments (50 μM [^{14}C]Gln but 2 mM [α-^{15}N]L-Gln; normal soil concentrations are in the μM range; see above) or to the different sensitivities of the techniques used (liquid scintillation counting and GC-MS; in the ^{14}C experiment only *c.* 7.5 μM Gln was taken up within 8 d).

The effect of root exudates on N uptake

Root exudates had no effect on the uptake and utilization of ^{15}N from NH_4^+ after 1 d, but significantly reduced the uptake after 7 d of germination (Table 2). This is consistent with the finding that the expression of a putative fungal AMT of *G. intraradices* was also not affected by root exudates (Fig. 5a). López-Pedrosa *et al.* (2006) characterized a functional high-affinity NH_4^+ transporter (GintAMT1) in the ERM of *G. intraradices*, whose expression was increased after the addition of 30 μM but decreased after the addition of 3 mM NH_4^+ (4 mM was used in these experiments). Numerous investigations have shown that root exudates from mycorrhizal host species stimulate spore germination, hyphal branching (e.g. Mosse, 1973; Graham, 1982; Gianinazzi-Pearson *et al.*, 1989; Nagahashi & Douds, 2000) and respiration (Tamasloukht *et al.*, 2003; Bücking *et al.*, 2008). The stimulation of presymbiotic growth and the strong increase in catabolic metabolism that could already be observed within hours after spores were exposed to root exudates (Tamasloukht *et al.*, 2003; Bücking *et al.*, 2008) could have induced an earlier return of the spores to the resting stage and could be responsible for the reduced uptake of NH_4^+ after 7 d. This possibility is supported by the finding that crude root exudates only stimulated the uptake of Gln and Gly at the earlier time-points (Fig. 4), and had no effect at the later time-points. The amino acid permease which has been described for the ERM of *Glomus mosseae*, GmosAAP1, showed a strong dependence on a proton gradient (Cappellazzo *et al.*, 2008) and root exudates have been shown to increase the H^+-ATPase activity at the fungal plasma membrane of germinating spores of *Gigaspora margarita* (Lei *et al.*, 1991). It seems to be unlikely, however, that the observed effects of root exudates on amino acid uptake were caused by changes in the polarization of the fungal plasma membrane in response to root exudates. The NH_4^+ transporter GintAMT1 (see above) also depends on the H^+-electrochemical gradient for its activity (López-Pedrosa *et al.*, 2006), but root exudates had no stimulatory effect on the uptake of NH_4^+ (Table 2).

Root exudates influence the expression of putative N metabolic genes

Partially purified root exudates have an effect on the transcript levels of genes that are putatively involved in the N metabolism of germinating spores. The same root exudates have also been

shown to stimulate the expression of genes that are involved in C metabolism during presymbiotic growth (Bücking *et al.*, 2008). The expression of UAP during presymbiotic growth (Fig. 5d) is consistent with the efficient utilization of urea (Fig. 2a). The positive effect of urea on amino acid concentrations may be mediated by generating intracellular NH_4^+ (Fig. 6). UAP and OAT, whose expression was also greatly increased by treatment with partially purified root exudates (Fig. 5e), have been suggested to be involved in utilizing the products of Arg catabolism in AM roots (Govindarajulu *et al.*, 2005). The results demonstrate that germinating spores break down stored Arg during presymbiotic growth and the increased expression of UAP, OAT and GDH in response to root exudates is consistent with an increased demand for NH_4^+ when presymbiotic growth is stimulated, but the uptake of exogenous NH_4^+ is not affected. During presymbiotic growth, Arg may also be involved in transporting N from the spore along the developing hyphae in a similar way to its translocation in the symbiotic stage (Govindarajulu *et al.*, 2005; Cruz *et al.*, 2007). GDH, which releases NH_4^+ and α-ketoglutarate from Glu, may function in anapleurosis and may release N from Glu produced by the breakdown of Orn made during the catabolic metabolism of Arg or by the degradation of Asp via AAT (Fig. 6).

By contrast, the expression of AMT and GS was not affected by root exudates (Fig. 5a,b). GS has been shown to be constitutively expressed during all stages of the fungal life cycle (germinating spores, ERM and IRM), and the activity of GS in the ERM of *G. intraradices* but not its expression was induced by a supply of exogenous N. A supply of NH_4^+ increased the GS activity in the ERM compared with hyphae grown on NO_3^- (Breuninger *et al.*, 2004). Similar effects during presymbiotic growth would be consistent with the lack of an effect of root exudates on NH_4^+ uptake and GS expression and with the observed increase in amino acid concentrations and high ^{15}N assimilation when NH_4^+ was supplied to germinating spores.

Conclusions

The spores of AM fungi do not depend on exogenous N to germinate or to develop, because they can remobilize internal N reserves such as amino acids and proteins during germination. The catabolism of Asp (via AAT) and Arg (via UAP and OAT) could provide the required amino groups and C skeletons for the biosynthesis of other amino acids (Fig. 6). However, while N is not needed for germination, spores of *G. intraradices* are also capable of taking up inorganic and organic forms of N and incorporating them into a broad range of amino acids. Exogenous N in the form of NH_4^+ or urea is readily taken up by germinating spores and utilized for *de novo* biosynthesis of amino acids. The capacity to use external NO_3^- is also significant and some amino acids can also be taken up, but to a lesser extent. Thus, the ability to synthesize amino acids does not limit the presymbiotic growth of AM fungal spores. Root exudates that increase the uptake of exogenous C resources by spores of *G. intraradices* (Bücking *et al.*, 2008) had no effect on the uptake of NH_4^+ but stimulated the uptake of some amino acids during the early stages of germination. The increase in the transcript levels of putative GDH, OAT, and UAP genes in response to root exudates may be associated with the higher demand for NH_4^+ and reflect an enhanced use of internal N stores following the stimulation of presymbiotic growth in preference to NH_4^+ uptake. Root exudates have also been shown to stimulate the utilization of C resources during germination (Tamasloukht *et al.*, 2003; Besserer *et al.*, 2006; Bücking *et al.*, 2008). The increased utilization of internal C and N stores by the fungus when host root signals are perceived would enable the fungus to utilize its resources more efficiently and could prevent its C and N reserves from being compromised when no host roots are present (Bécard *et al.*, 2004).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 The effect of crude and partially purified root exudates on the expression of genes putatively involved in the nitrogen (N) metabolism of germinating spores of *Glomus intraradices*

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