Characterization of an Amino Acid Permease from the Endomycorrhizal Fungus *Glomus mosseae*[1][W]

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Arbuscular mycorrhizal (AM) fungi are capable of exploiting organic nitrogen sources, but the molecular mechanisms that control such an uptake are still unknown. Polymerase chain reaction-based approaches, bioinformatic tools, and a heterologous expression system have been used to characterize a sequence coding for an amino acid permease (*GmosAAP1*) from the AM fungus *Glomus mosseae*. The GmosAAP1 shows primary and secondary structures that are similar to those of other fungal amino acid permeases. Functional complementation and uptake experiments in a yeast mutant that was defective in the multiple amino acid uptake system demonstrated that GmosAAP1 is able to transport proline through a proton-coupled, pH- and energy-dependent process. A competitive test showed that GmosAAP1 binds nonpolar and hydrophobic amino acids, thus indicating a relatively specific substrate spectrum. *GmosAAP1* mRNAs were detected in the extraradical fungal structures. Transcript abundance was increased upon exposure to organic nitrogen, in particular when supplied at 2 mM concentrations. These findings suggest that GmosAAP1 plays a role in the first steps of amino acid acquisition, allowing direct amino acid uptake from the soil and extending the molecular tools by which AM fungi exploit soil resources.

Amino acids reach a considerable quantity in soils of many ecosystems, and this could contribute significantly to the nitrogen (N) nutrition of plants. This is the case in soils in which mineralization processes are low, for example, arctic, boreal (Väre et al., 1997), and heathland soils (Read, 1996), or in poor sites such as wet mires and sand plains (Chen et al., 1999). It is also true for agricultural systems (Scheller, 1996).

Plants, with a few exceptions (Turnbull et al., 1996; Näsholm et al., 1998; Bennett and Prescott, 2004), do not possess the full machinery necessary to exploit such organic sources. Plants have adapted different strategies to access and compete for this key nutrient with the microbial communities of the rhizosphere. One of these is the establishment of symbiotic associations with mycorrhizal fungi (Girlanda et al., 2007).

Apart from the visionary speculation of Frank (1894), who proposed the ''organic nitrogen theory'' at the end of the 19th century (Read and Perez-Moreno, 2003), the possibility that fungal symbionts might be involved directly in the uptake of organic polymers was largely ignored until the mid-1980s. For a long time, the degradation of organic compounds, the uptake and transfer of organic N to the plant, was considered a prerogative of ectomycorrhizal fungi (Tibbett et al., 2000; Chalot et al., 2002; Tibbett and Sanders, 2002; Sawyer et al., 2003; Guidot et al., 2005; Müller et al., 2007). Unlike ectomycorrhizal fungi, arbuscular mycorrhizal (AM) fungi, which, under natural conditions, colonize the majority of root systems, have mainly been considered for their role in phosphate uptake and translocation (Bucher, 2007; Javot et al., 2007).

There is evidence that AM fungi can also contribute to the increase of N acquisition in host plants, and in recent years advances have been made in the understanding of the movement of N, in particular inorganic N, in AM symbiosis. Experiments based on radioactively labeled N, measurements of the activity of plant enzymes involved in N assimilation, and transcriptional studies of a nitrate reductase in the mycobiont have shown that AM fungi are capable of taking up nitrate and ammonium (Kaldorf et al., 1994, 1998; Johansen et al., 1996; Cliquet et al., 1997; Faure et al., 1998; Subramanian and Charest, 1998; Hawkins et al., 2000; Toussaint et al., 2004). Molecular evidence of ammonium uptake was obtained recently through the characterization of an ammonium transporter that is expressed in the extraradical mycelium of *Glomus intraradices* (López-Pedrosa et al., 2006). Inorganic N, taken up by the extraradical mycelium, is incorporated

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into amino acids and translocated to the intraradical mycelium mainly as Arg (Cruz et al., 2007). N is then transferred from the fungus to the plant as ammonium without any loss of carbon skeleton, thanks to the catabolic arm of the urea cycle that converts Arg into ammonium (Govindarajulu et al., 2005; Jin et al., 2005).

AM fungi could also be involved in the acquisition of organic N. Preliminary studies have demonstrated that the development of the extraradical mycelium of AM fungi is stimulated by external organic N sources (St. John et al., 1983a, 1983b; Joner and Jakobsen, 1995; Ravnskov et al., 1999). Näsholm et al. (1998) obtained indirect evidence of amino acid uptake from *Deschampsia flexuosa* colonized by AM fungi in field conditions. It has been shown that organic N uptake is greatly enhanced by AM colonization (Cliquet et al., 1997) and that AM symbiosis could both enhance the decomposition of N and increase N capture from organic patches (Hodge et al., 2001). More recently, Jin et al. (2005) demonstrated that the extraradical mycelium of AM fungi grown in in vitro cultures can take up and utilize exogenously supplied Arg.

Little is known about the genes involved in organic N metabolism in AM fungi. Until now, only a sequence coding for Gln synthetase has been characterized in *Glomus mosseae* and *G. intraradices* (Breuninger et al., 2004). No sequence responsible for organic N uptake has been described to date.

Amino acid transport systems have been studied extensively in higher plants (Okumoto et al., 2002), yeast, and filamentous fungi (Struck et al., 2002; Trip et al., 2002; Wipf et al., 2002b). The transporter classification groups all of the amino acid permeases into the amino acid/polyamine organocation superfamily (Jack et al., 2000). Up to 24 members of the amino acid permease family have been found in the yeast *Saccharomyces cerevisiae* (Wipf et al., 2002b), most of which have been functionally characterized (Regenberg et al., 1999). As far as mycorrhizal fungi are concerned, to date only two genes have been identified, both from ectomycorrhizal species, *Amanita muscaria* and *Hebeloma cylindrosporum* (Nehls et al., 1999; Wipf et al., 2002a).

In this work, we describe and functionally characterize in a yeast mutant an amino acid permease (GmosAAP1) from the AM fungus *G. mosseae*. *GmosAAP1* mRNA was detected in the extraradical mycelium, the fungal structure that explores soil resources. Organic N supplied as the amino acid pool at a concentration of 2 μM or 2 mM determined an increase in the *GmosAAP1* transcript levels.

AM fungi are traditionally acknowledged as the microbes that improve mineral supply to a plant, thanks to phosphate uptake, through their external mycelium (Bucher, 2007). Our findings suggest that AM fungi possess other molecular tools to exploit soil resources, since *GmosAAP1* may play a role in the first step of amino acid acquisition, allowing direct amino acid uptake from the environment.

RESULTS

Cloning and Sequence Analysis of *GmosAAP1*

In order to identify the genes involved in the uptake of N compounds in AM fungi, two oligonucleotides, designed on conserved amino acid domains (Supplemental Fig. S1), were used for PCR on cDNAs obtained from *Gigaspora margarita*, *Gigaspora rosea*, *G. mosseae*, and *G. intraradices*. A cDNA fragment of approximately 150 bp obtained from *G. mosseae* extraradical mycelium showed a similarity to previously described fungal amino acid permeases. A full-length cDNA, named *GmosAAP1* (accession no. AY882560), was then identified by means of 5′ and 3′ RACE-PCR (Supplemental Fig. S1). Twelve transmembrane domains were predicted for GmosAAP1 (Supplemental Fig. S1) using several programs available on the Web (HMMTOP, TMHMM, SOSUI, and TMPRED). This structure is consistent with that of other amino acid permeases (Van Belle and Andre, 2001).

The overall alignment of GmosAAP1 and vacuolar amino acid transporters described in *Schizosaccharomyces pombe* (accession no. Q10074) and in *S. cerevisiae* (accession no. NP_012534) showed a very low similarity level (approximately 12% of identical amino acids). In addition, the weakly conserved motif (T/I/K)LP(L/K/I), which works as a sorting signal for vacuole targeting (Stack et al., 1995), is not present in GmosAAP1. The alignment of a number of plasma membrane amino acid permease sequences from fungi and plants derived from the data bank coupled to a neighbor-joining phylogenetic analysis showed that the fungal transporters are clearly separated from those of the plants. GmosAAP1 clusters with fungal amino acid permease sequences (Supplemental Fig. S2).

In recent years, a number of sequences belonging to ascomycetes that were closely associated to AM spores, grown in pot cultures or from the field, were erroneously assigned to AM fungi (Redecker et al., 1999). For this reason, we wanted to confirm the authenticity of the GmosAAP1 sequence. A couple of specific primers for GmosAAP1 were used on the genomic DNA of a related species, *G. intraradices*, which was grown in sterile conditions on transformed roots. A PCR product of the expected size was obtained, cloned, and sequenced. The sequence, named *GintAAP1*, showed a clear similarity to GmosAAP1, with 96.2% identity at the nucleotide level (data not shown).

Functional Characterization of GmosAAP1 in Yeast

The yeast mutant *22Δ8AA*, which lacks eight endogenous amino acid transport systems, was transformed with plasmids containing *GmosAAP1* cDNA. The growth of *22Δ8AA* expressing GmosAAP1 was good on Pro, Asp, Glu, and γ-aminobutyric acid as a single N source (data not shown).

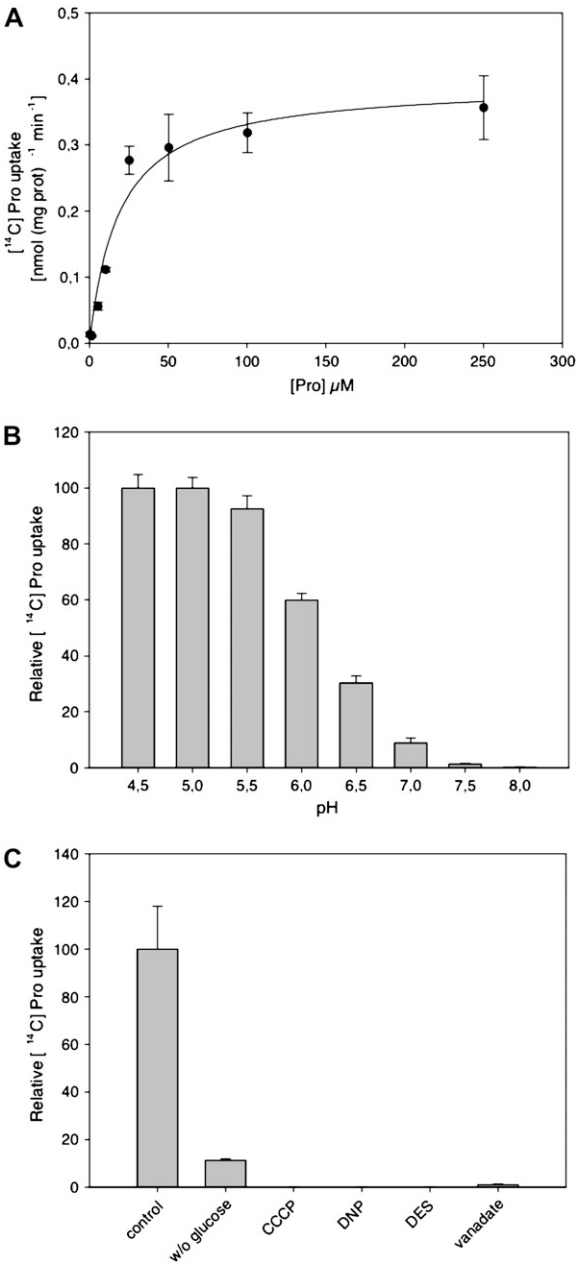

Figure 1. A, GmosAAP1-mediated [^{14}C]Pro uptake at different substrate concentrations. The experiments were performed at pH 4.5. The values represent means of three independent experiments ± SD. B, pH dependence of the uptake rate of [^{14}C]Pro in the yeast mutant *22Δ8AA* expressing GmosAAP1. Yeast expressing GmosAAP1 in pDR196 were measured at different pH values and an 18.8 μM substrate concentration. The values represent means of three independent experiments ± SD. C, Influence of plasma membrane energization on the uptake rate of [^{14}C]Pro in the yeast mutant *22Δ8AA* expressing GmosAAP1. The yeast cells were preincubated for 5 min in the presence of 100 mM Glc (control), without Glc, or with Glc and 0.1 mM 2,4-dinitrophenol (DNP), 0.1 mM diethylstilbestrol (DES), 0.1 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), or 0.1 mM vanadate. The values represent means of three independent experiments ± SD.

To directly determine the transport properties of GmosAAP1, radiotracer uptake studies were performed using [^{14}C]Pro. Under standard assay conditions, the [^{14}C]Pro uptake was linear for at least 4 min. The uptake rate was concentration dependent and displayed saturation kinetics (Fig. 1A). The K_m value for the transport of Pro was 18.8 μM (Fig. 1A). GmosAAP1 activity was clearly pH dependent, with an optimum at approximately pH 4.5 to 5.0 (Fig. 1B). The [^{14}C]Pro uptake depended on the presence of Glc and was sensitive to the protonophores 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone and the plasma membrane H^+-ATPase inhibitors diethylstilbestrol and vanadate, indicating that energization is required for transport (Fig. 1C). The range of the amino acids that bind to GmosAAP1, and therefore those most probably transported, was determined through their competitive effect on the uptake of the labeled Pro (Fig. 2). Negatively and positively charged amino acids were poorly recognized by GmosAAP1. Neutral, polar, and hydrophobic amino acids were better recognized, with the exception of Cys, which was an excellent competitor. All of the nonpolar, hydrophobic amino acids competed even more efficiently than Pro.

GmosAAP1 Gene Expression Profiles

Gene expression analysis was performed by reverse transcription (RT)-PCR assays on different stages of the *G. mosseae* life cycle: sporocarps germinated in water, extraradical mycelium, and mycorrhizal roots from which the external hyphae were removed. The last two samples were collected from pot cultures: *Cucumis sativus* mycorrhizal roots watered with a Long Ashton solution containing 1 mM nitrate. Three

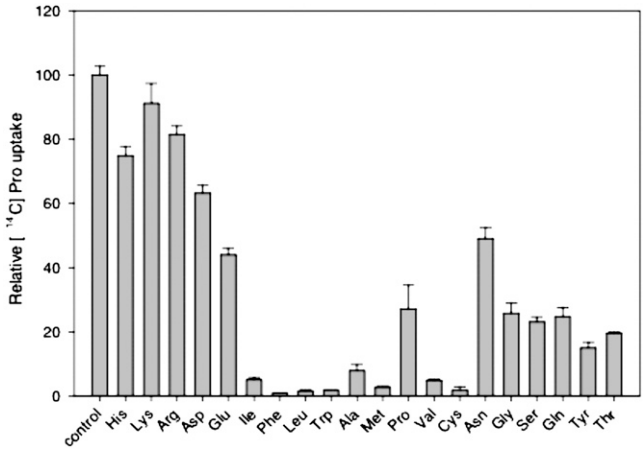

Figure 2. Substrate specificity of GmosAAP1. Inhibition of 18.8 μM [^{14}C]Pro uptake by a 5-fold molar excess of competing amino acids. The data are expressed as percentages of the uptake rate in the presence of 18.8 μM Pro. The values represent means of three independent experiments ± SD.

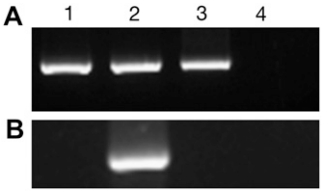

Figure 3. Gel electrophoresis of RT-PCR products obtained with oligonucleotides specific for the *G. mosseae* 28S rDNA (A) or *GmosAAP1* (B) on the following samples: lane 1, germinating spores; lane 2, extraradical mycelium; lane 3, intraradical mycelium; lane 4, no template.

months after inoculation, the roots presented the typical structures of AM symbiosis and a good mycorrhization level according to Trouvelot et al. (1986): F = 87% (frequency of mycorrhization of the root fragments), M = 52% (intensity of root cortex colonization), a = 49% (average presence of arbuscules within the infected areas), and A = 26% (arbuscule abundance in the root system).

In order to exclude cross-hybridization with the plant material, oligonucleotides for the *G. mosseae* 28S ribosomal gene (van Tuinen et al., 1998) and for *GmosAAP1* (G1/G2) were first tested on the *C. sativus* genomic DNA. No amplification product was obtained (data not shown).

The RT-PCR experiments with *G. mosseae* 28S ribosomal primers gave an amplified fragment of the expected size (380 bp) from the germinated sporocarps, extraradical mycelium, and intraradical fungal structure cDNAs (Fig. 3A). Amplifications with *GmosAAP1* primers generated a PCR product (780 bp), but only in the sample corresponding to extraradical mycelium cDNA (Fig. 3B).

To investigate whether *GmosAAP1* expression was modulated by organic N in the surrounding medium, semiquantitative and real-time RT-PCR assays were performed on pot culture extraradical mycelium treated for 72 h with a modified Long Ashton solution without N (0 M) or with a Long Ashton solution containing as N source a pool of amino acids (Leu, Ala, Asn, Lys, and Tyr) or NH_4^+ or NO_3^- at two different concentrations (2 μM or 2 mM). The amount of mRNA obtained from different samples was first calibrated using fungus-specific 28S rRNA primers. The mRNA samples were then amplified with *GmosAAP1*-specific oligonucleotides. The PCR product corresponding to *GmosAAP1* was considerably more abundant for the two samples treated with the amino acid pool at 2 μM and 2 mM (Fig. 4). Real-time RT-PCR assays were performed to obtain a quantitative measurement of this induction. In fact, an increase of the *GmosAAP1* transcripts level was observed on two independent biological samples after exposure to organic N. In spite of a certain variability in the independent samples, the *GmosAAP1* transcript levels were particularly abundant after the 2 mM treatment (Fig. 5).

DISCUSSION

The role of AM fungi in litter degradation and in the uptake of organic nutrients from the soil is an ecologically important issue that has particular relevance in plant nutrition (Hodge et al., 2001). As stated by Alexander (2007), ''In view of the worldwide distribution of arbuscular mycorrhizas, sometimes in highly organic soils, this is a topic ripe for further exploration.''

As a first step toward the identification of the molecular machinery that allows amino acid uptake and organic N transport in AM symbiosis, we have identified a cDNA sequence (GmosAAP1) from the AM fungus *G. mosseae* that shows a remarkable similarity to amino acid transporters. According to the transporter classification, GmosAAP1 belongs to the amino acid/polyamine organocation superfamily. Most of these transporters exhibit a uniform topology, with 12 putative α-helical transmembrane domains and cytoplasmically located N- and C-terminal hydrophilic regions (Wipf et al., 2002b). The number and distribution of the transmembrane domains of GmosAAP1 mirror an identical topology. The GmosAAP1 protein shows a limited similarity, in terms of primary sequence and structure, to vacuolar amino acid transporters described in yeast (Russnak et al., 2001). Together, the data suggest that GmosAAP1 is a plasma membrane protein. The identification of a partial sequence showing high similarity to GmosAAP1 from in vitro-grown *G. intraradices* clearly supports the authenticity of the sequence. Since in yeast and filamentous fungi AAPs usually belong to a multigene family (Wipf et al., 2002b), it is likely that *G. mosseae* also possesses additional AAPs.

GmosAAP1 Encodes a Functional Amino Acid Transporter

Yeast has provided a genuine heterologous expression system for the characterization of many nutrient and metabolite transporters from animals, plants, and nonyeast fungi. This instrument is particularly valuable for organisms that are currently recalcitrant to genetic transformation, such as AM fungi. In fact, the few transporters described in this group of fungi to

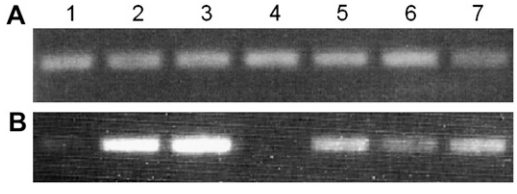

Figure 4. Gel electrophoresis of RT-PCR products obtained with oligonucleotides specific for the *G. mosseae* 28S rDNA (A) or *GmosAAP1* (B) on external mycelium treated as follows: lane 1, no N (0 M); lane 2, 2 μM of the amino acid pool (Leu, Ala, Asn, Lys, and Tyr); lane 3, 2 mM of the amino acid pool; lane 4, 2 μM KNO_3; lane 5, 2 mM KNO_3; lane 6, 2 μM $(NH_4)_2SO_4$; lane 7, 2 mM $(NH_4)_2SO_4$.

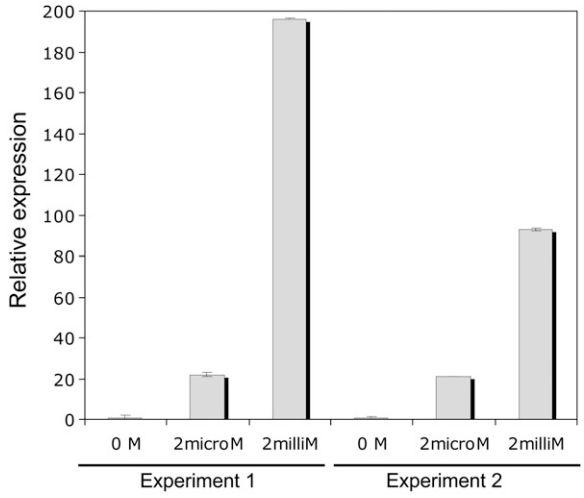

Figure 5. Real-time RT-PCR analysis of the *GmosAAP1* mRNA in extraradical mycelium treated with 0 M N or 2 μM or 2 mM of the amino acid pool. Relative expression levels were obtained with the comparative threshold cycle method (see "Materials and Methods" for details) and were normalized with respect to the *GmosAAP1* levels in the 0 M treatment.

date have been functionally characterized using yeast mutants (Harrison and van Buuren, 1995; Gonzalez-Guerrero et al., 2005; López Pedrosa et al., 2006).

The *22Δ8AA* mutant strain expressing GmosAAP1 was able to grow using some amino acids as a single N source, thus confirming the amino acid transport capability of GmosAAP1. The uptake rate for Pro was concentration dependent and displayed saturation kinetics, with a K_m value (18.8 μM) that is comparable with amino acid concentrations found in the soil (Scheller, 1996). The GmosAAP1 activity has an optimum at approximately pH 4.5 to 5.0, which is consistent with the pH optimum described for the uptake of Glu and Gln by mycelia of the ectomycorrhizal fungus *Paxillus involutus* (Chalot et al., 1995). The strong dependence on the presence of Glc and a proton gradient indicates that GmosAAP1-mediated transport requires a secondary active transport mechanism that is similar to those of its yeast homologs (Opekarová et al., 1993). Competition experiments indicate a preferential affinity of GmosAAP1 for all nonpolar and hydrophobic amino acids, suggesting a relatively narrow substrate specificity.

GmosAAP1 Is Expressed in Extraradical Structures

Qualitative RT-PCR assays indicated that *GmosAAP1* was expressed in the external mycelium but not in the intraradical fungal structures of plants treated with millimolar nitrate concentrations. *GmosAAP1* transcripts were not detected in sporocarps germinated in a water/agar medium; however, for a more comprehensive view, the expression in asymbiotic stages should be studied considering other growth conditions (e.g. exposure to different N sources).

GmosAAP1 expression in extraradical hyphae responded to the presence of different concentrations of organic N. In spite of a certain variability in the two independent biological samples, an induction was constantly observed after the two amino acid pool treatments in comparison with the 0 M treatment. In particular, *GmosAAP1* was strongly up-regulated after the 2 mM treatment. It is worth noting that the amino acid pool contained three amino acids, Leu, Ala, and Tyr, which, from the competition studies obtained in the yeast mutant (Fig. 2), are likely to be substrates of GmosAAP1. This might reflect a common mechanism of AAP regulation, that is, the transcriptional induction by the substrate (Grauslund et al., 1995).

The hypothesis that organic N acts as a signaling molecule in AM fungi is also supported by the observation that limiting organic N conditions induce a specific response at the transcriptional level in extraradical structures of *G. intraradices* (Cappellazzo et al., 2007).

The main function of *GmosAAP1*, as indicated by the putative localization on the plasma membrane, its expression in extraradical hyphae, and the biochemical properties in terms of K_m, may be the uptake of amino acids from the soil solution. A similar role has also been suggested for the gene identified in *H. cylindrosporum*, although detailed expression studies were not performed (Wipf et al., 2002a). *AmAAP1* gene expression has only been studied in *A. muscaria* mycelium grown in pure culture, and no data are available concerning its expression during its interaction with host plants (Nehls et al., 1999).

CONCLUSION

N nutrition of AM plants, in particular with regard to organic N sources, remains a largely unexplored area. With the identification of a gene that encodes a functional amino acid transporter, we offer experimental evidence that the AM fungus *G. mosseae*, which is extensively present in agricultural systems and often used as a component of commercial inocula, possesses molecular tools for the uptake not only of phosphate (Benedetto et al., 2005) but also of organic N from the soil. This finding could contribute to a better understanding of the organic N metabolism in AM fungi and lead to new important questions on its impact on host plant nutrition.

MATERIALS AND METHODS

Biological Material

The *Glomus mosseae* 'BEG 12' (International Bank for the Glomeromycota; http://www.kent.ac.uk/bio/beg/) inoculum (sporocarps and mycorrhizal roots) was obtained from Biorize. For germination, the sporocarps were collected with forceps, surface sterilized with 3% (w/v) chloramine-T, and placed in water-agar (1.5%, w/v) at 25°C in the dark. The inoculum was also used in pot culture to obtain mycorrhizal plants. *Cucumis sativus* 'Marketmore' seeds were previously surface sterilized for 30 s in 98% sulfuric acid and then

rinsed several times with distilled sterile water. The seeds were left to germinate for 7 d on water-agar plates at 24°C in the dark. The seedlings were then transferred to 0.3-L plastic pots containing heat-sterilized (3 h at 180°C) quartz sand and *G. mosseae* inoculum (1:10, v/v) and kept in a growth chamber for a 13-h photoperiod at 20°C/24°C dark/light. The plants were watered every second day with water and the other day with a Long Ashton solution (Hewitt, 1966) containing a low phosphorus concentration (3.2 μM $NaHPO_4 \cdot 12H_2O$). After 3 months, when the mycorrhization was fully established, the roots were carefully washed and then submerged for 72 h in a modified Long Ashton solution. According to the treatment, this solution was used directly (no N sample) or two different concentrations (2 μM or 2 mM) of KNO_3, $(NH_4)_2SO_4$, or a pool of amino acids with different biochemical properties (Leu, Ala, Asn, Lys, and Tyr) were added.

Two sets of independent mycorrhizal plants were treated. The extraradical mycelium and mycorrhizal root pieces, devoid of external hyphae, were collected with forceps under the stereomicroscope lens and immediately frozen in liquid N.

Root organ cultures of *Agrobacterium rhizogenes* (Ri T-DNA)-transformed carrot (*Daucus carota*) roots were used for monoxenic cultivation of the AM fungus *Glomus intraradices* 'MUCL 43194' (Declerck et al., 2005) obtained from GINCO (http://www.mbla.ucl.ac.be/ginco-bel). The carrot roots inoculated with *G. intraradices* were grown in two-compartment petri dishes on Strullu-Romand medium (Declerck et al., 1998) and solidified with 3 g L^{-1} Phytagel. The petri dishes were incubated horizontally in an inverted position at 27°C in the dark for 3 to 4 weeks. The *G. intraradices* extraradical structures were recovered by solubilizing the solid Strullu-Romand medium with sterile 50 mM Tris-HCl, pH 7. The extraradical structures were collected with forceps, rinsed with sterilized water, recovered by vacuum filtration on a sterilized polyvinylidene fluoride membrane (Durapore GVWP; Millipore), and immediately frozen in liquid N and stored at −80°C until used.

DNA and RNA Extractions

Genomic DNA was extracted from approximately 100 mg of roots using the hexadecyl-trimethyl-ammonium bromide protocol (Henrion et al., 1994). The roots were ground in liquid N to a fine powder, a hexadecyl-trimethyl-ammonium bromide extraction buffer was immediately added, and the samples were incubated at 65°C for 1 h. Crude lysates were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and once with chloroform. Aqueous phases were precipitated with 1.5 volume of isopropanol (−20°C overnight). The DNA pellet was washed with 70% ethanol, dried, and then resuspended in 20 μL of water.

The genomic DNA from *G. intraradices* extraradical mycelium from in vitro cultures was obtained with the DNAeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

RNA was extracted from approximately 100 sporocarps germinated in water-agar, 100 mg of mycorrhizal roots, 100 mg of nonmycorrhizal roots, and 0.2 to 0.3 mg of *G. mosseae* extraradical mycelium using the SV Total RNA Isolation System Kit (Promega). The RNA was precipitated with 6 M LiCl and resuspended in 20 μL of sterile water. The RNA samples were routinely checked for DNA contamination by RT-PCR analyses conducted using the 28S rRNA universal primers NS1/NS2 (White et al., 1990) and the One-Step RT-PCR Kit (Qiagen) according to Cappellazzo et al. (2007). The first strand of cDNA was synthesized using Sensiscript reverse transcriptase (Qiagen) and random primers according to the manufacturer's instructions.

PCR and RT-PCR

The PCR experiments were carried out with the oligonucleotides NITPLUS (5′-GCCCTGCGCTTCTTCATCGG-3′) and NITMINUS (5′-AAATGGCCGG-CATGACGAAG-3′). The primers were designed by Dr. E. Soragni (University of Parma, Italy). cDNAs from spores, germinated spores, or external mycelium of *Gigaspora margarita* 'BEG 34', *Gigaspora rosea* 'BEG 9', *G. mosseae* 'BEG 12', and *G. intraradices* 'DAOM181602', kindly provided by Prof. P. Franken (Institute for Vegetable and Ornamental Crops, Grossbeeren, Germany), were used as templates. PCR was carried out in a final volume of 30 μL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, 1 μM of each primer, 50 to 100 ng of cDNA, and 1 unit of RED*Taq*TM DNA polymerase (Sigma). A PCR program was conducted in a Perkin-Elmer GeneAmp 9700 thermal cycler according to these parameters: 95°C for 5 min (one cycle), 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min (35 cycles), and 72°C for 5 min (one cycle). The negative controls for all PCR experiments consisted of reaction mixtures from which template DNA was omitted. The PCR products were separated on a 1.2% Tris-acetate EDTA/0.5× agarose gel and visualized by ethidium bromide staining.

Specific primers for *G. mosseae* 28S rRNA (5.21/NDL22; van Tuinen et al., 1998) and *GmosAAP1* G1 (5′-CTGGAGAGAAGATATCAAC-3′) and G2 (5′-CATGCCCTGAGGAGCAGCG-3′) were tested on DNA and cDNA samples. The PCR program was conducted in a Perkin-Elmer GeneAmp 9700 thermal cycler according to these parameters: 95°C for 3 min (one cycle), 92°C for 45 s, annealing temperature (55°C for NS1/NS2, 64°C for 5.21/NDL22, and 54°C for G1/G2) for 45 s, 72°C for 45 s (35 cycles), and 72°C for 5 min (one cycle).

PCR was carried out on *G. intraradices* genomic DNA with APH1 (5′-GTCGTCGCTGCTTTCTCCTTCGG-3′) and APEF1 (5′-CAAGGAGACC-AAAGGCGATCTG-3′) primers using an annealing temperature of 48°C.

The PCR products were purified from agarose gels using the QIAEX II gel extraction kit (Qiagen) and directly cloned in the pGEM-T vector (Promega). Plasmid DNA was extracted using the Qiagen Mini kit and sequenced by Genelab (Enea).

RACE-PCR

The amplification of *GmosAAP1* 5′ and 3′ cDNA ends was performed by RACE using the SMART RACE cDNA amplification kit (BD Biosciences) utilizing a combination of specific forward primers, AT5 (5′-CCCTGCG-CTTCTTCATCGGCTATCTC-3′) and AT7 (5′-GCACACTGCTTGGCCCTCG-CTGAGATGG-3′), and reverse primers, AP1 (5′-GACCGACCATCTCAG-TACCACCG-3′) and APH2 (5′-CAATTCGCCAGGAGACCTGCTTG-3′), in nested PCR. The reactions were carried out in a GeneAmp 9700 thermal cycler according to the manufacturer's instructions.

Semiquantitative and Real-Time RT-PCR

Semiquantitative RT-PCR was performed on two independent biological samples. RNA samples were calibrated using ribosomal primers (5.21/NDL22); specific AAP primers (G1/G2) were then used to evaluate the *GmosAAP1* mRNA level in each treatment. PCR was allowed to proceed for a different number of cycles to determine the exponential amplification phase. Reactions were carried out in a final volume of 50 μL using the previously described conditions. RT-PCR experiments were conducted using two technical replicates.

Individual real-time reactions were assembled in a final volume of 20 μL with 0.15 μM of each oligonucleotide, 10 μL of 2× iQ SYBR Green Supermix (Bio-Rad), plus an appropriate volume of each cDNA preparation. The following primers were used: 5.21 and 28S1 (5′-CACTTCAGTACGAGATC-GAAG-3′) for the fungal 28S ribosomal gene, Tef1 (5′-GCAGAACGT-GAGCGTGGTAT-3′) and Tef2 (5′-ACCAGTACCGGCAGCAATAA-3′) for the fungal elongation factor gene, and AAP1 (5′-TACTCCTCCCACCGATT-ACG-3′) and AAP2 (5′-CCGATGATGAGATAGCCGAT-3′) for the *GmosAAP1* gene.

The PCR cycling program (15 s at 95°C followed by 30 s at 62°C for 28S rRNA and *Tef* genes and at 64°C for the *GmosAAP1* gene) included a heating step (3 min at 95°C) at the beginning of each run. Real-time RT-PCR was carried out with an ICycler apparatus (Bio-Rad). A melting curve (55°C–95°C, with a heating rate of 0.5°C per 10 s and continuous fluorescence measurement) was recorded at the end of each run to assess amplification product specificity (Ririe et al., 1997). All of the reactions were performed with at least two technical replicates, and only comparative threshold cycle values with a SD that did not exceed 0.3 were considered. The comparative threshold cycle method (Rasmussen, 2001) was used to calculate the *GmosAAP* relative expression level.

Sequence Analyses

Sequence analyses were performed with Sequencher (Gene Codes Corporation), BLASTX software available from the National Center for Biotechnology Information (Altschul et al., 1997), and ClustalW (Thompson et al., 1994). The secondary structure was predicted using HMMTOP (Tusnady and Simon, 2001), TMHMM (Sonnhammer et al., 1998), SOUSI (Hirokawa et al., 1998), and TMPRED (Hofmann and Stoffel, 1993). The protein family, domain, and functional sites were searched using the InterProScan program (Mulder et al., 2003).

Phylogenetic analyses were performed using version 3.1 of the MEGA (Molecular Evolutionary Genetic Analysis) program available on the Web

(http://www.megasoftware.net/mega.html) and analyzed by the neighbor-joining algorithm (Kumar et al., 2004).

Yeast Growth and Transformation

The yeast strain used was a mutant lacking multiple amino acid uptake systems, *22Δ8AA* (Matα gap1-1 put4-1 uga4-1 Δcan1 Δapl1 Δlyp1 Δhip1 Δdip5 ura3-1; Fischer et al., 2002). The full-length cDNA of *GmosAAP1* was cloned in the yeast expression vector pDR196 (Wipf et al., 2003), exploiting the *Saccharomyces cerevisiae* homologous recombination process. Approximately 100 ng of *Xho*I-linearized pDR196 vector was mixed with 100 ng of the PCR product of *GmosAAP1* obtained using specific primers (For, 5′-GCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCATGTACCACCGGGGAACCAAGAG-3′, and Rev, 5′-TACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCGTACATGCTTAGTAAAAGC-3′) containing an extension of 40 bp of sequence homology with the vector sequence flanking the *Xho*I restriction site. The mixture was used to transform the ΔYAP yeast (Wu et al., 1993). Colonies carrying recombinant plasmids (pDR196-GmosAAP1) were screened using a selective ura$^-$ medium.

The pDR196-GmosAAP1 construct was used to transform the *22Δ8AA* yeast strain. Transformants were selected on solid minimal synthetic defined medium supplemented with 10 mM $(NH_4)_2SO_4$. The plasmid DNA was isolated and reintroduced into the mutant strain *22Δ8AA*. The cDNA clone GmosAAP1 was able to restore the growth of the mutant under selective conditions. The empty vector pDR196 was used as a negative control.

Transport Measurements

S. cerevisiae cells were grown to a logarithmic phase for uptake studies. The cells were harvested at an optical density at 600 nm of 0.5, washed twice in water, and resuspended in buffer A (0.6 M sorbitol and 50 mM potassium phosphate at the desired pH) to a final optical density at 600 nm of 5. Prior to the uptake measurements, the cells were supplemented with 100 mM Glc and incubated for 5 min at 30°C. To start the reaction, 100 μL of this cell suspension was added to 100 μL of the same buffer containing at least 0.46 kBq [^{14}C]Pro with a specific activity of 8.58 GBq/mmol (Amersham) and unlabeled amino acid to the concentrations used in the experiments. Sample aliquots of 50 μL were removed after 30, 60, 120, and 240 s, transferred to 4 mL of ice-cold buffer A, filtered on glass fiber filters, and washed twice with 4 mL of buffer A. The uptake of carbon-14 was determined by liquid scintillation spectrometry. Competition for Pro uptake was performed by adding a 5-fold molar excess of the respective competitors to 18.8 μM Pro.

For analysis of the pH dependence, incubations were performed in 100 mM potassium phosphate buffer adjusted to the different pH values, 100 mM Glc, and 150 μM [^{14}C]Pro. The influence of plasma membrane energization on the uptake rate of [^{14}C]Pro was analyzed by incubating the yeast cells for 5 min in the presence of 100 mM Glc (control), without Glc, or with Glc and 0.1 mM 2,4-dinitrophenol, 0.1 mM diethylstilbestrol, 0.1 mM carbonyl cyanide *m*-chlorophenylhydrazone, or 0.1 mM vanadate. The transport measurements were repeated independently and represent means of at least three experiments.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY882560 (*GmosAAP1*) and AM940008 (*GintAAP1*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. ClustalW alignment and predicted hydrophobicity profile of GmosAAP1.

Supplemental Figure S2. Unrooted phylogram obtained using the neighbor-joining algorithm.

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