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Different nitrogen sources modulate activity but not expression of glutamine synthetase in arbuscular mycorrhizal fungi

Magdalene Breuninger,[a] Carmen G. Trujillo,[a] Esther Serrano,[a] Reinhard Fischer,[b] and Natalia Requena[a,*]

[a] *Physiological Ecology of Plants Department, University of Tübingen, Auf der Morgenstelle 1, Tübingen 72076, Germany*
[b] *Department of Microbiology, University of Marburg and Max-Planck-Institute for Terrestrial Microbiology, Karl-von-Frisch-Str., Marburg D-35043, Germany*

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Abstract

Glutamine synthetase (GS) is a central enzyme of nitrogen metabolism that allows assimilation of nitrogen and biosynthesis of glutamine. We isolated the cDNA encoding GS from two arbuscular mycorrhizal fungi, *Glomus mosseae* (*GmGln1*) and *Glomus intraradices* (*GiGln1*). The deduced protein orthologues have a high degree of similarity (92%) with each other as well as with GSs from other fungi. *GmGln1* was constitutively expressed during all stages of the fungal life cycle, i.e., spore germination, intraradical and extraradical mycelium. Feeding experiments with different nitrogen sources did not induce any change in the mRNA level of both genes independent of the symbiotic status of the fungus. However, GS activity of extraradical hypahe in *G. intraradices* was considerably modulated in response to different nitrogen sources. Thus, in a N re-supplementation time-course experiment, GS activity responded quickly to addition of nitrate, ammonium or glutamine. Re-feeding with ammonium produced a general increase in GS activity when compared with hyphae grown in nitrate as a sole N source.

Index descriptors: Mycorrhiza; Glutamine synthetase; Nitrogen metabolism; Symbiosis; Plant–fungal interactions

1. Introduction

The arbuscular mycorrhizal (AM) symbiosis formed between a group of soil fungi (the Glomeromycota) and the root of most vascular plants (Smith and Read, 1997) is characterized by the bi-directional exchange of nutrients between both symbiotic partners. The fungus colonizes the root cortex while extending also an extraradical mycelium into the soil. In that way a mycelial network is created that provides the plant with mineral elements located beyond the depletion area around the root. In turn, the plant provides its mycobiont with carbohydrates allocated to the root. Mycorrhizal plants have therefore an improved mineral nutrition in comparison to non-mycorrhizal plants. Although the role of AM fungi in improving the phosphate nutrition of their host plants has been extensively studied, much less is known about their contribution to the nitrogen nutrition. Although in a lower amount than phosphorus, nitrogen is taken up from the soil by the fungus and transferred to the host in substantial proportions. In contrast, the transfer of nitrogen from the host plant to the fungus and hence towards the soil is insignificant (Johansen et al., 1992, 1993). Therefore, in undisturbed soils the external mycelium of AM fungi play an important role in the turnover of inorganic N by competing efficiently with other soil microorganisms (Johansen et al., 1996). The nitrogen forms taken up from the soil by the AM extraradical mycelium are variable. Ammonium uptake is well documented but uptake of nitrate and amino acids has also been shown (Hawkins et al., 2000; Johansen et al., 1992, 1994a,b; Tobar et al., 1994). In particular, nitrate uptake might be of great significance in dry soils where the mobility of this ion might be largely reduced (Tobar et al., 1994). Once

* Corresponding author. Fax: +49-7071-295635.
E-mail address: natalia.requena@uni-tuebingen.de (N. Requena).

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inside the fungal cell, this nitrogen is possibly assimilated to satisfy internal demands prior to its transfer to the host plant. Asparagine, arginine, glutamate and glutamine are the most abundant amino acids present in extraradical mycelium of AM fungi (Bago et al., 1999; Johansen et al., 1996) and it is likely that the translocation of nitrogen towards the plant takes place in the form of these amino acids.

The assimilation of inorganic nitrogen into amino acids is a biochemical process critical to most soil microorganisms. The incorporation of ammonium (or nitrate after reduction to ammonium) into amino acids can follow either the route of the NADP-linked glutamate dehydrogenase (NADP–GDH) or the route of the glutamine synthetase-glutamate synthase (GS–GOGAT). NADP–GDH incorporates ammonium to a molecule of 2-oxoglutarate to produce a molecule of glutamate. In the GS–GOGAT pathway, GS incorporates first a molecule of ammonium into glutamate to produce glutamine and later the GOGAT enzyme converts glutamine and 2-oxoglutarate to two molecules of glutamate. Previous experiments using enzymatic methods showed that glutamine synthetase (GS) is possibly the main enzyme responsible for N assimilation in AM fungi (Smith et al., 1985). However, besides the isolation and characterization of a nitrate reductase enzyme (Kaldorf et al., 1994, 1998) no other protein or genes involved in the nitrogen assimilation pathway have been identified so far in these fungi.

In this work, we have isolated the full-length cDNA coding for glutamine synthetase from the arbuscular mycorrhizal fungus *Glomus mosseae* and named it *GmGln1*. We have also isolated a partial cDNA coding for GS from another AM fungus, *Glomus intraradices* (*GiGln1*). We have studied the expression of both genes during the symbiotic cycle and in response to different nitrogen sources. Here, we present evidence that in arbuscular mycorrhizal fungi GS is not regulated at the transcriptional level but post-transcriptionally in response to different nitrogen sources.

2. Materials and methods

2.1. Fungal strains

Glomus mosseae (BEG12) inoculum was purchased from Biorize (Dijon). Sporocarps were collected after wet-sieving and surface-sterilized according to Budi et al. (1999). They were germinated on Mes buffered water–agar plates as described elsewhere (Requena et al., 1999) and grown in an incubator at 27 °C in the dark. Ri T-DNA transformed carrot (*Daucus carota* L.) roots colonized by *Glomus intraradices* (line DC-1) were grown on modified M medium (Bécard and Fortin, 1988). The culture was maintained at 27 °C and transferred to fresh medium every two months.

2.2. Isolation of the full-length cDNA encoding GmGln1 by RACE

A partial cDNA sequence found in an expressed sequence tags (ESTs) library from germinated sporocarps of *G. mosseae* showed a strong similarity to the glutamine synthetase gene from several other fungi. In order to obtain the full-length cDNA sequence, germinated *G. mosseae* sporocarps were harvested after 10 days using fine forceps to recover all hyphae and immediately frozen in liquid nitrogen. Total RNA (100 ng) was extracted using the RNA easy kit (Qiagen). The corresponding full-length cDNA was obtained by rapid amplification of cDNA ends (RACEs) using the SMART RACE cDNA amplification kit (Clontech). Two pairs of primers based on the sequence of the original cDNA fragment were used GmGlnF1 5′-ccaagttggtccatgtgaagg-3′, GmGlnF2 5′-ttgatggttcttcaactaatcaagctc-3′, GmGlnR1 5′-ttgagcggcaacatgtcttgg-3′ and GmGlnup2 5′-gagcttcaacgacgtcgcgtcc-3′. The PCR reactions were performed using the proof-reading enzyme Advantage 2 (Clontech). Two overlapping fragments were obtained and directly cloned into the pCR2.1 TOPO vector and sequenced (MWG Biotech). A similar procedure was used to isolate the orthologue gene from *G. intraradices* using the *G. mosseae* primers GmGlnR1, GmGlnF2, and GmGlnF1 as degenerated primers.

2.3. Southern blot analysis

Genomic DNA from *G. intraradices* axenic hyphae grown in vitro was extracted using the Qiagen Plant DNAeasy kit. 15 μg of genomic DNA were digested overnight with *Eco*RI and electrophoresed in 0.8% agarose. The gel was blotted onto a nylon membrane and the membrane was hybridized with a radioactive labelled probe. The probe was generated by random labelling with Klenow enzyme (Sambrook et al., 1989) using as template plasmid DNA from *GiGln1* (993 bp).

2.4. Expression of GmGln1 during the fungal life cycle

The expression of the glutamine synthetase gene was studied during all different phases of the fungal life cycle, i.e., asymbiotic growth, in planta phase, extraradical mycelium. Mycorrhizal plants were established by inoculating parsley (*Petroselinum crispum*) seedlings with *G. mosseae* and grown in pots divided into compartments separated by a thin nylon mesh (50 μm) which allowed the passage of extraradical hyphae but not of roots. The hyphal compartment contained 2 mm glass beads from which the extraradical mycelium could be easily recovered. Plants were grown for two months in a phytochamber under controlled conditions of light (150 $\mu E\, m^{-2}\, s^{-1}$), temperature (20 °C), and photoperiod

(14 h light). Extraradical hyphae were then isolated from the glass bead compartment, washed several times in chilled sterile distilled water and frozen in liquid nitrogen. Colonized roots, free of extraradical hyphae were also collected, washed and immediately frozen in liquid nitrogen. Total RNA from germinated sporocarps (as described above), mycorrhizal roots and extraradical hyphae was extracted using the RNAeasy kit (Qiagen) and DNAase treated using the DNAse I enzyme from Invitrogen. RNA quality and quantity was assessed by Northern-blot using a DIG-labelled probe (Roche) containing the ribosomal genes from *G. mosseae* (Franken and Gianinazzi-Pearson, 1996). To calibrate the amount of fungal material inside the mycorrhizal plant, single stranded cDNA was synthesized from 50 ng of total RNA from spores and hyphae and from aproximately 250 ng of total RNA from mycorrhizal roots using hexamer oligonucleotides according to the protocol of the Superscript II Reverse Transcriptase from Gibco (Invitrogen). Calibration was carried out by PCR in the sscDNA synthesized from samples S, H, and M using the oligonucleotides VAGLO and VANS1 specific for the 18S rRNA gene from *G. mosseae*. One microliter of a 1:10,000 dilution of the ss cDNA was used as template.

cDNA for RT-PCR analyses of Gln1 expression was generated using oligo(dT) as primer according to the protocol of the Superscript II Reverse Transcriptase from Gibco (Invitrogen). Transcript levels were estimated by PCR using the primer combination GmGlnF2 (shown above) and GmGlnR0 5′-tccttcacatggaccaacttg gt-3′.

2.5. Nitrogen feeding experiment with germinated sporocarps

Sporocarps from *G. mosseae* were germinated on Mes buffered water–agar plates. After 12 days germinated sporocarps were transferred to Mes buffered water agar plates added of ammonium nitrate (2 mM), glutamine (10 mM) or to control plates with no N source. Sporocarps were harvested after 48 h using fine forceps to recover all hyphae and immediately frozen in liquid nitrogen. RNA was extracted using the Qiagen RNAeasy kit and cDNA and RT-PCR analyses performed as described above.

2.6. Expression of GmGln1 in mycorrhizal plants grown under different N regimes

Lettuce plants (*Lactuca sativa* L.) were inoculated with *G. mosseae* (BEG12) inoculum (including mycorrhizal roots, and spores) from Biorize (France) and grown in 500 ml pots containing the calcinated substrate terra-green. The substrate was rinsed thoroughly with tap water and autoclaved before use. Plants were daily watered with distilled water and received 100 ml of a complete Long–Ashton nutrient solution (Hewitt, 1966) at 1/10 of phosphate once a week. Plants were grown for 45 days in a phytochamber under controlled conditions of light (150 $\mu E\, m^2\, s^1$), temperature (20 °C) and photoperiod (14 h light). After this time, plants were repeatedly watered with distilled water for 1 week to wash out the remaining nutrient solution. One third of the plants (10) received then a complete Long–Ashton nutrient solution containing 5 mM of nitrate as a sole nitrogen source; another third received 5 mM of ammonium as a sole nitrogen source every two days while the rest was watered with nutrient solution lacking any nitrogen source (control). After 20 days, plants were harvested and mycorrhizal colonization was assessed by trypan blue staining (Phillips and Hayman, 1970). Roots were immediately frozen in liquid nitrogen after thorough tap water washing. Total RNA was extracted from control, NH_4^+ and NO_3^- fertilized mycorrhizal roots using TRIzol (Invitrogen). cDNA from both treatments was generated as explained above and the expression of *GmGln1* analysed by RT-PCR.

2.7. Time-course experiment of extraradical mycelium nitrogen feeding

The transformed mycorrhizal culture DC1 was established in bi-compartmental petri plates where *G. intraradices* mycelium was allowed to develop in a plant-free compartment containing liquid M medium (Bécard and Fortin, 1988). After 2 weeks of growth, the mycelium was washed three times using sterile M medium solution free of nitrogen and starved for N during two days. Then, one third of the plates (16) received fresh M medium (5 mM NO_3^-), another third received a modified M medium where nitrate was replaced by ammonium (5 mM) or by glutamine (10 mM). Mycelium was then harvested from the three treatments after 24, 48, 72, and 96 h. The mycelium was frozen in liquid nitrogen and RNA extracted using TRIzol (Invitrogen). cDNA was constructed using oligo(dT) as explained above.

2.8. Time-course GS enzyme activity in extraradical mycelium

A similar experiment set up as described above was used to assess GS activity in mycelium of *G. intraradices*. Hyphae were allowed to grow in liquid M medium for two weeks and then washed three times with the same solution free of N. Five plates were collected at this time before washing. After two days of N starvation, five plates corresponding to the control at time 0 were harvested and the rest of plates received three different N treatments: nitrate (5 mM), ammonium (5 mM), and glutamine (10 mM). Hyphae from five plates from each treatment were pooled and harvested at 24, 48, 72, and

96 h and frozen at −80 °C in protein extraction buffer. This buffer contained: 100 mM maleic buffer KOH, pH 6,8; saccharose 100 mM; β-mercaptoethanol 2% (v/v); ethylenglycol 15% (v/v); 5% polyvinylpolypyrolidone (PVPP); 1.5 mM PMSF. Samples were grinded in a mortar using liquid N_2. After homogeneization samples were centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatant transferred to eppendorf cups. Protein content was estimated according to Bradford using the BioRad protein assay. GS activity was estimated using the transferase method (Shapiro and Stadtman, 1970) in a reaction mixture as described in Smith et al. (1985). GS activity is expressed as specific activity in Units per mg of protein. One unit is defined as the amount required to catalyze the synthesis of 1 μmol of γ-glutamylhydroxamate per minute. Measurements were carried out in triplicate and the experiment was conducted twice. The same samples were used to determine NAD– and NADP–GDH (glutamate dehydrogenase) enzyme activity according to the protocol described by Pierleoni et al. (2001).

3. Results

3.1. Isolation of GmGln1

A cDNA fragment whose translation displayed high similarity to glutamine synthetases from other fungi was found in a cDNA library of germinated *G. mosseae* spores. The full-length cDNA (1615 bp) was obtained by 5′ and 3′ RACE technique using specific primers based on the known sequence (Fig. 1). The sequence around the putative start codon (GAAAA*AUG* GC) is almost identical to the consensus sequence for filamentous fungi (CAAAA*AUG*GC) (Ballance, 1986). Two in frame stop codons (TAA) located upstream of the ATG were found. The untranslated upstream and downstream sequences had a very low GC content of about 20% while the ORF had a GC content of 41% which is characteristic for AM fungi (Hosny et al., 1999). The 3′ untranslated sequence contained 337 nucleotides including several potential polyadenylation sequences (AAUAA). A partial cDNA (24 nucleotides missing to the putative ATG) coding for the orthologue gene in *Glomus intraradices* was also isolated (see Section 2).

The deduced amino acid sequence of the *G. mosseae* GS contains 354 aa including conserved signatures characteristics of the glutamine synthetase family to contribute to the activity of the enzyme (Kumada et al., 1993). The glutamine synthetase signature 1, including the conserved tetrapeptide DGSS located in the N-terminal part of the enzyme, as well as the putative ATP-binding region are boxed in Fig. 1. The active sites/catalytic residues strictly conserved among prokaryotic and eukaryotic GS with key roles in the catalysis or binding of substrates and other ligands were also found in GmGln1 and are indicated in Fig. 1 (Eisenberg et al., 2000). The calculated molecular mass of deduced GmGln1 protein is 39.15 kDa, which is in concordance with the monomer size of the typical eukaryotic enzyme GS type II (Eisenberg et al., 2000; Mathis et al., 2000). The protein deduced from the sequence of *GiGln1* partial cDNA is 92% homologue to GmGln1, including the conserved signatures of GS. Protein alignment of the two arbuscular mycorrhizal GS together with three ectomycorrhizal GS is shown in Fig. 2.

Comparative alignment of the GmGln1 and GiGln1 proteins with 15 other fungal glutamine synthetase sequences using the bacterial sequence from *Escherichia coli* as outgroup, showed that the endomycorrhizal proteins are highly conserved. This is in agreement with the attributed role of GS as molecular clock (Kumada et al., 1993; Pesole et al., 1991). The phylogenetic tree generated from the sequence alignment showed several main clusters of glutamine synthetase proteins corresponding to filamentous ascomycetes, yeast ascomycetes and basidiomycetes (Fig. 3A). Interestingly, *G. mosseae* and *G. intraradices* glutamine synthetases emerge from the same branch originating the basidiomycete cluster. An alignment of putative 14-3-3 interaction motifs from all these proteins (according to the motifs found in plant GS known to interact with these proteins) is presented in Fig. 3B, where the fourth amino acid would represent the phosphorylation site.

Southern blot analysis revealed that *GiGln1* is present as a single copy gene in the genome of *G. intraradices* (Fig. 3C), in concordance with the situation found in most fungi with the exception of *Neurospora crassa* where two different polypeptides (α and β) form the functional GS (Sánchez et al., 1980).

3.2. Gene expression analyses

GmGln1 expression is not highly regulated. RT-PCR analyses revealed that the *GmGln1* transcript was readily detected under all conditions tested. Transcript levels were similar during different stages of the life cycle, i.e., pre-symbiotic mycelia, extraradical hyphae, and intraradical mycelia (Fig. 4A). Similarly, levels of GS gene expression during pre-symbiotic growth remained constant independent of the nitrogen source supplied (Fig. 4B). In mycorrhizal roots, expression of *GmGln1* was similar between mycorrhizal plants treated with nitrate, ammonium or no nitrogen fertilization (Fig. 4C). Levels of mycorrhizal colonization were similar in all treatments as revealed by trypan blue staining (ca. 40% of root colonization).

The time-course expression of *GiGln1* in extraradical hyphae growing on different nitrogen sources showed also no difference in transcript levels. Neither time of incubation, neither nitrogen source (ammonium, nitrate

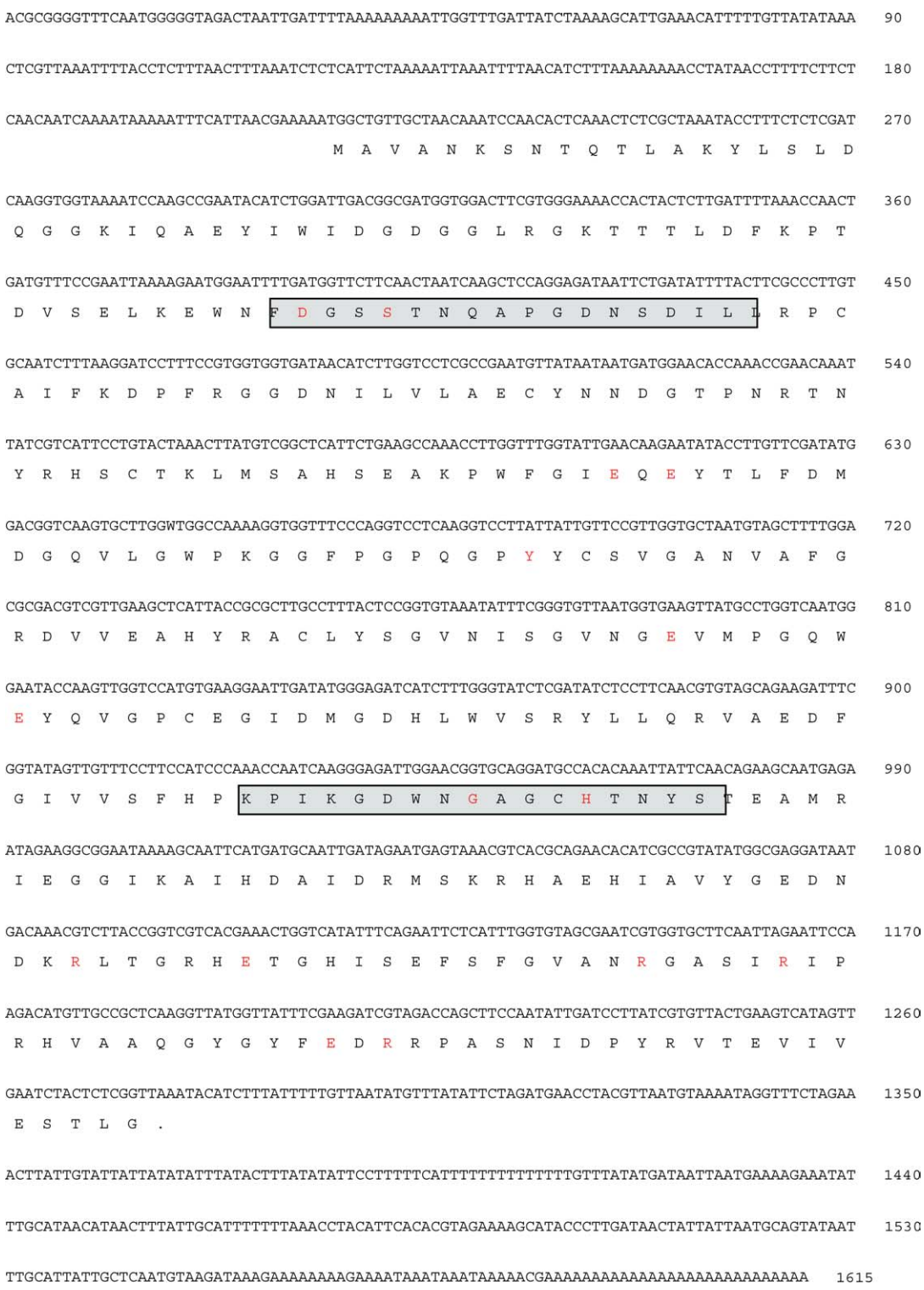

Fig. 1. Full-length cDNA sequence of *GmGln1* with the deduced GS protein. Boxes indicate the conserved signatures of GS type II characteristic of eukaryotes. The active site/catalytic residues, strictly conserved among prokaryotic and eukaryotic glutamine synthetases, are marked in red. These residues play key roles in the catalysis or binding of substrates and other ligands according to Eisenberg et al. (2000).

or glutamine) produced any significant change in expression as revealed by RT-PCR (Fig. 4D). Expression of the GS gene in hyphae grown for 96 h with glutamate (usually an inducer of GS) was also analyzed and similarly no change in the transcript level of *GiGln1* was observed.

Fig. 2. Amino acid alignment of the arbuscular mycorrhizal GS, GmGln1, and GiGln1, with two ectomycorrhizal GS from *T. borchii* and *H. cylindrosporum*. Amino acids shaded in grey represent conserved residues. The alignment was done with the MegAlign program of the DNAstar software package (Lasergene).

3.3. GS enzyme activity in AM mycelia grown under different N sources

In contrast to the expression levels of the GS gene, GS activity was considerably modulated upon the nitrogen source supplied to the extraradical hyphae. After 2 days of nitrogen starvation GS activity almost doubled. Re-supplementation of nitrate or glutamine to the growing mycelia resulted in an overall decrease of the GS activity, which finally returned to basal levels after 96 h. Interestingly, glutamine re-feeding after 24 h produced a temporal but dramatic increase in the GS activity. In contrast, ammonium re-feeding produced an increase of the specific GS mycelial activity with respect to hyphae grown on nitrate. Hyphae growing in ammonium had also a reduced growth rate when compared hyphae grown with nitrate or glutamine (Fig. 5). Measurement of NAD and NADP–GDH enzyme activity were also carried out in the same samples and very low or no activity could be detected even when using three times more protein than for GS activity measurements (data not shown).

4. Discussion

AM fungi can take up, assimilate, and translocate nitrogen to their host plants in a much significant

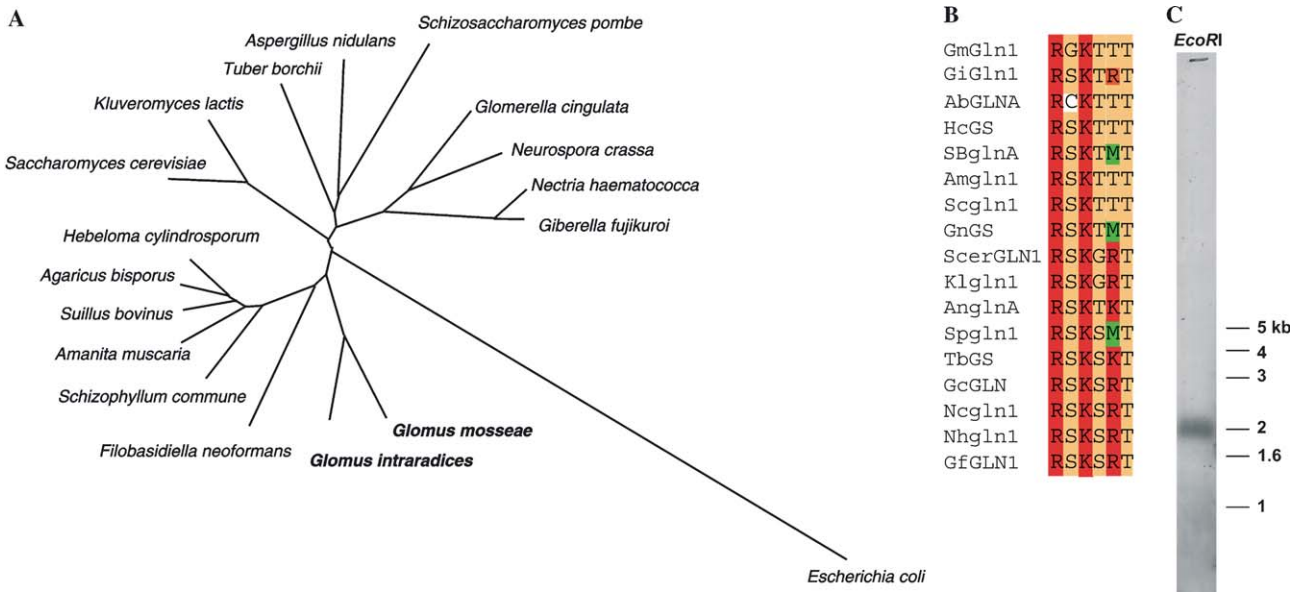

Fig. 3. (A) Phylogenetic relationships among fungal glutamine synthetases. A radial phylogenetic tree was constructed with the neighbor-joining algorithm implemented on the computer program ClustalX, on the basis of the GmGln1 and GiGln1 amino acid sequences together with other 14 sequences corresponding to different fungi. The sequence of *E. coli* was include as an outgroup; branches are drawn to scale (the scale bar correspond to 0.1 changes per site). The accession numbers of the sequences used in the alignment are: AY360451 (GmGln1 *G. mosseae*); P06711 (GlnA *E. coli*); AF462037 (*T. borchii*); O00088 (*A. bisporus*); CAD22045 (*A. muscaria*); AAK70354 (*A. nidulans*); CAD10037 (*Filobasidiella neoformans*); CAC27836 (*Giberella fujikuroi*); Q12613 (*Glomerella cingulata*); AAK96111 (*H. cylindrosporum*); AAD52617 (*Nectria haematococca*); NCU06724.1 (*N. crassa*); P32288 (*S. cerevisiae*); NP_593400 (*Schizosaccharomyces pombe*); AAF27660 (*Schizophyllum commune*); CAD48934 (*Suillus bovinus*). (B) Alignment of the putative 14-3-3 interacting motif from all the fungal GS sequences represented in the tree above. Color code indicates functional classification of amino acids; red is basic, beige is polar; green is hydrophobic. (C) Genomic Southern blot of *G. intraradices* digested with *Eco*RI and probed with a ^{32}P-labelled cDNA fragment from *GiGln1*.

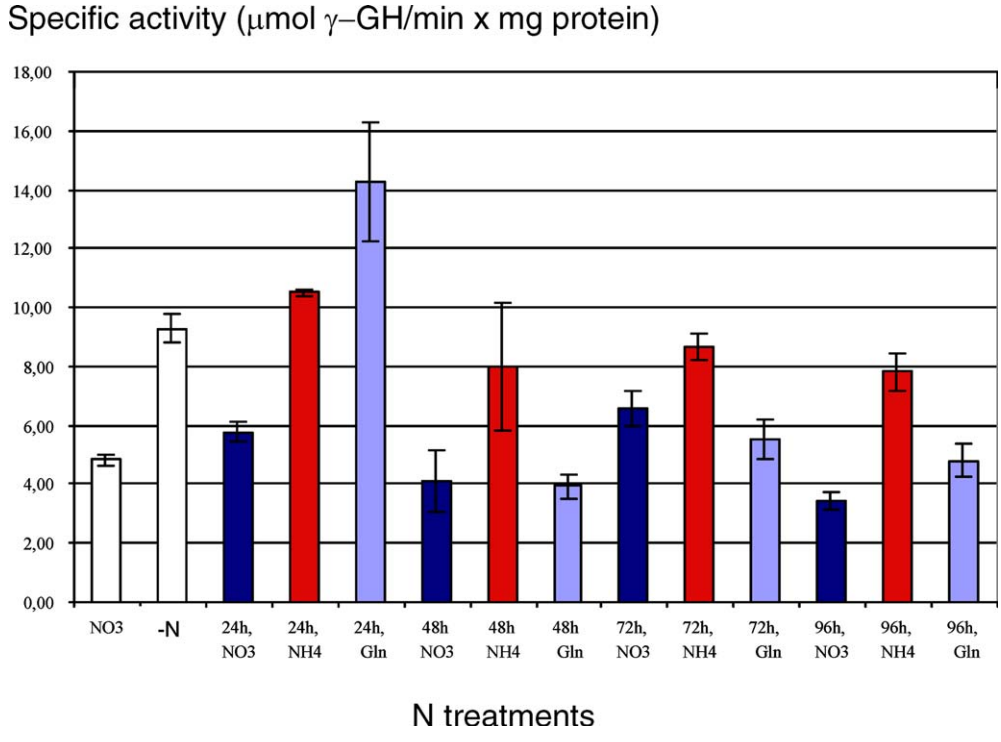

Fig. 5. Glutamine synthetase activity determined by the transferase method in extraradical hyphae of *G. intraradices* from in vitro cultures. Activity was compared between cultures starved for two days (N) and re-supplemented with different N sources (5 mM NO_3^-, 5 mM NH_4^+ or 10 mM Gln) over a time course of 96 h. Activity was expressed as specific activity in micromoles of γ-glutamylhydroxamate per minute and per mg of protein.

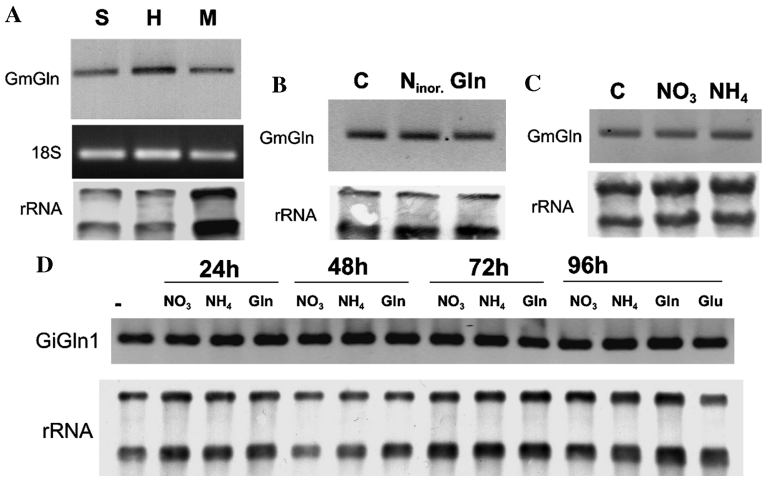

Fig. 4. Expression analyses of *GmGln1* and *GiGln1* analyzed by RT-PCR. (A) Expression of *GmGln1* during different stages of the *G. mosseae* life cycle, i.e., spore germination (S), extraradical hyphae (H), and intraradical hyphae in mycorrhizal roots (M). Samples were calibrated according to the fungal 18S rRNA to contain similar amounts of fungal mRNA. (B) Expression of *GmGln1* during spore germination on media containing either no nitrogen (C) or inorganic nitrogen (N_{inor}, 2 mM NH_4NO_3) or organic nitrogen (10 mM Gln). (C) Expression of *GmGln1* in intraradical hyphae from mycorrhizal roots of *L. sativa* exposed to three different nitrogen fertilization regimes (C, no nitrogen, 5 mM NO_3^- or 5 mM NH_4^+). (D) Time-course expression of *GiGln1* in extraradical hyphae of *G. intraradices* from in vitro cultures. Hyphae were starved for N during two days and at that time *GiGln1* expression was studied and compared with expression after re-supplementation with different N sources (5 mM NO_3^-, 5 mM NH_4^+, 10 mM Gln or 10 mM Glu) over a period of 96 h.

proportion than the flux in the opposite direction (Johansen et al., 1992, 1993). Therefore, the symbiosis with these fungi might represent, depending on the ecological circumstances, an important mechanism for plants to cope with nitrogen deficient situations such as spatial unavailability. The mechanisms of uptake, assimilation, and translocation of different nitrogen sources are however still very obscure. Evidence for the uptake of ammonium as well as nitrate by the extraradical hyphae of AM fungi has been demonstrated in several experimental conditions (Ames et al., 1983; Bago et al., 1996; Frey and Schüepp, 1993; Johansen et al., 1992; Tobar et al., 1994). However, much less is known about the ability of AM hyphae to take up and metabolize organic nitrogen. Hawkins et al. (2000) showed organic nitrogen uptake by extraradical hyphae of different AM fungi, as well as transport to the host plant when supplied in the form of ^{15}N labelled glycine or glutamic acid. Here, we present further indirect evidence of organic nitrogen uptake and metabolism by extraradical hyphae from the arbuscular mycorrhizal fungus *G. intraradices* when applied as glutamine as a single nitrogen source. From all these experiments, it can be postulated that ammonium, nitrate, and amino acid transporters/permeases as well as proton pumping ATPases might be functioning at the extraradical mycelium to make possible the uptake of these different nitrogen sources. A similar picture has already emerged for ectomycorrhizal fungi (Javelle et al., 2003; Montanini et al., 2002; Nehls et al., 1999; Wipf et al., 2002). However, none of these systems have been described up to now in endomycorrhizal fungi, with the exception of two H^+-ATPase isozymes identified in *G. mosseae* (Requena et al., 2003).

Once inside the fungal cell, inorganic nitrogen has to be assimilated in order to be used for internal consume or eventually to be translocated towards the host root. In contrast to direct incorporation of glutamine, inorganic nitrogen has to be assimilated into key nitrogen donors such as glutamate or glutamine by means of either the NADP-linked glutamate dehydrogenase (NADP–GDH) or the glutamine synthetase/glutamate synthase (GOGAT) enzymes. Glutamine is an essential amino acid playing a central role in the nitrogen metabolism of many fungi. It is the precursor of many essential metabolites such as nucleic acids, amino sugars, and other amino acids as histidine, tyrosine, and asparagine. In AM fungi, evidence of the implication of the GS/GOGAT pathway has been reported (Cliquet and Stewart, 1993; Johansen et al., 1996; Smith et al., 1985) pointing towards the glutamine synthetase as the main enzyme involved in nitrogen assimilation. However, a possible involvement of NADP–GDH was not excluded for extraradical hyphae (Cliquet and Stewart, 1993). Our results clearly show that AM extraradical hyphae assimilate inorganic nitrogen mainly through the GS–GOGAT pathway. However, our results do not allow discriminating whether assimilated nitrogen is only used to fulfill fungal needs or also to be translocated in this form towards the intraradical hyphae.

It is known that in other fungi glutamine synthesis occurs even in cells growing on glutamine and that nitrogen is cycled between glutamine and glutamate via the GS–GOGAT cycle (Mora, 1990). The lack of GS activity would result in the accumulation of glutamate and derivatives with toxic effects for the cell (Margelis et al., 2001). This seems to be the case also for the AM fungus

G. intraradices, where even after 96 h of growth in media containing glutamine as a single nitrogen source, glutamine activity was still present and showed values similar to the activity observed for hyphae growing on nitrate. These results would provide further evidence for the involvement of the GS–GOGAT pathway as the main route for ammonium assimilation in AM fungi. This seems to be also the case for the ectomycorrhizal fungus *Tuber borchii* (Montanini et al., 2003). Given the coenocytic nature of the arbuscular mycorrhizal mycelium, it is tempting to speculate on the fate of the nitrogen taken up by the extraradical hyphae.

The eukaryotic glutamine synthetase (GSII) is believed to consist of eight subunit oligomers, as a difference with the GSI characteristic of prokaryotes that is dodecameric (Eisenberg et al., 2000). However, the conservation of many important amino acid residues with a role in the catalysis as well as binding of substrates and other ligands among prokaryotic and eukaryotic GS indicates a similar mechanism of action. All these residues were strictly conserved in the *GmGln1* sequence in contrast to the results found for the ectomycorrhizal fungus *T. borchii* (Montanini et al., 2003) where several substitutions were found. The authors attributed to these differences some of the functional peculiarities observed for the *T. borchii* enzyme such as the tetrameric quaternary structure observed in vitro in addition to the more characteristic octameric structure. The regulation of the eukaryotic enzyme can take place at multiple levels. That is, by changes in the expression levels, metal ion binding, association-dissociation of oligomers, by effectors as well as by post-translational modifications of the protein. In contrast to the GSI from bacteria, GSII lacks the adenylation loop at the carboxy-terminus and therefore, no significant regulation at the enzyme level would be expected (Eisenberg et al., 2000). The best studied GSII are the mammalian enzymes. In that system, several isozymes with different biochemical properties have been described. The high similarity in their amino acid sequences seems to favor post-translational regulation as the mechanism controlling their different properties. In plants, the cytosolic GS is encoded by a small gene family with different isozymes with a developmental- and organ-specific pattern of expression (Stanford et al., 1993). In addition a chloroplastic GS, usually encoded by only one gene, exists. Plant GS have been shown to be regulated by gene expression, association of heteromeric polypeptides, reversible phosporylation, oxidative modifications and interaction with 14-3-3 proteins (Carvalho et al., 1997; Finnemann and Schjoerring, 2000; Moorhead et al., 1999; Ortega et al., 1999; Riedel et al., 2001; Stanford et al., 1993). Among fungi, the regulation of GS activity is rather diverse. Thus, in some species such as *Agaricus bisporus* or the ectomycorrhizal fungus *T. borchii* the expression is mainly controlled at the gene level, where ammonium exerts an important repressing effect (Kersten et al., 1997; Montanini et al., 2003). In yeast, nitrogen starvation induces expression of genes encoding enzymes for the synthesis of Glu and Gln and by increasing the activity of permeases responsible for the uptake of amino acids as a source of nitrogen (Magasanik and Kaiser, 2002). *GLN1* the gene encoding GS in yeast is rapidly induced through the Gln3p GATA factor upon shift from glutamine containing media to glutamate (Benjamin et al., 1989). In contrast, in other fungi such as *Aspergillus nidulans* or the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*, glutamine synthetase is a highly expressed gene but not highly regulated at the transcriptional level (Javelle et al., 2003; Margelis et al., 2001). In this study, we show that in two arbuscular mycorrhizal fungi variations observed in GS activity in response to different nitrogen sources were not correlated with similarly regulated mRNA levels. GS transcript levels appear alike among all treatments assayed in both fungi *G. mosseae* and *G. intraradices*. In contrast, enzyme activity was modulated upon nitrogen source in extraradical hyphae of *G. intraradices*. Therefore it follows that GS activity in AM fungi is possibly subjected to post-transcriptional regulation in response to different nutritional conditions. A putative 14-3-3 interaction motif was detected in the N-terminal part of the protein, in a similar location to the putative 14-3-3 interaction motifs from the GS-2 from *Nicotiana tabacum* (Riedel et al., 2001). Interestingly, we have recently isolated several ESTs corresponding to 14-3-3 proteins from *G. mosseae* (Breuninger and Requena, unpublished results). It is tempting to speculate that, similarly to plants, in AM fungi GS enzyme activity could be regulated by phosphorylation-driven interaction with 14-3-3 proteins.

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