Enzymatic Evidence for the Key Role of Arginine in Nitrogen Translocation by Arbuscular Mycorrhizal Fungi[1][OA]

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Key enzymes of the urea cycle and ^{15}N-labeling patterns of arginine (Arg) were measured to elucidate the involvement of Arg in nitrogen translocation by arbuscular mycorrhizal (AM) fungi. Mycorrhiza was established between transformed carrot (*Daucus carota*) roots and *Glomus intraradices* in two-compartment petri dishes and three ammonium levels were supplied to the compartment containing the extraradical mycelium (ERM), but no roots. Time courses of specific enzyme activity were obtained for glutamine synthetase, argininosuccinate synthetase, arginase, and urease in the ERM and AM roots. $^{15}NH_4^+$ was used to follow the dynamics of nitrogen incorporation into and turnover of Arg. Both the absence of external nitrogen and the presence of L-norvaline, an inhibitor of Arg synthesis, prevented the synthesis of Arg in the ERM and resulted in decreased activity of arginase and urease in the AM root. The catabolic activity of the urea cycle in the roots therefore depends on Arg translocation from the ERM. ^{15}N labeling of Arg in the ERM was very fast and analysis of its time course and isotopomer pattern allowed estimation of the translocation rate of Arg along the mycelium as 0.13 μg Arg mg^{-1} fresh weight h^{-1}. The results highlight the synchronization of the spatially separated reactions involved in the anabolic and catabolic arms of the urea cycle. This synchronization is a prerequisite for Arg to be a key component in nitrogen translocation in the AM mycelium.

Symbiotic associations with arbuscular mycorrhizal (AM) fungi enhance the acquisition of several mineral nutrients by plants. Nutrient acquisition via the fungal partner involves transfer across two interfaces: one between the soil and the extraradical mycelium (ERM) of the fungus and one between the intraradical mycelium (IRM) of the fungus and the root cortex cells. Soil-to-plant nitrogen transport by the ERM of AM fungi was first demonstrated using compartmented pots where ^{15}N-labeled nitrogen sources were applied to soil containing the ERM, but no roots (Ames et al., 1983; Johansen et al., 1992; Frey and Schüepp, 1993). The ERM can take up ammonium (NH_4^+), nitrate (NO_3^-), and amino acids (Ames et al., 1983; George et al., 1992; Frey and Schüepp, 1993; Johansen et al., 1994; Tobar et al., 1994; Bago et al., 1996; Hawkins et al., 2000; Toussaint et al., 2004; Govindarajulu et al., 2005) and nitrogen uptake from root-free compartments (RFCs) could account for as much as 30% to 80% of total plant nitrogen uptake in compartmented growth systems (Frey and Schüepp, 1993; Johansen et al., 1994). High nitrogen uptake ability by AM fungi was confirmed using in vitro model systems (Govindarajulu et al., 2005).

A high-affinity NH_4^+ transporter was recently characterized in *Glomus intraradices* (López-Pedrosa et al., 2006) and AM hyphae possess the enzymes required for uptake of NO_3^- and NH_4^+ and their assimilation into amino acids (Kaldorf et al., 1994; Bago et al., 1996; Johansen et al., 1996; Toussaint et al., 2004). There is no evidence for AM fungal translocation of NO_3^- and NH_4^+ and it is more likely that nitrogen is translocated in the form of amino acids (Bago et al., 2001). Analysis of the levels of free amino acids in the ERM by HPLC revealed that Arg is by far the most abundant amino acid in the ERM. Arg levels were reported in the range of 50 to 200 nmol mg^{-1} dry weight, depending on the development stage, and this represented more than 90% of the total free amino acids in the ERM (Johansen et al., 1996; Govindarajulu et al., 2005; Jin et al., 2005). Vacuoles are probably the major storage compartment for Arg, which is an efficient storage molecule for nitrogen due to its low carbon-to-nitrogen ratio (6:4)

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and, for example, yeast (*Saccharomyces cerevisiae*) fed with NH_4^+ contained only 10% of its Arg in the cytoplasm (Jennings, 1995).

Characteristics of the ERM, such as its coenocytic nature (Smith et al., 1994), high rates of cytoplasmic streaming (Cox et al., 1980), and high Arg content (Johansen et al., 1996), were integrated in a model for nitrogen flow from soil to host plant via the fungus (Bago et al., 2001). The model suggests that the anabolic arm of the urea cycle in the ERM converts inorganic nitrogen into Arg, which is subsequently accumulated and translocated in tubular vacuoles. The catabolic arm of the urea cycle is suggested to operate in the IRM generating urea and ultimately NH_4^+ is made available to the roots. The transfer of nitrogen as inorganic rather than organic forms was recently confirmed in a study showing that ^{13}C labeling of the free amino acids in the ERM never led to the appearance of ^{13}C in the metabolic pools of the plant (Govindarajulu et al., 2005). In this work, it was shown that high Arg content in AM fungi was associated with the expression of putative nitrogen metabolic genes, suggesting that the enzymatic pathways are involved, which emphasizes the need for direct measurements of urea cycle key enzyme activities in response to external nitrogen supply. The proposed model requires that a number of enzymes and transporters are present at specific locations in the AM. It implies that exposure of the ERM to an appropriate inorganic nitrogen source should increase the activity of enzymes required for nitrogen assimilation (e.g. Gln synthetase [GS] and argininosuccinate synthetase [ASS]). Furthermore, the activity of enzymes involved in Arg catabolism (e.g. arginase and urease) should increase in the IRM. The magnitude of such nitrogen supply-induced changes in AM fungal enzyme activity would probably increase with increasing root sink strength. The nature and site of nitrogen transport across the fungus-plant interface has not yet been determined, but have been suggested to involve an Ato-like fungal NH_4^+ efflux system and plant Amt NH_4^+ transporters (Chalot et al., 2006).

Few studies have investigated the nitrogen metabolic pathway (Hawkins et al., 2000; Bago et al., 2001; Pfeffer et al., 2001) or investigated the key enzymes of nitrogen metabolism in AM symbiosis (Subramanian and Charest, 1997, 1998, 1999). Increased activity of GS has been reported in AM roots from pot experiments (Azcón et al., 1982; Barea et al., 1987; Cliquet and Stewart, 1993; Subramanian and Charest, 1998, 1999). One of the possible routes of Arg synthesis in fungi starts with the synthesis of Orn from Glu and carbamoyl phosphate from CO_2 and Gln/ammonia. The subsequent reaction of formed citrulline with Asp leads to Arg formation (Jennings, 1995).

More recent experiments used the in vitro monoxenic system of root organ culture of AM fungi (Bécard and Fortin, 1988; St-Arnaud et al., 1996) to allow growth of the ERM into a RFC. It was shown that the ERM of *G. intraradices* has a GS monomer distinct from that of roots (Toussaint et al., 2004) and that genes with high similarity to known Orn aminotransferase, urease accessory protein, and an NH_4^+ transporter are preferentially expressed in the IRM (Govindarajulu et al., 2005). The observed expression of genes with a putative role in nitrogen metabolism in the IRM and ERM significantly advances our understanding of nitrogen translocation in AM. However, enzymes are subject to complex posttranslational regulation (Jennings, 1995) and determination of the activity of key enzymes of the urea cycle in ERM and in AM roots would provide more direct evidence for model validation.

The objective of this work was to obtain such direct evidence for the function of Arg in nitrogen translocation by AM fungi. Time-course studies focused on the activity of GS and key enzymes of the urea cycle and in response to enhanced NH_4^+ concentrations in the medium and on Arg synthesis and turnover based on experiments utilizing $^{15}NH_4^+$. We hypothesized that external supply of nitrogen would have contrasting effects on enzymes of the urea cycle in different parts of the mycelium: Enzymes of the anabolic arm would become more active in the ERM, whereas enzymes of the catabolic arm would become more active in the IRM. Such a response pattern would require that Arg be synthesized in the ERM and subsequently translocated to the IRM.

RESULTS

Mycorrhizas established well in the monoxenic cultures between root-inducing transferred (Ri T)-DNA transformed carrot (*Daucus carota*) roots and *G. intraradices* Schenck and Smith (DAOM 181602) and 32% to 48% of the root length was colonized at the end of the sampling period. Mycelium of *G. intraradices* developed in RFCs of all 160 cultures with fresh weights in the range 4 to 25 mg. Amounts of mycelium did not differ significantly between treatments, but mean values per sampling time increased from 8.3 to 12.7 mg fresh weight over the 96-h sampling period. Root growth was also intense and roots had to be frequently trimmed so as not to invade RFCs. Symptoms of senescence (coloration) or dehydration of root cultures were absent.

Enzyme-specific activities for GS, ASS, arginase, and urease were determined in AM roots and the ERM in time after addition of $(NH_4)_2SO_4$ to RFCs. The root compartment received no more nitrogen than that originally present in M medium. Specific activity of GS in the ERM showed no response over the time course of the experiment, whereas the activity in AM roots increased in response to the supply of nitrogen to the RFC (Fig. 1). The highest NH_4^+ supply (5 mM) resulted in GS activity in roots being more than twice that measured without NH_4^+ and levels remained high throughout the experiment. Roots from treatments with 0.1 mM NH_4^+ had somewhat lower GS specific activity, which reached a maximum at 24 to 48 h and

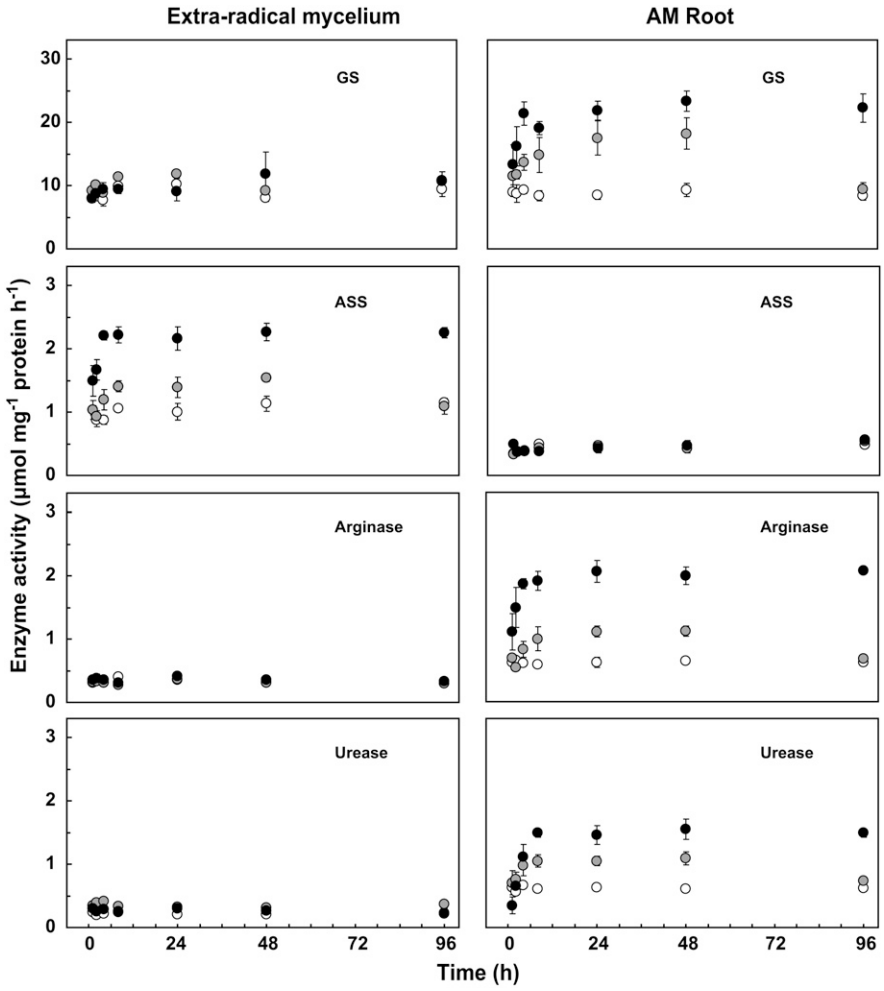

Figure 1. Specific enzyme activity in the ERM of *G. intraradices* and AM roots of carrot grown in an in vitro compartmented system after exposing the ERM to $(NH_4)_2SO_4$ at nil (white symbols), 0.1 (gray symbol), or 5 mM (black symbols). Enzyme activity was normalized against protein concentration of the sample. Symbols with vertical bars represent means and SD ($n = 3$).

then decreased. Without additional nitrogen, GS specific activity was similar in roots and in the ERM; it should be noted that root measurements also include a possible contribution from intraradical fungal mycelium. Specific activity of ASS remained around 1 μmol mg^{-1} protein h^{-1} for the ERM in the absence of NH_4^+ in the RFC. This activity increased in response to the addition of 5 mM NH_4^+ and, after 8 to 12 h, had reached a plateau about 2.5 times the initial values (Fig. 1). The plateau was somewhat lower in the 0.1 mM NH_4^+ treatment and had decreased to the nil treatment level at 96 h. Specific activity of ASS was restricted to constant residual levels in the AM root and did not respond to NH_4^+ availability in the RFC.

The specific activity of arginase and urease was consistently low in the ERM and neither responded to additional nitrogen nor changed over time. Corresponding activity in roots was considerably higher and, consequently, distribution of activity between AM roots and the ERM differed from that of ASS (Fig. 1). In roots, both arginase and urease remained low without additional nitrogen, but responded strongly to 0.1 and 5 mM NH_4^+ supplied to the RFC (Fig. 1). Maximal activity was reached with 5 mM NH_4^+, being of the same order of magnitude for the two enzymes (i.e. twice the initial levels). A plateau was reached in the 5 mM treatment, whereas activity had decreased toward the initial levels in cultures receiving 0.1 mM NH_4^+. Enzyme activity was also measured at three time points in root cultures in the absence of a mycorrhizal fungus. The specific activity of the four enzymes in uncolonized roots was similar to levels measured in mycorrhizal roots from treatments receiving nil NH_4^+ in the ERM compartment.

The specificity of L-norvaline as an ASS inhibitor and of phenyl phosphorodiamidate (PPDA) as a urease inhibitor was tested by incubation of the inhibitor in enzyme extracts from the ERM and AM roots. Once added to the extracts, inhibitors were shown to be specific, such that L-norvaline had significant effects only on ASS activity, whereas PPDA had significant effects only on urease activity (Table I).

Activity of GS, ASS, arginase, and urease was determined over time both in the ERM and in the AM root in cultures fed with 5 mM NH_4^+ to the RFC and supplied with either L-norvaline or PPDA (Fig. 2).

Table I. *GS, ASS, arginase, and urease specific activity in AM roots of carrot and in the ERM of G. intraradices grown in an in vitro compartmented system in the presence of 5 mM $(NH_4)_2SO_4$ in the RFC of the ERM*

Enzyme activity was determined in crude extracts in the absence of inhibitors or after addition of 500 μM phenyl PPDA (an inhibitor of the urease activity) or 20 mM L-norvaline (an inhibitor of ASS) to the reaction media followed by 30-min incubation. Numbers represent mean values of enzyme activity expressed in terms of protein content ± SD ($n = 6$).

Enzyme	Inhibitors Added to Reaction Media	Activity	
		ERM	AM Roots
		μmol mg protein^{-1} h^{-1}	
GS	None	27.4 ± 3.12	27.8 ± 3.23
	0.5 mM PPDA	31.2 ± 4.14	23.6 ± 2.1
	20 mM L-norvaline	25.6 ± 2.70	27.2 ± 2.2
ASS	None	2.94 ± 2.12	0.51 ± 0.32
	0.5 mM PPDA	2.98 ± 3.21	0.43 ± 0.32
	20 mM L-norvaline	0.17 ± 0.10	0.12 ± 0.10
Arginase	None	0.60 ± 0.10	2.44 ± 0.55
	0.5 mM PPDA	0.50 ± 0.11	1.99 ± 0.2
	20 mM L-norvaline	0.43 ± 0.12	2.18 ± 0.23
Urease	None	0.82 ± 0.24	4.91 ± 0.75
	0.5 mM PPDA	0.41 ± 0.23	0.73 ± 0.23
	20 mM L-norvaline	0.87 ± 0.33	4.81 ± 0.54

L-norvaline or PPDA supplied to the RFC had no detectable effects on GS specific activity in the ERM, whereas levels in AM roots were strongly decreased. In the ERM, PPDA slightly decreased ASS activity, whereas L-norvaline was strongly inhibitory, as expected, reducing ASS activity to the residual values similar to those detected in AM roots. None of the inhibitors influenced the low arginase activity in the ERM. Addition of PPDA to the RFC resulted in a slight decrease of arginase activity in the AM root, whereas L-norvaline reduced arginase activity by approximately 65%. Residual urease activity in the ERM also was not altered by the inhibitors, whereas addition of PPDA to the RFC resulted in nearly complete inhibition of urease specific activity in roots. L-norvaline reduced urease activity by approximately 50% (Fig. 2).

Nitrogen Pools in the ERM in Relation to External NH_4^+ Level

Nitrogen concentration in the ERM at 96 h after exposure to NH_4^+ increased from 2.8% to 3.6% nitrogen (dry weight) in response to a supply of 0.1 mM NH_4^+, but only by an additional 0.2% by further increasing the supply to 5 mM NH_4^+ (Table II). Nitrogen uptake by the ERM was therefore not proportional to nitrogen availability in the RFC. Correspondingly, concentration of Arg in the ERM increased from 2.1 to 6.2 mg g^{-1} fresh weight in response to external levels of NH_4^+ (Table II). In the nitrogen turnover study, exposure of the ERM to 5 mM $^{15}NH_4^+$ resulted in increases in Arg concentration from 1 to 3 μg mg^{-1} fresh weight over the 172-h labeling period (Fig. 3). Analysis of the amino acid composition of the ERM revealed that Arg was the most abundant amino acid present in the ERM (data not shown). The presence of L-norvaline (ASS inhibitor) or PPDA (urease inhibitor) slightly reduced Arg concentration in the ERM, but had no noticeable effect on nitrogen concentration (Table II).

When ^{15}N was added in the form of $(^{15}NH_4)_2SO_4$ to the RFC, the abundance of ^{15}N rapidly approached 25% to 30% in the ERM, illustrating that the fungus efficiently takes up NH_4^+ (Fig. 4). ^{15}N enrichment of roots was slower and reached a plateau corresponding to 7% ^{15}N after 80 h (Fig. 4). In particular, the ^{15}N abundance of Arg (ERM) rapidly increased to 75% to 80% and, hence, approached that of supplied nitrogen (Fig. 4). Interestingly, the distribution of the ^{15}N label of Arg in the ERM reached the statistical pattern already 12 h after the addition of the $(^{15}NH_4)_2SO_4$ to the RFC (Fig. 5).

The intramolecular distribution of the ^{15}N label was addressed using tandem mass spectrometry (MS/MS) analysis of selected isotopomers (e.g. mass-to-charge ratio [m/z] 177 corresponding to Arg labeled by two ^{15}N atoms). The MS/MS spectrum of m/z 177 reveals a number of fragments (Fig. 6). At low mass, abundant signals were found corresponding to the guanidinium moiety [i.e. m/z 60 ($CH_6{}^{14}N_3^+$) − m/z 63 ($CH_6{}^{15}N_3^+$)]. This pattern reflects the distribution of the label between the α-amino and guanidinium groups. Deviations from the statistical predicted pattern were observed only at the beginning of the experiments. The observed trend corresponds to deficiency of ^{15}N in the guanidinium group.

The rate of allocation of Arg from the RFC to the AM root compartment was determined from the equation $C_t = C_0 \exp(\lambda t)$. The concentration in the hyphae was taken as constant within the 24-h frame and, hence, the sum of unlabeled and labeled Arg remains equal to that of the initial concentration (C_0). Thus, the rate of allocation equals dC/dt, for $t = 0$, leading to $C_0\lambda = 0.13$ μg Arg mg^{-1} fresh weight h^{-1} (Fig. 7).

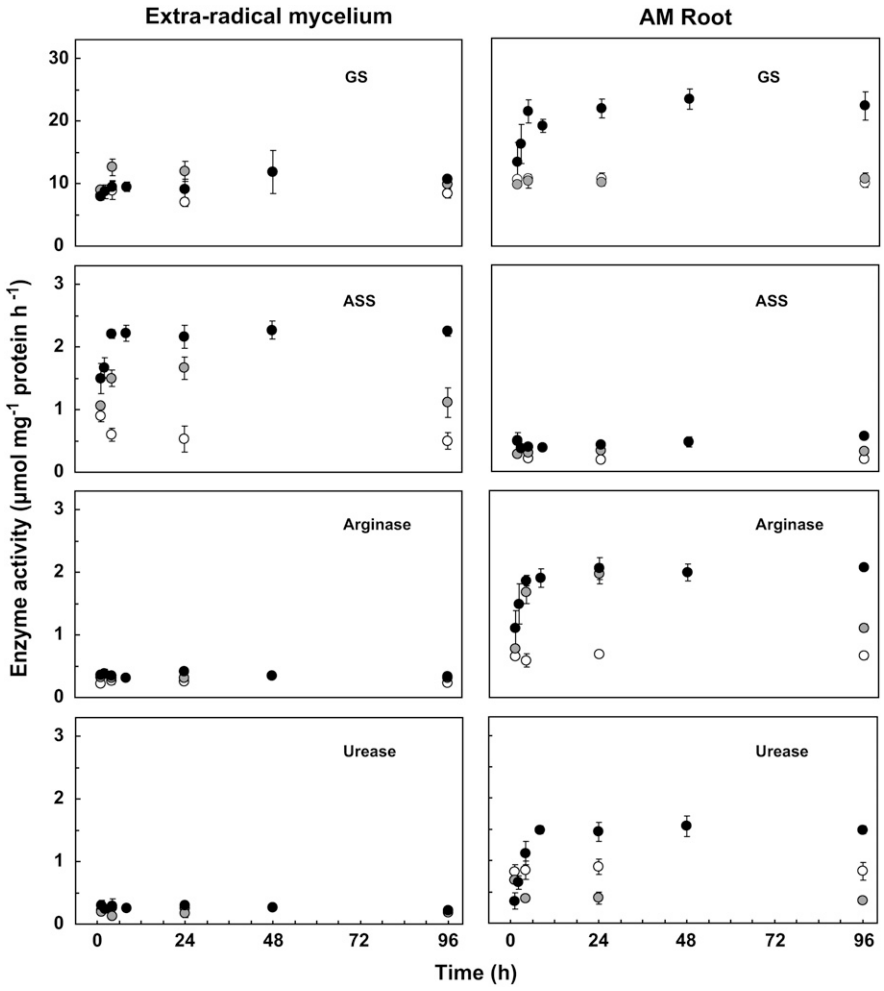

Figure 2. Specific enzyme activity in the ERM of *G. intraradices* and AM roots of carrot grown in an in vitro compartmented system after exposing the ERM to 5 mM $(NH_4)_2SO_4$ (black symbols), 5 mM $(NH_4)_2SO_4$ plus 20 mM L-norvaline (an inhibitor of Arg synthesis; white symbols), and 5 mM $(NH_4)_2SO_4$ plus 500 μM PPDA (an inhibitor of urease activity; gray symbols). Enzyme activity was normalized against protein concentration of the sample. Symbols with vertical bars represent means and SD ($n = 3$).

DISCUSSION

This study shows that enzyme activity of the catabolic arm of the urea cycle in roots colonized by *G. intraradices* is synchronized with enzyme activity of the anabolic arm in the ERM. Addition of NH_4^+ to the ERM induced increased activity not only of ASS in the ERM, but also of arginase and urease in colonized roots (see outline in Fig. 8). This synchronization resulted from translocation of Arg from the site of anabolism in the ERM to the site of catabolism in the IRM: Norvaline-induced inhibition of Arg synthesis in the ERM was accompanied by strong inhibition of arginase in colonized roots. Our combined time-course studies of enzyme activity and short-term ^{15}N-labeling patterns of Arg support a model describing nitrogen metabolism and translocation in AM fungi (Bago et al., 2001). Furthermore, our enzyme activity-based evidence for the importance of the urea cycle and for Arg translocation in AM fungi strongly complements previous evidence based on expression levels of putative genes of nitrogen metabolism and longer term

Table II. *Nitrogen and Arg concentrations in the ERM of G. intraradices grown in an in vitro compartmented system 96 h after exposing the mycelium in the RFC to the following treatments: A, none; B, 0.1 mM $(NH_4)_2SO_4$; C, 5 mM $(NH_4)_2SO_4$; D, 5 mM $(NH_4)_2SO_4$ + 20 mM L-norvaline (inhibitor of Arg synthesis); and E, 5 mM $(NH_4)_2SO_4$ + 0.5 mM PPDA (inhibitor of urease activity)*

Two individual samples were analyzed per treatment. n.d., Not determined.

	Concentrations in the ERM of *G. intraradices*				
Treatment	A	B	C	D	E
Nitrogen (% dry weight)	2.65	3.38	3.79	3.79	2.21
	2.86	3.72	n.d.	3.81	3.47
Arg (mg g^{-1} fresh weight)	2.03	3.08	6.20	5.30	4.82
	2.17	3.67	6.20	4.43	4.24

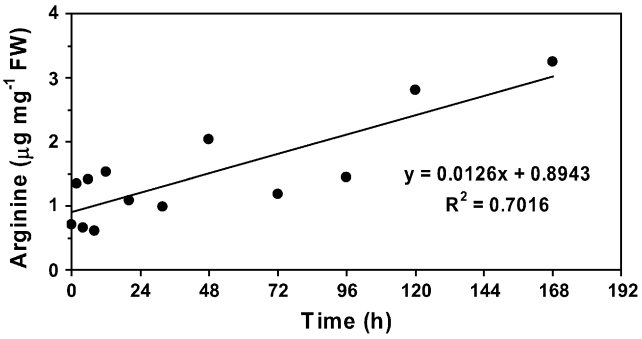

Figure 3. Time course of Arg concentration in the ERM of *G. intraradices* after exposure to 5 mM $(NH_4)_2SO_4$ in an in vitro compartmented root culture system.

^{15}N-labeling patterns of amino acids (Govindarajulu et al., 2005; Jin et al., 2005).

NH_4^+ Uptake and Synthesis of Arg by *G. intraradices*

The rapid ^{15}N enrichment of ERM proves its ability to take up NH_4^+, in agreement with previous studies using similar model systems (Bago et al., 1996; Toussaint et al., 2004) and with the identification of a high-affinity NH_4^+ transporter in *G. intraradices* (López-Pedrosa et al., 2006). Assimilation of inorganic nitrogen can follow either the route of the NADP-linked Glu dehydrogenase or the route of GS-Glu synthase. A key step in the transformation of assimilated nitrogen into Arg is the synthesis of the reactive intermediate carbamoyl phosphate. The enzymes catalyzing this series of reactions preferentially use Gln as a nitrogen source (Thoden et al., 1997). ^{15}N tracer studies and inhibition of nitrogen assimilation provided some evidence for the operation of a GS-Glu synthase enzyme system in AM fungi (Cliquet and Stewart, 1993; Johansen et al., 1996; Toussaint et al., 2004). Our results showed no relation between GS activity in the ERM and the external availability of NH_4^+ in the range 0 to 5 mM (Fig. 1), and are therefore in agreement with previous data for enzyme activity and transcript level of GS (Breuninger et al., 2004). However, under similar experimental conditions, expression of GS genes increased markedly in response to resupply of nitrogen to the fungal compartment (Govindarajulu et al., 2005). Because all endogenous or exogenous nitrogen sources are eventually converted to NH_4^+, a constitutive level of GS activity was to be expected, even in the absence of exogenous NH_4^+. In fact, GS activity tends to be higher under nitrogen starvation than in treatments where exogenous nitrogen was supplied (Jennings, 1995; Breuninger et al., 2004). This would imply that the relatively high levels of specific activity of GS observed in the absence of external NH_4^+ (Fig. 1) are sufficient to assimilate the additional NH_4^+ taken up at 5 mM.

In that case, the GS specific activity of the ERM would represent a potential for NH_4^+ assimilation and the actual rate of NH_4^+ metabolism would be better assessed by the activity of enzymes functioning downstream in the metabolic pathway. This interpretation is in agreement with the increase in specific activity of ASS in response to higher NH_4^+ concentrations in the RFC in contrast to GS (Fig. 1). The pattern of ASS specific activity reflects the content of Arg in the ERM as a function of NH_4^+ concentration in the RFC and the time course of Arg accumulation after exposure of the ERM to 5 mM NH_4^+ (Table II).

The fast and abundant incorporation of ^{15}N in Arg observed after the addition of $(^{15}NH_4)_2SO_4$ indicates that direct assimilation, as well as synthesis of the amino acids used by the urea cycle, is very efficient. The statistical distribution of the labeled N for Arg also reflects these conditions and implies that steady-state concentrations of the reactants Orn and Asp are low compared to the amount of NH_4^+ assimilated. The apparent deficiency of ^{15}N in the guanidinium group observed early in the labeling experiment indicates that the δ-amino and α-amino group of Orn and Asp, respectively, are reached relatively late by the label. These results are completely in line with observations by Jin et al. (2005), where Glu, Gln, and Asn were labeled to more that 90% after 1 week. Surprisingly, Asp was only labeled to a moderate degree. The reason for this deviation is not known.

Accumulation and Translocation of Arg in the ERM of *G. intraradices*

Patterns of ^{15}N enrichment and Arg accumulation in the ERM strongly suggest the involvement of Arg in NH_4^+ assimilation by the ERM (Figs. 3 and 4). If the high levels of Arg observed were homogeneously distributed in the cell, cytoplasmic Arg concentrations would be unrealistically high. However, in fungi, the majority (about 90%) of the basic amino acids are present in vacuoles (Messenguy et al., 1980). Vacuolar accumulation of Arg is regarded as a storage mechanism because it decreases when the cytoplasm

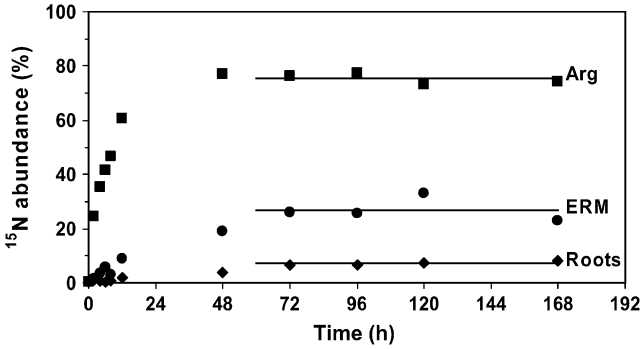

Figure 4. Time course of ^{15}N abundance in AM roots of carrot, in the ERM of *G. intraradices*, and in Arg from the ERM. The ERM was exposed to 5 mM $(NH_4)_2SO_4$ with 82% ^{15}N at 0 h. Solid lines indicate the stationary levels attained.

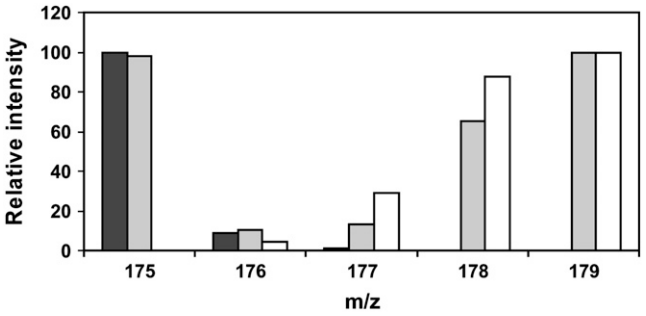

Figure 5. Isotopomer distribution ($^{15}N_0$ [*m/z* 175], $^{15}N_1$ [*m/z* 176], $^{15}N_2$ [*m/z* 177], $^{15}N_3$ [*m/z* 178], and $^{15}N_4$ [*m/z* 179]) of Arg in the ERM after 12 h (gray). The distribution is compared with that of unlabeled Arg (black) and the binomial distribution probability calculated on the basis of 82% ^{15}N (white).

is depleted (Davis, 1986). This compartmentation of Arg could be visualized based on the ^{15}N experiments where the labeled Arg rapidly approached a statistical distribution (Fig. 5). Based on the time dependency of the unlabeled Arg pool, and assuming a relatively slow rate of Arg transport from the IRM to the ERM, it was possible to estimate a net rate of Arg translocation from the ERM to the root compartment of 0.13 μg Arg mg^{-1} fresh weight h^{-1}, equivalent to 3 nmol nitrogen mg^{-1} fresh weight h^{-1} (Fig. 6). Such a high rate implies that Arg apparently plays a central role in nitrogen translocation by AM fungi. The high translocation rate of Arg from the ERM into the IRM is a key feature for the validity of the model proposed by Bago et al. (2001) for nitrogen translocation in AM symbiosis. It was highlighted by the enrichment of the AM root in ^{15}N (Fig. 4), which can only be provided from the ERM because only the ERM was in contact with the ^{15}N substrate. Jin et al. (2005) also found evidence of the transport of Arg from the ERM into the IRM, but the translocation rate was not estimated.

Catabolism of Arg in the IRM of *G. intraradices*

Any transfer of nitrogen from the IRM into root cells needs to occur in inorganic forms (Jin et al., 2005). The catabolic arm of the urea cycle is required for this purpose and arginase and urease were accordingly highly active in AM roots (Fig. 1). Arg catabolism in yeast is an adaptive mechanism that confers a hierarchy to nitrogen sources, allowing the most abundant to be used first (Lohse et al., 2005). Therefore, maximal arginase activity is seen only when Arg is present and NH_4^+ or other preferred nitrogen sources are absent. In this respect, the experimental design was ideal because the presence of NH_4^+only in the RFC would inhibit Arg catabolism in the external root mycelium, whereas the absence of ammonium in the AM root compartment would allow maximal activity of the catabolic arm of the urea cycle, as assessed by the specific activity of key enzymes involved in the pathway (Fig. 1).

However, enzyme activity in AM roots is more difficult to interpret than those obtained for the ERM because we are analyzing metabolic changes in a mixture of two distinct components, the IRM and the root tissue. Our use of cultures with well-developed mycorrhiza reduces the heterogeneity of the plant material. However, given the high root-to-fungus biomass ratio, it is likely that enzyme activity contributed by the IRM was underestimated. Nevertheless, the observation of concomitant changes in specific enzyme activity (Fig. 1) and in transcript levels of genes with high similarity to known Orn aminotransferase, urease accessory protein, and NH_4^+ transporters in the IRM (Govindarajulu et al., 2005) supports the involvement of the catabolic arm of the urea cycle. The increase in GS specific activity in roots with increasing NH_4^+ availability in the RFC (Fig. 1) would accordingly correspond to the metabolism of NH_4^+ produced during urea degradation in the IRM, after being transported into root cytoplasm.

Synchronization between ASS in the ERM and Arginase in the AM Root

According to our starting hypothesis, any event preventing synthesis of Arg in the ERM would result in decreased activity of the enzymes involved in Arg catabolism. In agreement with this, we show that inhibition of ASS activity due to exposure of the ERM to L-norvaline resulted in the inhibition of arginase and urease in the AM root in a separate compartment (Fig. 2). This could not have been caused by a direct effect of L-norvaline on arginase or urease in the roots (Table I), but only by decreased Arg production in the ERM and subsequent reduction in Arg supply to the IRM. The urease inhibitor had only a small effect on arginase activity; this may be related to the reposition of substrates or side effects of the inhibitor. These results highlight the necessary synchronization between the spatially separate anabolic and catabolic arms of the urea cycle, which allow the translocation of nitrogen through the fungal mycelium.

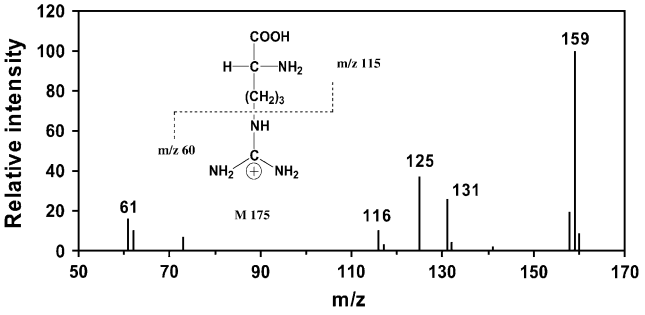

Figure 6. MS/MS analysis of $^{15}N_2$-Arg (*m/z* 177) in the ERM after 4 h. Principal fragmentations are indicated on the formula inserted; the *m/z* values indicated correspond to fragments observed from nonlabeled Arg (*m/z* 175).

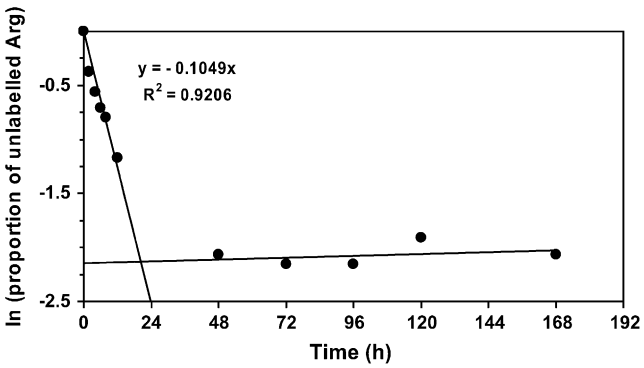

Figure 7. Time course of depletion of unlabeled Arg from the ERM of *G. intraradices* grown in an in vitro compartmented system. The ERM was exposed to 5 mM $(NH_4)_2SO_4$ with 82% ^{15}N at 0 h. The proportions of unlabeled Arg were log transformed.

Specific enzyme activity in the ERM and AM root as influenced by nitrogen availability in the medium leads us to suggest that regulation of the urea cycle involves a negative effect of external NH_4^+ availability on the catabolic arm in the ERM and a negative effect of internal NH_4^+ or a downstream metabolite, possibly Arg, on NH_4^+ uptake and assimilation in the IRM. Further evidence for synchronization between Arg synthesis in the ERM and degradation in the IRM is the pattern of ERM ASS, and AM root arginase and urease when the ERM was exposed to 0.1 mM nitrogen (Fig. 1). Their specific activity initially increased, then decreased toward activity at 96 h similar to that observed when the ERM received no nitrogen. This most likely resulted from nitrogen deficiency because such a pattern was absent from the 5 mM NH_4^+ treatment.

Assuming that both Arg and polyphosphate (poly-P) are translocated in vacuoles, it has been suggested that one may be counterion for the other (Jennings, 1995). Consequently, it seems feasible that the amount of Arg translocated could depend on the poly-P content of the hyphae and hence on the external inorganic phosphate (Pi) supply. We have previously used root culture model systems and in vivo ^{31}P-NMR to show that poly-P content in the ERM of *G. intraradices* increased 3.6- or 3.4-fold by increasing the external Pi supply from 40 to 200 or 5,000 μM (N. Viereck and I. Jakobsen, unpublished data). We investigated the possible influence of Pi status on Arg in the ERM in a separate experiment where the ERM was exposed to 5 mM $(^{15}NH_4)_2SO_4$ at 37, 370, or 3,700 μM KH_2PO_4 (data not shown). The Pi concentration had only moderate, if any, effect on the Arg concentration in the mycelium based on following values (means $\pm$ SE): 3.7 $\pm$ 0.3, 4.3 $\pm$ 0.5, and 5.2 $\pm$ 0.2 μg Arg mg^{-1} fresh weight for the 37, 370, and 3,700 μM KH_2PO_4 treatments. This is further supported by determining the ^{15}N content of Arg in the ERM as a function of Pi status. No differences in ^{15}N abundance could be found during exposure to 37, 370, and 3,700 μM Pi, which indicates that translocation of Arg is independent of Pi status, but does not disprove that Arg and poly-P are counterions.

Concluding Remarks

Although we have now achieved a more complete understanding of the mechanism by which AM fungi can contribute to plant nitrogen acquisition, there are several questions that persist and should be the focus of further research: (1) What is the counterion for Arg in the vacuole? (2) What is the fate of Orn produced in the IRM? (3) If ammonium is to be transported from the IRM to the root cytoplasm, the sites of NH_4^+ transfer should be enriched in NH_4^+ transporters; are these NH_4^+ transporters identical to those induced when NH_4^+ is taken up by the root? (4) Is this mechanism of NH_4^+ uptake by the ERM active when the AM root has direct access to NH_4^+? (5) Carbon limitation occurred and influenced Pi transport in mycorrhizal culture systems of similar design and age as used here (Bücking and Shachar-Hill, 2005); how

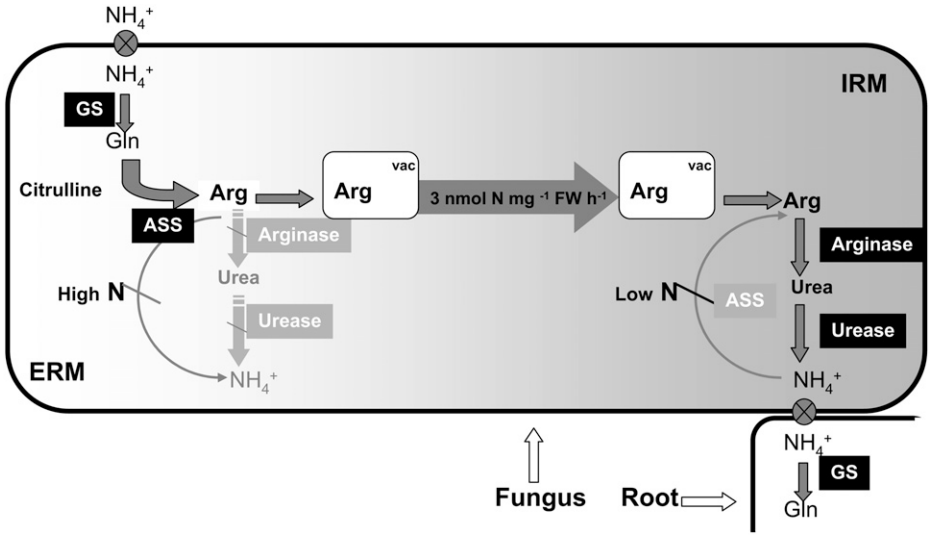

Figure 8. Schematic outline of the results obtained. Ammonium is taken up by the ERM and assimilated via GS into Gln. ASS is the key enzyme for the biosynthesis of Arg. The presence of ammonium in the ERM inhibits the activity of arginase and urease. Arg concentration in the cytoplasm is maintained by compartmentation into the vacuole, where Arg can be transported toward the IRM. The transfer of nitrogen from the ERM into the IRM was estimated as 3 nmol nitrogen g^{-1} fresh weight h^{-1}. The IRM is deficient in nitrogen, which favors the activity of arginase and urease leading to the production of ammonium, which is transferred to the root where it is integrated into Gln through the action of GS.

would changes in the carbon-to-nitrogen ratio in roots influence activity of key enzymes of nitrogen metabolism and rates of nitrogen transfer?

MATERIALS AND METHODS

Root and Fungal Cultures

Experimental systems consisted of Ri T-DNA (*Agrobacterium rhizogenes*)-transformed carrot (*Daucus carota*) roots colonized with *Glomus intraradices* Schenck and Smith (DAOM 181602). Root cultures were started from root segments already inoculated with *G. intraradices*. Two-compartment petri dishes (100-mm diameter, 15-mm depth) contained 20 mL of M medium (Bécard and Fortin, 1988) in the root + hyphae compartment and 20 mL of the same medium without Suc in the RFC. Petri dishes were incubated in the dark at 25°C and the phytagel of the RFC was heavily colonized by *G. intraradices* after 8 weeks. This phytagel was then removed and replaced by liquid M medium (14 mL) at double concentration and without Suc. The mycelium was allowed to colonize this medium over the subsequent 2 weeks. Petri dishes were examined regularly and roots were trimmed as required to prevent crossing into the RFC. Ten-week-old cultures with vigorous roots and densely colonized RFCs were selected for the experiments.

Activity of Enzymes in the ERM and AM Roots

GS, ASS, arginase, and urease activities in AM roots and the ERM were measured as a function of nitrogen availability in the RFC. Liquid medium in the RFC was replaced by fresh medium containing $(NH_4)_2SO_4$ at three levels: 0, 0.1, and 5 mM nitrogen. Cultures with the highest nitrogen level either remained untreated or were treated with inhibitors of ASS (20 mM L-norvaline) or urease (0.5 mM PPDA). Each of the five treatments had 32 replicate petri dishes, resulting in a total of 160. Root compartments received neither additional nitrogen nor inhibitors.

Four petri dishes per treatment were collected at each sampling time: 0, 1, 2, 4, 8, 24, 48, or 96 h after treatment initiation, or as otherwise indicated. Liquid medium was removed and the ERM was rinsed twice with miliQ water. The mycelium was collected, blotted on absorbent paper, weighed, and frozen at −80°C. AM roots removed from the phytogel were rinsed twice with miliQ water, blotted, weighed, and frozen as ERM samples.

AM roots and the ERM were used for determination of protein concentration (Bradford, 1976) and enzyme measurements. Root colonization by *G. intraradices* was determined on roots from 10 randomly selected cultures using a clearing and staining procedure modified after Phillips and Hayman (1970), omitting phenol from the reagents and HCl from the rinse. Two hundred intersects per sample were scored for the presence of intercellular hyphae, arbuscules, vesicles, and hyphal or arbusculate coils (McGonigle et al., 1990). Observations were performed with a microscope at 100-fold magnification.

Enzyme Assays

GS (EC 6.3.1.2) activity was determined by the synthetase assay (Magalhães and Huber, 1991). Roots and hyphae were ground in liquid nitrogen and extracted using 1 mL buffer to 10 mg biological sample. The extraction buffer (pH 8.0) contained 50 mM Tris-HCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 5 mM dithiothreitol, 10 mM $MgSO_4$, 1 mM cystein, 0.6% (w/v) polyvinylpyrrolidone, 1 mM reduced glutathione, and 5 mM Glu. Extracts were centrifuged at 35,000*g* for 15 min at 4°C and supernatants were used for the assay. The GS reaction mixture contained 3.8 μmol ATP, 5 μmol $MgSO_4$, 1.5 μmol hydroxylamine, 15 μmol L-Glu, and 10 μmol Tris-HCl in a final volume of 180 μL, pH 7.6. The reaction was started by adding 60 μL of enzyme extract and stopped after 30 min by the addition of 80 μL of ferric chloride reagent (4 mL $FeCl_3$ 10% [w/v]), 1 mL TCA 24% (w/v), and 500 μL HCl 2 mM in 6.5 mL water. After centrifuging at 5,000*g* for 10 min, the absorbance was read at 546 nm for each sample; a blank was made containing all the compounds, except that the ferric chloride solution was added before the enzyme extract to prevent any reaction. GS activity was assessed by the γ-glutamylhydroxamate produced per mg protein per hour, based on a γ-glutamylhydroxamate standard curve.

Other frozen subsamples were ground in liquid nitrogen and extracted, also using 1 mL of buffer to 10 mg of sample. The extraction buffer (pH 7.5) contained 100 mM Tris-HCl, 1% (v/v) 2-mercaptoethanol, and 0.1 mM phenylmethylsulphonylfluoride. Homogenates were centrifuged at 20,000*g* for 15 min at 4°C and the supernatants were used as the enzyme source for determination of ASS (EC 6.3.4.5), arginase (EC 3.5.3.1), and urease (EC 3.5.1.5) activity.

ASS was determined using 100 μL of enzyme extract in 100 μL of reaction mixture containing 50 mM HEPES, pH 7.5, 16 mM ATP, 30 mM citrulline, 90 mM Asp, and 5 mM $MgCl_2$. Reactions were incubated at 27°C for 60 min, stopped by addition of 70% TCA, and centrifuged at 7,000*g* for 2 min. ASS activity was determined by substrate disappearance (citrulline), which was colorimetrically determined at 490 nm by the reaction of diacetylmonoxime (McLean et al., 1965; Brasse-Lagnel et al., 2005). The reaction was performed under dark conditions. Citrulline calibration curves were constructed with different concentrations of urea.

Arginase activity was measured in a spectrophotometric assay for detection of urea, with minor modifications (Alabadí et al., 1996). The enzyme solution was activated with 1 mM $MnCl_2$ at 37°C for 40 min. The reaction mixture (250 μL) contained 10 μL of the enzyme source in assay buffer (50 mM CHES buffer [pH 9.6], 250 mM L-Arg, 2 mM $MnCl_2$). Reactions were carried out at 37°C for 20 min and stopped by the addition of 100 μL of 15% (v/v) perchloric acid. A 100-μL aliquot was mixed vigorously with 1.5 mL of acid mixture (9% [v/v] of phosphoric acid and 27% [v/v] of sulfuric acid) and 50 μL of 3% (w/v) α-isonitrosopropiophenone (Sigma) in 95% ethanol. This mixture was heated at 95°C for 60 min in a water bath placed in the dark and then cooled for 10 min to room temperature. Absorbance was read at 535 nm. Arginase activity was calculated on the basis of urea accumulation in the reaction medium.

Urease activity was determined using 100 μL of enzyme extract in 100 μL 0.1 M Tris-maleate buffer containing 1 mM EDTA (pH 7.0) and 50 mM urea, according to Miksch and Eberhardt (1994). Samples were incubated for 30 min at 37°C, and NH_3 concentration was determined spectrophotometrically by modification of the Berthelot reaction (Willis et al., 1993; Yang et al., 1998).

Specificity of Inhibitors of ASS and Urease

Additional 12 dishes were maintained for 72 h for testing inhibitor specificity on extracts of roots and hyphae. AM roots and the ERM were used to determine protein concentrations, and GS, ASS, arginase, and urease activity in the (1) absence of inhibitors; (2) presence of 20 mM L-norvaline; and (3) presence of 0.5 mM PPDA. L-Norvaline or PPDA was added to the extraction medium and allowed to incubate for 30 min at 25°C in the dark, after which enzyme activity was determined as described above.

^{15}N-Labeling Study to Analyze Turnover of Arg in the ERM

Monoxenic cultures of carrot roots and *G. intraradices* were established as described above. After establishment of the ERM for 2 weeks in liquid M medium, the medium was replaced by fresh medium containing 5 mM $(NH_4)_2SO_4$ of 82% ^{15}N enrichment and triplicate cultures were harvested sequentially over the subsequent 168 h. Mycelium and roots from one replicate culture were dried and total nitrogen and ^{15}N abundance were determined on an isotope ratio mass spectrometer (Finnigan MAT Delta E; Thermo Electron) coupled to an EA 1110 elemental analyzer (Thermo Electron).

Amino Acid Extraction and Analyses

The free amino acid pool was extracted from frozen samples of the ERM and AM root using the procedure described by Johansen et al. (1996). The frozen ERM samples (5–10 mg) received 300 μL of the extraction solution (MeOH:$CHCl_3$:water 12:5:3 [v/v/v]) in an Eppendorf vial. The sample was kept at ambient temperature for 1 h followed by sonication for 15 min; 150 μL of water was added after an additional hour and repeated sonication (15 min). The tube was vortexed and centrifuged (2,000*g*, 2 min) to facilitate phase separation. Root samples were extracted in a similar way, except that sample sizes of 0.5 to 1.0 g were used in connection with 1-mL extraction solution. Phase separation was obtained by the addition of 500 μL of water. The methanol-water phase was isolated and directly analyzed by liquid chromatography (LC)-MS.

Stable isotope dilution analysis was performed by using an extraction solution containing approximately 50 μg mL^{-1} of $^{15}N_2$-Arg (minimum 98 atom% ^{15}N) obtained from Isotec (Sigma).

LC-MS analyses were carried out using a LCQ (Classic) MS^n system (Finnigan) bundled with an electrospray ionization source and a complete Thermo Separation Products HPLC system.

Underivatized amino acids were separated using a Purospher RP-18e (125 × 4 mm, 5 μm) column (Merck) with 0.1% (v/v) nonafluoropentanoic acid/acetonitrile gradient at ambient temperature and a flow rate of 0.50 mL/min. In all cases, a sample size of 20 μL was used. The LCQ system was operated in the low mass mode (m/z 10–200) with a maximal ion injection time of 200 ms. Source parameters: spray voltage, 4.5 kV; heated capillary, 200°C; sheath gas flow rate, 80; and auxiliary gas flow rate, 10 (arbitrary units).

The ^{15}N content of Arg was determined using single ion monitoring of the protonated amino acid [i.e. m/z 175 ($^{15}N_0$), m/z 176 ($^{15}N_1$), m/z 177 ($^{15}N_2$), m/z 178 ($^{15}N_3$), and m/z 179 ($^{15}N_4$)]. The content of Arg in the ERM and AM root material was determined by stable isotope dilution analysis based on $^{15}N_2$-Arg. The ion current at m/z 175 and m/z 177 was acquired by single ion monitoring for this purpose.

The isotopomers of Arg (i.e. m/z 175, m/z 176, m/z 177, m/z 178, and m/z 179) was investigated by MS/MS. The ion in question was trapped with an isolation width of 1.5 units and activated by collisions with He using a relative amplitude of 22% for 30 ms. In all cases, a stabilizing Q value of 0.25 was used.

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