GintAMT2, a new member of the ammonium transporter family in the arbuscular mycorrhizal fungus *Glomus intraradices*

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**Abstract**

In the symbiotic association of plants and arbuscular mycorrhizal (AM) fungi, the fungus delivers mineral nutrients, such as phosphate and nitrogen, to the plant while receiving carbon. Previously, we identified an NH\(_4^+\) transporter in the AM fungus *Glomus intraradices* (GintAMT1) involved in NH\(_4^+\) uptake from the soil when present at low concentrations. Here, we report the isolation and characterization of a new G. intraradices NH\(_4^+\) transporter gene (GintAMT2). Yeast mutant complementation assays showed that GintAMT2 encodes a functional NH\(_4^+\) transporter. The use of an anti-GintAMT2 polyclonal antibody revealed a plasma membrane location of GintAMT2. GintAMT1 and GintAMT2 were differentially expressed during the fungal life cycle and in response to N. In contrast to GintAMT1, GintAMT2 transcript levels were higher in the intraradical than in the extraradical fungal structures. However, transcripts of both genes were detected in arbuscule-colonized cortical cells. GintAMT1 expression was induced under low N conditions. Constitutive expression of GintAMT2 in N-limiting conditions and transitory induction after N re-supply suggests a role for GintAMT2 to retrieve NH\(_4^+\) leaked out during fungal metabolism.

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**1. Introduction**

Under natural conditions the roots of most vascular plants form arbuscular mycorrhiza, an ancestral type of mutualistic plant-fungal interaction, which is based on the bi-directional exchange of nutrients between both symbiotic partners (Wang and Qiu, 2006; Ferro and Pérez-Tienda, 2009). Arbuscular mycorrhizal (AM) fungi, belonging to the monophyletic phylum Glomeromycota (Schüepp et al., 2001), are obligate biotrophs that depend entirely on the plant for their carbon supply (Bago and Becard, 2002). In return, the fungus supplies the plant with mineral nutrients directly taken up from the soil by the extraradical mycelium (Smith and Read, 2008).

Although the uptake of inorganic phosphate (Pi) from the soil is considered to be the key physiological process by which AM fungi improve plant growth (Bucher, 2007), there is increasing evidence that AM fungi also play an important role in plant nitrogen (N) nutrition (He et al., 2003). In fact, N uptake by the extraradical mycelium can account for more than 30% of total plant N uptake (Frey and Schüepp, 1993; Tanaka and Yano, 2005). The extraradical hyphae of AM fungi are able to take up and assimilate both inorganic (nitrate and ammonium) and organic N forms from the soil, and to translocate N from these different sources to the plant (Tobar et al., 1994; Hawkins et al., 2000;Govindarajulu et al., 2005). Moreover, AM fungi can accelerate the decomposition and transfer of substantial amounts of N to their host plant from organic material (Hodge et al., 2001; Leigh et al., 2009), playing an important role in N turnover in ecosystems.

N is a major nutrient for all organisms, being needed for the synthesis of many compounds, including amino acids, purines, pyrimidines, some carbohydrates and lipids, enzyme cofactors and proteins, all of which are essential for growth processes. In the fungal partner, a few studies have highlighted the importance of N both in the asymbiotic and symbiotic stages. It has been recently shown that although the spores of AM fungi are able to take up and assimilate inorganic and organic forms of N, they do not depend on exogenous N to germinate since they can remobilize their internal N sources (Gachomo et al., 2009). Moreover, germination and growth of the germ tube of *Gigaspora margarita* spores were shown to be stimulated by nitrate, and a role for N on polyphosphate accumulation in the germ tubes has been proposed (Yao et al., 2010). In the symbiotic stage, low N levels induced changes in fungal development and hyphal morphogenesis, leading to a well organized and economic developmental pattern (Bago...
et al., 2004). Despite the importance of N for both the plant and the fungal partners, current knowledge on the mechanisms of N transport and metabolism in the symbiosis is scarce.

The first molecular evidence for N uptake by AM fungi was obtained through the characterization of an ammonium transporter of Glomus intraradices (López-Pedrosa et al., 2006). More recently, an amino acid transporter (Cappellazzo et al., 2008) and a nitrate transporter (Tian et al., 2010) have been also described in Glomus mosseae and G. intraradices, respectively. Inside the fungus, inorganic N is assimilated in order to be used for internal consumption or to be translocated towards the host root. Radiotracer experiments and gene expression analyses have demonstrated that: (i) inorganic N taken up by the extraradical hyphae is assimilated through the GS/GOGAT cycle, asparagine synthase and the urea cycle, (ii) arginine is the main form in which N is transported from the extraradical to the intraradical mycelium, and (iii) NH4+ released from transported arginine through the catabolic arm of the urea cycle is the N form transferred to the host (Breuninger et al., 2004; Govindarajulu et al., 2005; Cruz et al., 2007; Guether et al., 2009a; Kobae et al., 2010; Tian et al., 2010).

Ammonium (NH4+) is a primary N source for plants and microorganisms and a key metabolite in N metabolism. Although AM fungi are able to take up both NO3- and NH4+, a clear preference for NH4+ has been demonstrated (Villégas et al., 1996; Hawkins et al., 2000; Toussaint et al., 2004), which is explained, at least in part, by the extra energy the fungus must expend in reducing NO3- to NH4+ before it can be incorporated into organic compounds (Marzluf, 1996). Given the central role NH4+ plays in N metabolism and in N transport processes in AM, the aim of our work was to characterize AM fungal proteins involved in NH4+ uptake to get further insights into the regulatory mechanisms of N transport in the symbiosis. Transport of NH4+ across biological membranes is mediated by NH4+ transporters of the ammonium transporter/methylamine permease/rhesus (AMT/Mep/Rh) protein family (TC#1.A.11), which is spread throughout the whole tree of life (Andrade and Einsle, 2007). In a previous study, we identified the first AMT in an AM fungus: GintAMT1, a G. intraradices high-affinity NH4+ transporter involved in NH4+ uptake by the extraradical mycelium from the surrounding environment when present at low concentrations (López-Pedrosa et al., 2006). Here, we report the cloning and characterization of a new G. intraradices AMT that seems to be functionally different to GintAMT1.

2. Materials and methods

2.1. Arbuscular mycorrhizal monoxenic cultures and treatments

Arbuscular mycorrhizal monoxenic cultures consisted of Ri T-DNA (Agrobacterium rhizogenes)-transformed carrot (Daucus carota L. clone DC2) roots colonized with the AM fungus Glomus intraradices Schenck and Smith DAOM 197198. Cultures were established in bi-compartmental Petri plates to allow separating the root compartment from the hyphal compartment (St-Arnaud et al., 1996).

Cultures were started by placing a mycorrhizal carrot root segment in the root compartment containing M medium (Chabot et al., 1992). Petri plates were incubated in the dark at 24 °C until the hyphal compartment, which contained M medium without sucrose (M–C medium), was profusely colonized by the fungus (approximately 6 weeks). The content of the hyphal compartment was then removed and replaced by liquid M–C medium (15 ml) containing either 3.2 mM (100% N) or 0.8 mM (25% N) NO3-, Ca2+ and K+ losses, resulting from the reduction of NO3- salts, were compensated by the addition of the corresponding Cl- and SO42- salts, respectively. 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 hyphal compartment. Only cultures with vigorous roots and densely colonized hyphal compartments were selected for the experiments. At this point, the medium was removed and replaced by fresh liquid M–C medium without NO3-. The time of medium exchange was referred as time 0 for the N starvation treatment, and mycelia were harvested 2 and 7 days later. For the N re-supply experiments, mycelia grown in 25% N media and N-starved for 48 h were supplemented with different N sources and concentrations or water (control plates). Casein hydrolysate (N–Z amine HD; Sigma) was used as amino acid mixture. Extraradical mycelia were harvested 24 and 48 h later.

For the carbon-supplementation experiments, three different treatments were prepared: (1) Mycelia from the hyphal compartment of a control plate (M–C medium), (2) mycelia from the hyphal compartment of a control plate supplemented with 4 mM acetate in the hyphal compartment, and (3) mycelia from the hyphal compartment of a control plate supplemented with 25 mM glucose in the root compartment. Mycelia were harvested 2 and 7 days after supplementation.

In all experiments, mycelia were collected with forceps, rinsed with sterilized water, dried with sterilized filter paper, immediately frozen in liquid N and stored at −80 °C until used.

To analyze intraradical gene expression, hyphae growing in the hyphal compartment were used as a source of mycorrhizal inoculum. Carrot roots were placed on top of a densely colonized hyphal compartment and collected 15 days later. Extraradical hyphae attached to the roots were removed with forceps under a binocular microscope. Roots were then frozen in liquid N and stored at −80 °C until used. Spores of the AM fungus G. intrarradices were collected from the hyphal compartment of monoxenic cultures by blending the culture medium in 10 mM sodium citrate (pH 6) and sieving. The spores were recovered with a pipette under a binocular microscope. Spores were immediately frozen in liquid N and stored at −80 °C until used.

2.2. Nucleic acids extraction and cDNA synthesis

Total RNA was extracted from extraradical mycelia from the different treatments, spores of G. intraradices, and mycorrhizal carrot roots using the Plant RNAeasy Kit (Qiagen), according to the manufacturer’s instructions. DNase treatment was performed using RNA-free DNase Set (Qiagen) following the manufacturer’s instructions. The RNA samples were routinely checked for DNA contamination and their integrity was examined with Experion (Bio-Rad). cDNAs were obtained from 1 μg of total DNase-treated RNA in a 20 μl reaction containing 200 units of Super-Script III Reverse Transcriptase (Invitrogen) and 50 pmol oligo(dT)20 (Invitrogen), according to the manufacturer’s instructions.

Total genomic DNA was extracted from G. intraradices extraradical mycelia developed in a control plate using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer’s instructions.

2.3. Isolation of the GintAMT2 gene

Two degenerated primers AMT2.1 and AMT2.2 (Table 1), designed in base to conserved motifs present in NH4+ transporters of other organisms, were used for PCR amplification of first strand cDNA from extraradical mycelia of G. intraradices grown in M–C medium. The PCR product was cloned in the pCR2.1 vector (Invitrogen). Sequence analysis of positive clones allowed identification of a 173 bp cDNA fragment with high homology to previously described fungal AMTs. The full-length cDNA sequence was obtained by RACE using the SMART RACE cDNA amplification kit (BD Biosciences) according to the manufacturer’s protocol. The primers used...
for the 3’ and 5’ RACE reactions were AMT2.3 and AMT2.4 (Table 1), respectively. PCR products were cloned into pCR2.1 vector and sequenced.

The genomic and the full-length cDNA clones of GintAMT2 were obtained by PCR amplification of G. intraradices genomic DNA and cDNA, respectively, using a set of primers flanking the complete open reading frame (AMT2.5 and AMT2.6; Table 1). PCR products were cloned into the pCR2.1 vector and sequenced. For yeast complementation assays the full-length cDNA clone of GintAMT2 was subcloned into the pFL61 vector (Minet et al., 1992).

Table 1
List of primers used in this study.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Primer sequence [5’–3’]</th>
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<tr>
<td>AMT2.1</td>
<td>GGGGTGGTTGCTGGG</td>
</tr>
<tr>
<td>AMT2.2</td>
<td>CTCGACGCGATGCTATTTG</td>
</tr>
<tr>
<td>AMT2.3</td>
<td>CTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>AMT2.4</td>
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<td>AMT2.7</td>
<td>AAAGGCGAAGAAGACGACGAC</td>
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<tr>
<td>AMT2.8</td>
<td>AGTGCCTCTGGCTGCTGCTG</td>
</tr>
<tr>
<td>AMT2.9</td>
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<tr>
<td>MltPfr</td>
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</tr>
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<tr>
<td>GintPfr</td>
<td>AAACGCAATGCTGCAACAGAC</td>
</tr>
<tr>
<td>GintPfr</td>
<td>AAACGCAATGCTGCAACAGAC</td>
</tr>
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* Not cloning site is underlined.

2.6. Real-Time quantitative RT-PCR

Real-Time RT-PCR experiments were run on an IQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad). Gene-specific primers were used to amplify GintAMT1 (AMT2/AMT3; López-Pedrosa et al., 2006) and GintAMT2 (AMT2.8 and AMT2.9; Table 1) in the synthesized cDNAs. Real Time RT-PCR reactions were carried out in a final volume of 20 µl containing 10 µl of IQ™ SYBR Green Supermix 2 x (Bio-Rad), 0.2 µM of each primer and 1 µl of a 1:10 dilution of cDNA template. The PCR program consisted in an initial incubation at 95 °C for 3 min, followed by 36 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat-dissociation protocol (from 58 to 95 °C) after the final cycle of the PCR. The efficiency of the primer sets was evaluated by performing Real-Time PCR on several dilutions of DNA. The results obtained for the different treatments were standardized to the elongation factor 1-alpha (GintEF1α) gene levels (Benabdellah et al., 2009). Real Time RT-PCR determinations were performed on at least two independent biological samples from two replicate experiments. Real Time RT-PCR reactions were carried out three times for each biological sample, with the threshold cycle (Ct) determined in duplicate. Only Ct values leading to a Ct mean with a standard deviation below 0.3 were considered. The relative levels of transcription were calculated by using the 2−ΔΔct method (Livak and Schmittgen 2001). Data were subjected to ANOVA and then to Fisher’s protected least-significant difference (PLSD) or Duncan’s multiple range tests when appropriate by using SPSS.

2.7. Antibody production

A rabbit polyclonal anti-GintAMT2 antibody was produced from a 16 amino acid synthetic peptide designed from the deduced amino acid sequence of GintAMT2 (Biomedal, Sevilla, Spain). The selected peptide was PLDSDMVLKENKYFPGD (amino acids 6–21), located at the extracellular N-terminus of the protein. The serum was analyzed for the presence of antibodies that recognized the peptide by protein gel blot analyses. The antibodies were then purified by affinity chromatography using an affinity resin containing the peptide (Biomedal, Sevilla, Spain). The affinity purified antibodies cross-reacted with immunoblots of the GintAMT2 peptide (data not shown), whereas the antibodies pre-blocked with the synthetic peptide (1:3, v/v) showed no cross-reaction.
**2.8. Isolation of microsomes**

A microsomal protein fraction of *G. intraradices* was extracted as previously described (Benabedellah et al., 2009). Briefly, extracellular mycelium of *G. intraradices* was homogenized with a pestle and mortar in a cold homogenization buffer (1:2, w/v) consisting of 25 mM Tris–HCl pH 7.5, 250 mM sucrose, 2 mM EDTA pH 8, 1 mM PMSF and 10% PVPP (w/v). The homogenate was loaded on to a QiAShredder spin column (Qiagen) and centrifuged at 13,000 g for 40,000 rpm (Beckman 50 Ti rotor) for 1 h at 4 °C to sediment the “total membrane fraction”. The pellet was resuspended in keeping buffer containing 2 mM Tris–HCl pH 7.5, 250 mM sucrose, 2 mM EDTA pH 8 and 1 mM PMSF, and stored at −80 °C until analysis.

Yeast microsomal proteins were extracted as described by Pérez-Castiñeira et al. (2002) with minor modifications. Briefly, cells were grown in selective medium, collected by centrifugation, resuspended in cold homogenization buffer (150 mM Tris–HCl pH 7.5, 150 mM KCl, 15 mM EDTA pH 8, 2.5 mM DTT and 0.5 mM PMSF), and disrupted with glass beads by vortexing for 1 min three times with intervals of 30 s on ice. Cell homogenates were diluted 1:4 (v/v) with ice-cold GTED20 buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8, 1 mM DTT, 20% glycerol, 5 μg/ml pepstatin and 5 μg/ml chymostatin) and centrifuged for 15 min at 3000 rpm (Sorvall SS-34 rotor). The resulting supernatant was centrifuged for 1 h at 16,000 rpm (Sorvall SS-34 rotor) to sediment the microsomes. The pellet was resuspended in 150 μl of ice-cold GTED20 buffer supplemented with 1 mM PMSF and 10 μg/ml pepstatin and stored at −80 °C until analysis.

Protein contents were determined using the Bradford assay (Bradford, 1976), with bovine serum albumin (BSA) as standard.

**2.9. SDS/PAGE and Western-blot analysis**

Microsomal membrane proteins were incubated at 65 °C for 15 min in SDS–PAGE sample buffer, separated by 10% SDS–PAGE electrophoresis and transferred to a PVDF membrane by electroblotting. Blots were blocked in blocking solution (5% (w/v) BSA, 0.1% (v/v) Tween-20 in PBS) for 1 h at room temperature under gentle agitation on an orbital shaker, washed twice with PBS-T (PBS containing 0.1% (v/v) Tween-20) and incubated overnight with a 1:10,000 dilution of the affinity purified anti-GintAMT2 in PBS containing 0.5% (w/v) BSA at 4 °C under gentle agitation. After washing three times in PBS-T, the membrane was incubated with a 1:10,000 dilution peroxidase-conjugated goat anti-rabbit IgG (Sigma) in PBS containing 0.5% (w/v) BSA for 1 h at room temperature. Finally, the membrane was washed twice with PBS-T for 15 min and once with milli-Q water, and detected using the ECL Western Blotting Detection Kit (Amersham), following the manufacturer’s instructions.

**2.10. Immunolocalization of GintAMT2**

Freshly harvested extraradical mycelium from the hyphal compartment of a control plate was immediately fixed in 4% (v/v) paraformaldehyde in PBS. After vacuum infiltration, fixation was performed overnight at 4 °C. The fixed samples were hand sectioned using a double-edged razor blade. Sections were washed three times in PBS and left dry onto APTES (Sigma)-coated multiwell slides for 1 h at room temperature. After fixation, samples were firstly permeabilized by dehydration–rehydration through a graded series of methanol (5 min each at 30%, 50%, 70% and 100% methanol in PBS, and 70%, 50%, 30% and 0% methanol in PBS). A second permeabilization step was the partial digestion of the fungal cell walls with 0.05 U chitinase from *Streptomyces griseus* (Sigma) in 30 μl PBS for 30 min at 37 °C. After digestion, samples were treated with 0.3% (v/v) Triton X-100 in PBS at 37 °C for 10 min, washed three times with PBS (5 min each), and blocked with 5% (w/v) BSA in PBS at room temperature for 30 min. The slides were incubated for 1 h at 37 °C in a humid chamber with the affinity purified anti-GintAMT2 (1:50 in PBS containing 1% BSA). After three washes with 1% (w/v) BSA in PBS (5 min each) and blocking with 5% (w/v) BSA in PBS at room temperature for 10 min, slides were exposed to secondary antibodies (Alexa Fluor 568 anti-rabbit IgG; Molecular Probes) applied 1:25 in PBS for 45 min at room temperature in the dark. After five more washings in PBS (5 min each), samples were incubated in 0.05 mg/ml WGA conjugated to Alexa Fluor 488 (Molecular Probes) in PBS for 30 min at room temperature in the dark to stain the fungal wall. Finally, samples were washed twice with PBS (5 min each) and once with milli-Q water, counterstained with DAPI (1 μg/ml in water) for 10 min at room temperature in the dark, washed twice with milli-Q water (10 min each) and mounted with Mowiol 4–88 (Polysciences).

Observations were performed on a confocal laser scanning microscope (CLSM) (Leica TCS-SP2-A0BS) at the appropriate channels of excitation and emission corresponding to DAPI (to visualize the nuclei), WGA-Alexa488 (to visualize the fungal wall), and Anti-GintAMT2-Alexa568 (to visualize the immunofluorescence signal). Z-series of optical sections of 0.5–1.0 μm intervals were collected. Images were taken from the projection of series of 15–20 optical sections. Differential Interference Contrast (DIC, Nomarski) images were also taken.

**2.11. Laser microdissection (LMD)**

Medicago truncatula seedlings were inoculated with *G. intraradices* using the Millipore sandwich method described by Guether et al. (2009b). Mycorrhizal roots were dissected into about 5 mm pieces and placed in RNase-free tubes containing freshly prepared Farmer’s fixative (absolute ethanol/glacial acetic acid, 3:1, v/v). After a step under vacuum at room temperature for 20 min, fixative solution was changed and root samples were incubated at 4 °C overnight. Roots were subsequently dehydrated in a graded series of ethanol (50%, 70%, 90%, 100% and 100% in sterilized water) followed by two incubations with Neoclear (Merck), with each step on ice for 30 min. Neoclear was gradually replaced with paraffin (Paraplast Plus; Sigma). In detail, about 10 Paraplast Plus chips were added to 20 ml of fresh Neoclear and samples were leaved for 2 h at room temperature and then for 3 h at 58 °C. Once the chips dissolved at 58 °C, the mixture was replaced with molten Paraplast Plus at 58 °C, and the medium was changed twice approximately at 4.5 h intervals before embedding in pure paraffin. Root samples were embedded in paraffin in Petri dishes and stored at 4 °C. A Leica AS LMD system (Leica Microsystems, Inc.) was used to collect arbuscule–colonized cortical cells from paraffin root sections as described by Balestrini et al. (2007). RNA was extracted following the Pico Pure kit (Arcturus Engineering) protocol. DNase treatment was performed using RNA-free DNase Set (Qiagen) in Pico Pure column, following the manufacturer’s instructions. RNA was then quantified using a NanoDrop 1000 spectrophotometer. All RT–PCR assays were carried out using the One Step RT–PCR kit (Qiagen), DNA contamination in RNA samples was evaluated using GintEF1α specific primers of *G. intraradices*. For the RT–PCR analyses, the samples were incubated for 30 min at 50 °C, followed by 15 min of incubation at 95 °C. Amplification reactions were run for 40 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s using the GintAMT1 and GintAMT2 specific primers used for the Real-Time PCR assays, and specific primers for the mycorrhiza-specific plant phosphate transporter MtPT4 (Zocco et al., 2011) and the fungal phosphate transporter GintPT (Table 1). The RT–PCR experiments were conducted on two independent
biological replicates. The PCR products were visualized by agarose gel electrophoresis.

3. Results

3.1. Sequence analysis of GintAMT2

A G. intraradices full-length cDNA clone showing high similarity to previously described AMT genes from other fungi was isolated. This gene (GenBank Accession No. FM993985) was named GintAMT2 according to the nomenclature proposed for AM fungal genes (Franken, 2002). The full-length cDNA contains an open reading frame of 1413 nucleotides, a 5' untranslated region of 206 bp and a 3' untranslated region of 96 bp. The deduced protein has a length of 471 amino acids with a calculated molecular mass of 50 kDa. In silico analysis of protein topology reveals 11 transmembrane domains in the deduced amino acid sequence of GintAMT2 according to the prediction of the TMHMM algorithm, with an extracellular N-terminus and a cytosolic C-terminus (Fig. 1A). This structure is typical of the Mep/AMT proteins (Thomas et al., 2000). GintAMT2 contains all residues that are required or predicted to be essential for NH$_4^+$ transport, such as the aspartic acid D175, positioned at the outer pore of the NH$_4^+$ channel (Khademi et al., 2004), and the two histidine residues H183 and H332 within the NH$_4^+$ channel (Javelle et al., 2006). Moreover, the GintAMT2 protein contains the Ammonium Transporter Signature

Fig. 1. (A) Proposed topological model of G. intraradices AMT2 according to the prediction of the TMHMM algorithm. Putative transmembrane domains are labeled in white, residues corresponding to the ammonium transporter signature (ATS) are shown in black and residues that are required or predicted to be essential for ammonium transport activity are shown in white in a dark grey box. (B) Alignment of the ATS sequences. The boxed amino acid in the GintAMT2 sequence represents a residue that does not match the signature sequence. (C) Phylogenetic relationships between the deduced amino acid sequence of GintAMT2 (G. intraradices) and other fungal ammonium transporters. Sequences were obtained from the GenBank database with the following accession numbers: Glomus intraradices (GintAMT1: CA542776; GintAMT2: CA524900), Hebeloma cylindrosporum (HcAMT1: AAM21092, HcAMT2: AAH2416, HcAMT3: AAK82417), Tuber borchii ( TbAMT1: AAL1032), Ustilago maydis (UmMEP1: AAL08424, UmMEP2: AAO42611), Saccharomyces cerevisiae (ScMEP1: P40260, ScMEP2: P41948, ScMEP3: P53390), Schizosaccharomyces pombe (SpAMT1: NP_588424, SpAMT2: NP_593462), Aspergillus nidulans (AnMEAA: AA731317, AnMEPA: AA73118), Fusarium fujikuroi (FfMEPA: CAJ44733, FfMEPB: CAJ44734, FfMEPC: CAK55531), Cryptococcus neoformans (CnAMT1: XP_566614, CnAMT2: XP_567361), Synechocystis sp. (NP_442561).
BLAST searches in the protein sequence database indicated that GintAMT2 shows the highest similarity to the functionally characterized AMTs of mycorrhizal fungi, such as GintAMT1 of G. intraradices (61% identity; López-Pedrosa et al., 2006), and HcAMT1 and TbAMT1 of the ectomycorrhizal fungi Hebeloma cylindrosporum (60% identity; Javelle et al., 2001) and Tuber borchii (57% identity, Montanini et al., 2002), respectively. A phylogenetic analysis of GintAMT2 with a number of functionally characterized AMTs from other fungi showed that GintAMT2 clusters with the high-affinity NH\textsubscript{4}\textsuperscript{+} transporters in subgroup I (Fig. 1C) (Javelle et al., 2003).

To get further insights into the structure of the GintAMT2 gene, the genomic clone was also isolated. The genomic sequence was 2985 bp long (GenBank Accession No. FR744743). Comparison between genomic and cDNA sequences showed that the transcription start site was located at position -206 (relative to ATG) and that GintAMT2 contains three introns at positions 106, 315, 1227, with a size of 107, 86 and 77 bp, respectively. The first two introns are flanked by the characteristic splicing sequences GT and AG at the 5' and 3' ends, respectively. The 5' flanking region of GintAMT2 (1381 bp upstream of the translation initiator ATG) was searched for putative regulatory elements present in the promoter sequences of the nitrogen-regulated genes of yeast and filamentous fungi (Wong et al., 2008). Two GATA core sequences acting as putative DNA binding domains of the GATA transcription factors, key regulators of N metabolism in all fungal species studies thus far, were found at positions -422 and -28 (relative to the transcription start site). Additionally, the carbon-response elements CWTCtC and CGGANNtA that in Saccharomyces cerevisiae recognize Gcr1p, a transcriptional activator of genes involved in glycolysis (Huie and Baker, 1996), and Rtg1 which plays a central role in the glucose-induced expression of hexose transporter genes (Ozcan et al., 1996; Kim and Johnston, 2006), were found at the positions -985 and -927, respectively.

3.2. GintAMT2 is a functional AMT

To investigate whether GintAMT2 encodes a functional AMT, the open reading frame of the cDNA was expressed in the yeast triple mep mutant 31019b under the control of the constitutive yeast phosphoglycerate kinase gene promoter (Minet et al., 1992). This strain is defective in all three endogenous NH\textsubscript{4}\textsuperscript{+} transporters (Mep1, Mep2 and Mep3) and is thus unable to grow on a medium containing ≤5 mM NH\textsubscript{4}\textsuperscript{+} as the sole N source (Marini et al., 1997). To compare GintAMT2 with the already described G. intraradices NH\textsubscript{4}\textsuperscript{+} transporter GintAMT1 (López-Pedrosa et al., 2006), the coding sequence of GintAMT1 was also expressed in the mutant yeast. As found for GintAMT1, GintAMT2 was able to complement the growth defect of the mutant yeast strain to grow in the presence of 1 mM NH\textsubscript{4}\textsuperscript{+} as the sole N source, indicating that GintAMT2 encodes a functional NH\textsubscript{4}\textsuperscript{+} transporter. However, GintAMT1 restored more efficiently than GintAMT2 the growth defect of triple mep.A cells (Fig. 2). Since the GintAMT2-expressing yeast cells were unable to take up the NH\textsubscript{4}\textsuperscript{+} analogue methylammonium, the kinetics of NH\textsubscript{4}\textsuperscript{+} transport by GintAMT2 could not be determined.

To assess if GintAMT2 complementation depended on external pH, growth tests were performed at initial pH values ranging from 4.5 to 7.5 on minimal medium containing 1 mM NH\textsubscript{4}\textsuperscript{+} as the sole N source. Although yeast cells transformed with the empty vector showed better growth at high pH, growth complementation by either GintAMT1 or GintAMT2 was not affected by external pH (data not shown).

3.3. GintAMT2 response to nitrogen

To validate the in silico analysis of the GintAMT2 promoter, that is, to determine whether GintAMT2 gene expression is regulated by N, its expression was analyzed in the extraradical mycelium of G. intraradices developed in liquid M–C medium in the presence of either 3.6 or 0.8 mM NO\textsubscript{3}\textsuperscript{-} and then exposed for different periods of time to a N-free medium. In contrast with what we observed for GintAMT1, whose transcript levels increased when the fungus was grown in a low-N M medium, GintAMT2 expression levels were not affected by the N content of the culture media. While exposure of the mycelia grown either in standard- or low-nitrogen M–C media to a N-free medium induced a transient accumulation of GintAMT1 transcript levels, those of GintAMT2 were not affected (Fig. 3A).

To further investigate the effect of N on GintAMT2, we also determined whether the addition of different N sources to the N-deprived mycelia had an effect on its expression. An accumulation of GintAMT2 transcripts was observed 24 h after the addition of either 30 μM NH\textsubscript{4}\textsuperscript{+} or NO\textsubscript{3}\textsuperscript{-}. Addition of 3 mM NH\textsubscript{4}\textsuperscript{+} did not affect GintAMT2 expression at any of the two analyzed time-points, whereas GintAMT2 was up-regulated 24 and 48 h upon exposure of the N-deprived mycelia to 3 mM NO\textsubscript{3}\textsuperscript{-}. GintAMT2 transcript levels also increased 48 h after supplementation of the N-free media with 2.5% of an amino acid-mixture (Fig. 3B).

3.4. Effect of different carbon sources on gene expression

Our in silico analysis of the GintAMT2 promoter suggested that this gene might be regulated by carbon. To investigate if transcription of the G. intraradices ammonium transporters was affected by a carbon supply, GintAMT1 and GintAMT2 expression were assessed in the extraradical mycelia of G. intraradices that had been either supplemented with glucose in the root compartment of a split-petri dish or with acetate, a carbon source taken up and assimilated by the extraradical mycelia (Pfeffer et al., 1999), in the hyphal compartment. Gene expression analyses were performed 2 and 7 days after carbon supplementation. When glucose was added to the root compartment, GintAMT1 and GintAMT2 expression were transiently down-regulated. However, a different gene expression pattern was observed when the G. intraradices extraradical mycelia had been directly exposed to acetate. While GintAMT2 expression was down-regulated at the two time-points analyzed, GintAMT1 was just slightly reduced 7 days after the addition of acetate (Fig. 4).
intraradices-colonized carrot roots lacking extraradical mycelia and on RNA isolated from quiescent spores. Mycorrhizal colonization of the carrot roots was 8–10%. Specific amplification of fungal material with the primers designed to amplify GintAMT1 and GintAMT2 was confirmed by performing conventional PCR reactions on G. intraradices and carrot genomic DNAs and on cDNAs from G. intraradices extraradical mycelia and from carrot and *Medicago* non-mycorrhizal roots (data not shown). Transcript levels of GintAMT1 and GintAMT2 accumulated differentially in the different

### 3.5. GintAMT2 is up-regulated during the intraradical symbiotic phase

As a step toward understanding the role of GintAMT1 and GintAMT2 in *G. intraradices*, we also investigated whether GintAMT1 and GintAMT2 were differentially expressed during the fungal life cycle. This was initially done by analyzing GintAMT1 and GintAMT2 expression on RNA from extraradical hyphae developed in the hyphal compartment of the split-petri dishes, on RNA from extraradical mycelia developed in the hyphal compartment of the control split-petri dishes and on RNA isolated from quiescent spores. Mycorrhizal colonization of the carrot roots was 8–10%. Specific amplification of fungal material with the primers designed to amplify GintAMT1 and GintAMT2 was confirmed by performing conventional PCR reactions on *G. intraradices* and carrot genomic DNAs and on cDNAs from *G. intraradices* extraradical mycelia and from carrot and *Medicago* non-mycorrhizal roots (data not shown). Transcript levels of GintAMT1 and GintAMT2 accumulated differentially in the different
fungal structures. In the symbiotic intraradical phase, that is, the mycorrhizal roots lacking extraradical mycelium, the expression levels of GintAMT2 were higher than those of GintAMT1; however, GintAMT1 was more highly expressed than GintAMT2 in the extraradical hyphae and in the spores (Fig. 5).

3.6. Immunolocalization of GintAMT2

Having established that GintAMT2 was more highly expressed in the intraradical fungal structures, we decided to investigate the location of the encoded protein by using polyclonal antibodies raised against a peptide corresponding to the 6–21 amino acids at the N-terminus of GintAMT2. Affinity-purified antibodies cross-reacted with the peptide, whereas the preimmune serum showed no cross-reaction (data not shown). Although our in silico analyses revealed that the amino acid stretch selected to raise the antibodies against GintAMT2 was absent in the coding sequence of the other AMTs found in the G. intraradices transcriptome (Tisserant et al., submitted), specificity of the affinity-purified antibodies for GintAMT2 was checked by protein gel blot analysis of microsomal membrane fractions isolated from the 31019b yeast cells expressing the available sequences of GintAMT1 (López-Pedrosa et al., 2006) or GintAMT2, and from G. intraradices extraradical mycelia. A protein with an apparent molecular mass of 50 kDa was detected in the microsomal fractions of G. intraradices and of the GintAMT2-expressing yeast cells. However, no protein signal was detected in the microsomal fraction isolated from the GintAMT1-expressing cells, the yeast cells transformed with the empty vector (Fig. 6A) and in the blots incubated with the preimmune serum (data not shown).

With the specificity of the antibody confirmed we performed immunofluorescence detection of GintAMT2 in the extraradical mycelia of G. intraradices and in mycorrhizal carrot roots. The GintAMT2 antibody was detected with a secondary antibody conjugated to Alexa Fluor 568 (red signal) and the sections were counterstained with a wheat germ agglutinin (WGA)-Alexa Fluor 488 conjugate to localize chitin and to expose the fungus as a green fluorescent signal and with DAPI to reveal the nuclei. The red fluorescence pattern obtained by confocal microscopy of the immunostained extraradical fungal structures indicated a plasma membrane location of GintAMT2 in the extraradical hyphae (Fig. 6B.9) and in the spores (Fig. 6C.4). Controls with the antibodies pre-blocked with the synthetic peptide showed no labeling (Fig. 6B.4). Unfortunately, cross-reactivity of the antibodies with the plant proteins hampered detection of the GintAMT2 protein in the intraradical fungal structures (data not shown).
AMT homologs of a fungal species is needed to understand the specific contribution of each AMT to the adaptation of the fungus to particular growth conditions and/or to overall \( \text{NH}_4^+ \) uptake.

In this study, the cloning and characterization of the \( G. \) intraradices gene \( GintAMT2 \), a second member of the AMT family of this AM fungal species, is reported. The encoded protein shares a high level of sequence identity to other AMTs of the AMT/MEP family and is able to complement a \( S. \) cerevisiae \( \text{mepl}\text{mep2}\text{mep3} \) triple mutant. \( GintAMT2 \)-mediated \( \text{NH}_4^+ \) uptake into the yeast cells presumed a localization of the corresponding protein in the plasma membrane, which was further confirmed with anti-\( GintAMT2 \) specific polyclonal antibodies. In \( G. \) intraradices, \( GintAMT2 \) was indeed shown to be located in the plasma membrane. As it has been reported for the \( A. \) nidulans ammonium permease \( \text{MepC} \) (Monahan et al., 2006), no methylammonium transport attributable to \( GintAMT2 \) activity was detected under the conditions tested. Although the kinetic properties of \( GintAMT2 \) could not be determined, its close phylogenetic relationship with the high affinity AMTs of other fungi suggests that it might encode a high-affinity and low capacity AMT. Despite the high sequence identity between \( GintAMT1 \) and \( GintAMT2 \), the improved growth of the \( GintAMT1 \)-expressing yeast cells compared with the \( GintAMT2 \)-expressing cells and the finding that \( GintAMT2 \) does not transport methylammonium suggest that these two AMTs are functionally different.

Differential accumulation of \( GintAMT1 \) and \( GintAMT2 \) transcripts in the different fungal structures also suggests that their encoded proteins might play different physiological roles in the symbiosis. The higher expression levels of \( GintAMT1 \) in the extraradical mycelium suggests a preferential role for its gene product in fungal \( \text{N} \) acquisition from the soil, while increased expression of \( GintAMT2 \) in the intraradical fungal structures suggests a preferential role in the symbiotic association. However, detection of \( GintAMT1 \) and \( GintAMT2 \) transcripts in the LMD arbuscule–colonized cortical cells indicates that both transporters might have overlapping physiological functions in the symbiotic interface. Given that current experimental evidence supports a role for AMT proteins in \( \text{NH}_4^+ \) uptake (Khamedi et al., 2004; Lamoureux et al., 2010) and that \( \text{NH}_4^+ \) is the \( \text{N} \) form taken up by the plant at the arbuscular interface (Govindarajulu et al., 2005; Tian et al., 2010), expression of AMT genes in the arbuscules indicates that there might exist a competition between the plant and the fungus for the nitrogen that is present in the interfacial apoplasm (Guether et al., 2009a). Since our gene expression analyses on LMD cells are not quantitative, the relative contribution of each individual transporter in the arbuscules remains to be determined. Expression of \( GintAMT2 \) in the intercellular hyphae the fungus develops inside the root could also account for the higher expression level of this gene in the symbiotic intraradical phase. Unfortunately, cross-reactivity of the \( GintAMT2 \) antibodies with the plant proteins precluded determining the precise intraradical fungal structures where the protein is located.

Detection of the \( GintAMT2 \) protein in the fungal structures the fungus develops outside the root, that is, hyphae and spores, and accumulation of \( GintAMT1 \) and \( GintAMT2 \) transcripts not only in the extraradical hyphae, the fungal structures involved in nutrient uptake from the soil, but also in arbuscules and spores suggests a role for these AMTs in processes other than \( \text{N} \) uptake for nutrition. Interestingly, some fungal AMTs (\( \text{Mep2} \) in \( S. \) cerevisiae, \( \text{UmMep2} \) in \( U. \) maydis, \( \text{CaAMT2} \) in \( C. \) albicans, \( \text{SpAMT1} \) in \( S. \) pombe and \( \text{MepB} \) in \( F. \) fujikuroi) have been shown to be required for a switch from single-cell growth to filamentous growth in response to \( \text{N} \) limitation and a role for these transporters as nitrogen-sensors has been proposed (Lorenz and Heitman, 1998; Smith et al., 2003; Biswas and Morschhauser, 2005; Mitsuazawa, 2006; Teichert et al., 2008). In the plant pathogen \( C. \) gloeosporioides, it has been recently reported that \( \text{NH}_4^+ \) accumulation has a direct effect on germ-tube differentiation, fungal appressorium formation

**Fig. 7.** Gel electrophoresis of RT-PCR products obtained from RNAs isolated from LMD arbuscule–colonized cortical cells (Arb.), using specific primers for \( GintAMT1 \) and \( GintAMT2 \). \( GintTEF \), \( GintPT \) and \( MlPT4 \) amplicons, obtained from parallel control reactions, were used as internal standards.

### 3.7. \( GintAMT1 \) and \( GintAMT2 \) are expressed in arbuscules

Given the importance of the arbuscule–cortical cellular interface in nutrient exchange and to get further insights into the potential role of the \( G. \) intraradices AMTs in the symbiosis, RT-PCR experiments were performed in RNA from microdissected (LMD) cortical cells containing arbuscules. Approximately 2000 \( M. \) truncatula cortical root cells containing arbuscules were collected and two independent biological samples were considered. As a positive control for the presence of the fungus, expression of \( GintEF1a \) was monitored (Fig. 7). LMD samples were validated by analyzing expression of the mycorrhiza-specific plant phosphate-transporter \( MlPT4 \) (Harrison et al., 2002) and of the fungal phosphate transporter \( GintPT \), which can be considered positive controls for arbusculated cells. RT-PCR assays showed that both \( GintAMT1 \) and \( GintAMT2 \) were expressed in arbuscules, suggesting that both transporters are active in the arbuscular interface.

### 4. Discussion

Fungi frequently encode multiple AMT proteins. Of the sequenced fungal genomes, for example, \( S. \) cerevisiae contains three homologs (Marini et al., 1994, 1997). \( H. \) cylindrosporum has three (Javelle et al., 2001, 2003), \( L. \) bicolor has eight (Lucic et al., 2008), and \( U. \) maydis has two (Smith et al., 2003). The main function of the fungal AMTs is to act as \( \text{NH}_4^+ \) permeases to import \( \text{NH}_4^+ \) either to obtain \( \text{N} \) as a nutrient source or to retrieve \( \text{NH}_4^+ \) that has leaked out during metabolism (Marini et al., 1997). However, some fungal AMTs have evolved a regulatory function. Examples include \( \text{Mep2} \) of \( S. \) cerevisiae (Lorenz and Heitman, 1998), \( \text{SpAMT1} \) of \( S. \) pombe (Mitsuazawa, 2006) and \( \text{UmMep2} \) of \( U. \) maydis (Smith et al., 2003), which are required for the induction of filamentous growth under low-nitrogen conditions. Therefore, fully characterization of individual
and differentiation responses that affect pathogenicity (Miya
ta et al., 2010). These authors have hypothesized that modulation of processes regulating NH₄⁺ accumulation, as found in host plants (Pageau et al., 2006), may contribute to the rapid colonization of the host and the transition from biotrophic-quesicent to active necrotrophic infections. Unfortunately, the lack of mutant screen-
ing and stable transformation systems for AM fungi (Harrier and Millam, 2001; Helber and Requena, 2008) hampers functional characterization of the individual AMT proteins. However, it is tempting to speculate that the spacer and arbuscule AMTs might play a role in intracellular NH₄⁺ retention leading to regulation of fungal development.

Differential transcript accumulation of GintAMT1 and GintAMT2 in response to N availability supports previous findings suggesting that the AMT genes are differentially regulated within a particular fungal species. In the G. intraradices extraradical mycelium, GintAMT1 was induced under low N conditions while GintAMT2 was constitutively expressed. This expression pattern is similar to that of the Aspergillus nidulans genes encoding the NH₄⁺ permease MeaA and MepA and of the Cryptococcus neoformans AMT1 and AMT2 genes, with MeaA and AMT1 being constitutively expressed and MepA and AMT2 induced by N limitation (Monahan et al., 2002; Rutherford et al., 2008). In S. cerevisiae and some filamentous fungi, such as A. nidulans, N. crassa and Fusarium fujikuroi, expression of the NH₄⁺ permeases is regulated by the GATA transcription fac-
tors, a family of transcription factors that activate gene expression when levels of preferred N sources become limiting (Marzulj, 1997). This regulatory system is known as N metabolite repression in filamentous fungi (Wiam et al., 1985) or N catabolite repression in S. cerevisiae (Cooper and Sumrada, 1983). The identification of two GATA core sequences in the promoter sequences of GintAMT1 and the observation that GintAMT1 presents an expression profile typical of genes subjected to N metabolite repression suggests that these mechanisms might also operate in AM fungi and supports the view that the N regulatory mechanisms may be conserved within fungi. Although in the absence of knock out mutants it is difficult to define the precise physiological role of the different G. intraradices AMTs, by analogy with what happens in other fungal species, induction of the high-affinity AMT GintAMT1 in response to N limitation suggests that it probably acts to scavenge NH₄⁺. However, constitutive expression of GintAMT2 in N-limiting condi-
tions and transitory induction after the addition to the N-starved mycelia of different N sources suggests a role for this transporter in the processes regulating the differentiation of different from the metabolism of different nitrogen sources, as it has been shown for the A. nidulans MeaA permease (Monahan et al., 2006).

Transient induction of GintAMT2 mRNA levels in response to low concentrations of NH₄⁺ is in agreement with previous data using a similar model system showing transient induction of the enzymatic activity of key enzymes of the anabolic arm of the urea cycle (Cruz et al., 2007). The lack of response to high NH₄⁺ concen-
trations supports our hypothesis that GintAMT2 is a high-affinity transporter and suggests that under these conditions NH₄⁺ uptake might be mediated by an as yet uncharacterized low affinity transporter. Because all endogenous or exogenous nitrogen sources are eventually converted to NH₄⁺ and/or amino acids and since it has been proven that the addition of a high nitrate concentration to the extraradical mycelium up-regulates a nitrate transporter and several nitrogen metabolizing enzymes at shorter times than used in this study (Tian et al., 2010), induction of GintAMT2 expression 24 h after the addition of nitrate could occur through changes in the NH₄⁺ or amino acid intracellular levels. This hypothesis is sup-
ported by our finding that the addition of low NH₄⁺ concentrations or an amino acid pool induced GintAMT2 expression. In this sense, it was found that the intracellular level of glutamine regulates AMT gene expression in H. cylindrosporum (Javelle et al., 2003).

In addition to the regulation by nitrogen, GintAMT1 and Gin-
tAMT2 were regulated in response to carbon. A carbon supply would have been expected to increase the amount of carbon skel-
etons available for the synthesis of amino acids and, therefore, the fungal N demand and uptake. However, GintAMT1 and GintAMT2 expression was down-regulated after the addition of either glucose to the root compartment or acetate to the hyphal compartment of the split Petri. Further experiments, such as determining the intra-
cellular NH₄⁺ and amino acids concentrations or the existence of carbon-dependent mechanisms of gene regulation in AM fungi, are needed to elucidate the underlying regulatory mechanisms.

In conclusion, data presented in this work show that the G. intraradices AMT family is comprised of at least two members. While GintAMT1 appears to act mainly as an NH₄⁺ scavenger under N limiting conditions, GintAMT2 seems to play a role in NH₄⁺ reten-
tion. Our gene expression data also suggest that, like in other fun-
gal species, the G. intraradices AMT proteins play a role not only in N uptake for fungal nutrition but also to retrieve NH₄⁺ that has leaked out during plant metabolism. Development of a transformation system for AM fungi is needed to determine whether there is cer-
tain redundancy between the G. intraradices AMT genes, to define their precise physiological role and to determine whether they might also have a regulatory function.

Acknowledgments

We are grateful to Ascensión Valderas for excellent technical assistance. This research was supported by the Spanish Ministry of Science and Education (Project AGL2006-08218/AGR and AGL2009-08868). Jacob Pérez-Tienda was supported by a PhD con-
tact (ISP) from the Spanish National Research Council (CSIC) and Valentina Fiorilli by a grant from the BIOBIT-CIPE (Piedmont Re-
gion Project).

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