Available online at www.sciencedirect.com
SCIENCE DIRECT
Fungal Genetics and Biology 43 (2006) 102–110
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

# *GintAMT1* encodes a functional high-affinity ammonium transporter that is expressed in the extraradical mycelium of *Glomus intraradices*

Agustín López-Pedrosa [1], Manuel González-Guerrero [1], Ascensión Valderas, Concepción Azcón-Aguilar, Nuria Ferrol *

*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*

Received 14 July 2005; accepted 31 October 2005
Available online 28 December 2005

**Abstract**

We report the cloning and characterization of the first $NH_4^+$ transporter gene (*GintAMT1*) in an arbuscular mycorrhizal fungus. *GintAMT1* encodes a polypeptide of 479 amino acids sharing high sequence similarity with previously characterized $NH_4^+$ transporters from other fungi. Heterologous expression of *GintAMT1* in the yeast triple *mep* mutant complemented the defect of this strain to grow in the presence of less than 1 mM $NH_4^+$. As revealed by [$^{14}$C]methylammonium uptake experiments carried out in yeast, *GintAMT1* encodes a high-affinity $NH_4^+$ transporter. In mycelia developed in the presence of 0.9 mM $NO_3^-$, *GintAMT1* transcription was increased after the addition of 30 μM $NH_4^+$ but decreased after the addition of 3 mM $NH_4^+$. However, in mycelia grown in the presence of higher N concentrations, *GintAMT1* transcripts decreased after the addition of $NH_4^+$, irrespective of the concentration used. These data suggest that *GintAMT1* is involved in $NH_4^+$ uptake by the extraradical mycelia from the surrounding media when it is present at micromolar concentrations.


*Keywords:* Arbuscular mycorrhizal fungi; Nitrogen nutrition; Ammonium transport; Root organ culture

## 1. Introduction

The roots of most plant species are colonized by arbuscular mycorrhizal (AM) fungi to form mutually beneficial symbiotic associations called mycorrhizas. AM fungi, belonging to the fungal phylum Glomeromycota (Schüßler et al., 2001), are obligate biotrophs which depend entirely on the plant for their carbon supply (Bago and Bécard, 2002). The fungus in return provides the plant with essential mineral nutrients that are taken up from the soil by the extraradical mycelium and translocated to the plant (Smith and Read, 1997). Within the cortical cells of the root the fungus forms finely branched hyphal structures, the arbuscules, which are surrounded by a specialized plant membrane and which are believed to be the site of bi-directional nutrient transfer (Ferrol et al., 2002).

The main benefit of the AM association is an improved P status of the mycorrhizal plant, but there is increasing evidence that AM fungi also contribute to the N-budget of the plant. Different authors have shown the ability of the extraradical mycelium of AM fungi to deplete the inorganic $^{15}N$, added as $^{15}NH_4^+$ to the soil of a root-free compartment (Frey and Schüepp, 1993; Johansen et al., 1992, 1993) and that AM extraradical hyphae were able to transport this N-isotope to a plant which was growing several centimetres away from the nitrogen source. The capability of AM extraradical hyphae to take up (Bago et al., 1996) and transport $^{15}NO_3^-$ to the host plant has also been reported (Tobar et al., 1994a,b). Much less is known about the uptake of organic nitrogen, but Hawkins et al. (2000) showed that AM hyphae are able to take up glycine and glutamic acid and transport nitrogen from these sources to the plant roots in a form that is presently unknown.

* Corresponding author. Fax: +34 958 129600.
*E-mail address:* nferrol@eez.csic.es (N. Ferrol).
[1] The two first authors have contributed equally to this work.

1087-1845/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2005.10.005

Moreover, Hodge et al. (2001) reported that the AM symbiosis can both enhance decomposition of and increase nitrogen capture from complex organic material in soil.

Although AM fungi are able to take up both $NO_3^-$ and $NH_4^+$, a clear preference for $NH_4^+$ has been demonstrated (Hawkins et al., 2000; Toussaint et al., 2004; Villegas et al., 1996), which is explained, at least in part, by the extra energy the fungus must expend in reducing $NO_3^-$ to $NH_4^+$ before it can be incorporated into organic compounds (Marzluf, 1996). Ammonium is a ubiquitous intermediate in nitrogen metabolism and one of the major nutrients for plants and microorganisms. The whole process of $NH_4^+$ assimilation in the AM symbiosis involves $NH_4^+$ uptake and assimilation by the AM fungus and transfer to the plant. Several lines of evidence indicate that the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle is responsible for $NH_4^+$ assimilation in AM extraradical hyphae (Breuninger et al., 2004; Johansen et al., 1996), although the involvement of glutamate dehydrogenase has not been experimentally excluded. Recently, Govindarajulu et al. (2005) have shown that inorganic nitrogen taken up by the external mycelium is incorporated into amino acids, translocated from the extraradical to the intraradical mycelium as arginine, but transferred to the plant as $NH_4^+$.

In most organisms, ammonium uptake is mediated by members of the $NH_4^+$ transporter family (AMT/MEP). The first of the genes involved in $NH_4^+$ transport were cloned from yeast (Marini et al., 1994) and *Arabidopsis* (Ninnemann et al., 1994). Since then, these genes have been found in a variety of bacteria (Thomas et al., 2000), fungi (Javelle et al., 2001, 2003b; Montanini et al., 2002) and homologues have been found in humans (Marini et al., 2000). Aside from their role in $NH_4^+$ uptake, $NH_4^+$ transporters can also act as $NH_4^+$ sensors. In yeast, the high-affinity transporter MEP2 has been considered to act as the sensor for low $NH_4^+$ availability and evidence suggests that it is associated with the signal transduction cascades leading to filamentous growth (Lorenz and Heitman, 1998). The objective of the present work was to characterize an $NH_4^+$ transporter of *Glomus intraradices* in order to get some insights into the mechanisms of nitrogen acquisition and sensing in AM fungi.

## 2. Materials and methods

### 2.1. Arbuscular mycorrhizal monoxenic cultures and N treatments

Arbuscular mycorrhizal monoxenic cultures were established as described by St-Arnaud et al. (1996). Briefly, clone DC2 of carrot (*Daucus carota* L.) Ri-T DNA transformed roots were cultured with the AM fungus *G. intraradices* Smith & Schenck (DAOM 197198, Biosystematic Research Center, Ottawa, Canada) in two-compartment Petri dishes. Cultures were initiated in one compartment ("root compartment") of each plate, which contained M medium (Chabot et al., 1992). Fungal hyphae, but not roots, were allowed to grow over to the second compartment ("hyphal compartment"), which contained M medium without sucrose and modified to provide different $NO_3^-$ concentrations (0.9, 1.8, or 3.6 mM). $Ca^{2+}$ and $K^+$ losses, resulting from the reduction of $NO_3^-$ salts, were compensated by the addition of the corresponding $Cl^-$ and $SO_4^{2-}$ salts, respectively. Plates were incubated in the dark at 24 °C until the transition from absorptive to sporulative phase was initiated (approximately 2–3 months from the establishment of the cultures). At this stage, 0, 30 μM, or 3 mM $(NH_4)_2SO_4$ pulses were applied to the hyphal compartment of the plates of the different $NO_3^-$ treatments by placing 500 μL of stock solutions in droplets dispersed throughout the compartment, allowing diffusion to distribute the added solution evenly. The time of $(NH_4)_2SO_4$ addition was referred as time 0 and mycelia were harvested 24 and 48 h after the pulse.

### 2.2. Isolation of the GintAMT1 cDNA

The 3′ end of *GintAMT1* was obtained by chance while trying to isolate the 3′ end of a different *G. intraradices* gene by rapid amplification of cDNA ends (RACE). The 5′ end of *GintAMT1* was obtained by RACE by using the SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA) and the *GintAMT1* specific primer AMT1 (5′-CTAAGAGCAGCAGCACCAGAAGAAA TG-3′) following the manufacturer's instructions. 5′ RACE reactions were performed using 1 μg total RNA extracted from *G. intraradices* extraradical mycelium grown in MC plates at the standard nitrate concentration (3.6 mM). PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced. The full-length clone was obtained by ligating the 3′ and 5′ fragments in a *Sac*II/*Mef*I-digested pGEM-T Easy vector.

### 2.3. Sequence analyses

Nucleotide sequences were determined by *Taq* polymerase cycle sequencing and using an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Computer database comparisons were performed using BLAST algorithm (Altschul et al., 1990) and computer translation by using the Translate tool from EXPASY Molecular Biology Server. Amino acid sequence comparisons were made with the BESTFIT program of the Genetics Computer Group (Madison, WI, USA), and multiple sequence alignments of translated gene sequences were carried out with the program CLUSTALW (version 1.5; Thompson et al., 1994). Genetic distances were estimated by using the Kimura's two-parameter method employed by PHYLIP (Felstein, 1993). The tree was displayed with the help of the TREEVIEW program (Page, 1996). Transmembrane domains of GintAMT1 were predicted by using SOSUI (Hirokawa et al., 1998).

### 2.4. Functional complementation assays

The full-length of *GintAMT1* was cloned into the *Not*I site of the yeast expression vector pFL61 (Minet et al., 1992).

The *Saccharomyces cerevisiae* strain 31019b (*mep1Δ mep2Δ*::*LEU2 mep3Δ*::*KanMX2 ura3*; Marini et al., 1997) was transformed with pFL61–GintAMT1 or with the empty vector, using a lithium acetate-based method (Schiestl and Gietz, 1989). Yeast transformants were first selected in SD medium by autotrophy to uracil and subsequently transferred to yeast nitrogen base without ammonium sulphate and amino acids (YNB-N)-glucose medium supplemented with various $(NH_4)_2SO_4$ concentrations as the sole nitrogen source (20, 5, 1, 0.5, and 0.1 mM).

### 2.5. [$^{14}$C]Methylammonium uptake assays

Initial rates of [$^{14}$C]methylammonium (Amersham Biosciences, Piscataway, NJ, USA) uptake were measured as described by Marini et al. (1994). Briefly, pFL61–*GintAMT1* and pFL61 transformed *mepΔ* cells were grown to the logarithmic phase in YNB-N medium supplemented with 2% glucose and 500 μg/mL L-proline. Cells were harvested at $OD_{620\,nm}$ of 0.5–0.7, washed, and resuspended in 20 mM sodium phosphate buffer, pH 7, to a final $OD_{620\,nm}$ of 8. Five minutes before the uptake measurements, cells were supplemented with 100 mM glucose and incubated at 30 °C. To start the reaction, 100 μL of this cell suspension was added to 100 μL of the same buffer containing different concentrations (3–400 μM) of [$^{14}$C]methylamine–HCl (57 mCi/mmol; Amersham Biosciences, Piscataway, NJ, USA), and after 0.5, 1, 2, and 4 min, aliquots were withdrawn, diluted in 4 mL of ice-cold sodium phosphate buffer containing 100 mM unlabeled methylammonium, and filtered through glass fibre filters (GF/C; Whatman International, Maidstone, UK). Filters were washed twice with 4 mL of distilled water and counted in a liquid scintillation spectrometer (TRI CARB 1500, Packard). For inhibition studies, different concentrations of $(NH_4)_2SO_4$ or 200 μM *N*,*N*′-dicyclohexylcarbodiimide (CCCP) were added to standard uptake assay mixtures containing 25 μM [$^{14}$C]methylammonium, which were then processed and counted as described above.

### 2.6. Pseudohyphal tests

Pseudohyphal growth test was performed as previously described by Gimeno et al. (1992). Diploid *mep2Δ* (ZAM37) cells were transformed with pFL61 or pFL61–GintAMT1 and transformants were tested on YNB-N medium containing 100 μM $NH_4^+$, and grown for 4 days at 29 °C. The diploid wild-type strain Σ1278b was used as a positive control.

### 2.7. RNA extractions and gene expression analysis

Extraradical mycelium grown under the different conditions tested was recovered from the hyphal compartment by blending (5 s high speed, 5 min with occasional low-speed pulses) the phytagel in 10 mM sodium citrate (pH 6) and collecting the extracted mycelium with a 50 μm sieve under sterile conditions. The extraradical mycelium was immediately liquid nitrogen-frozen and stored at −80 °C until used. RNAs were extracted using the RNeasy Plant Mini Kit (Qiagen, Maryland, USA) following the manufacturer's instructions.

*GintAMT1* expression was studied by real-time RT-PCR by using iCycler iQ (Bio-Rad, Hercules, CA, USA). cDNAs were obtained from 1 μg of total DNase-treated RNA in a 20 μL reaction containing 200 units of SuperScript II Reverse Transcriptase (Gibco, BRL), according to the manufacturer's protocol. The primer set used to amplify a coding region of *GintAMT1* in the synthesized cDNAs were AMT2 (5′-TGTGTCAGCATTGTCTTCAGT-3′) and AMT3 (5′-GGCAAGTGCGGGTGTAATAG-3′). Each 25 μL reaction contained 1 μL of a 1:10 dilution of the cDNA, 200 μM dNTPs, 200 nM each primer, 3 mM $MgCl_2$, 2.5 μL 1× SyBR Green (Molecular Probes, Eugene, OR, USA), and 0.5 U Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) in 1× PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl).

The PCR program consisted in a 5-min incubation at 95 °C to activate the hot-start recombinant *Taq* DNA polymerase, followed by 35 cycles of 30 s at 95 °C, 45 s at 55 °C, and 45 s at 70 °C, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat-dissociation protocol (from 70 to 100 °C) after the final cycle of the PCR. The efficiency of the primer set was evaluated by performing real-time PCR on several dilutions of plasmid DNA. The results obtained for the different treatments were standardized to the 18S rRNA levels, which were amplified with the following primers: RMF (5′-TGTTAATAAAAATCGGTGCGTTGC-3′) and RMR (5′-AAAACGCAAATGATCAACCGGAC-3′). Real-time PCR experiments were carried out three times, with the threshold cycle ($C_T$) determined in triplicate. The average for the triplicate of one representative experiment was used in all subsequent analysis. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

## 3. Results

### 3.1. Sequence analysis of GintAMT1

The full-length *GintAMT1* cDNA contains a 1437 nucleotide-long open reading frame with 142 nucleotides before the putative ATG start codon and a tail of 46 bp. The translation product of this open reading frame was 479 amino acids long, with a predicted molecular weight of 50 kDa. The alignment shown in Fig. 1A indicates that the predicted sequence from cDNA shows high homology to the functionally characterized $NH_4^+$ transporter AMT1 of *Tuber borchii* (61% identity) and to the *Hebeloma cylindrosporum* AMT1 and AMT2 proteins (60% identity). Analysis of the hydrophilicity shows that the predicted protein is highly hydrophobic and contains 11 putative membrane spanning regions (Fig. 1B), which is in

**A**

```
AMT1Hebeloma    MVN---VTYDDSGSLVAYTPDGT---------PIVYNLGDMAWIAASMALVWIMIPGVGL
AMT2Hebeloma    MVN---VTYDASQPLVWTDAAGN---------TGVYSPGDIAWVLASTALVWIMVPGVGF
AMT1Tuber       MNDPDHLNYVPLVPFNGTKPFGGDS--LTQDLNIWYESGNVAWVMTSTALVLLMIPGVGF
GintAMT1        MSAPAAAPAAEPNPFDGVNITELIANVSALSSVNDIDQGHTAWIMMSTALVFIMIPGVGY
                *            .:                      . *. **:  * *** :*:****

AMT1Hebeloma    FYSGLLRRKNALSMIWTSLASIAVVSFQWFFWGYSLAFSETGNAFIGDLKYFGLKGVLDQ
AMT2Hebeloma    FYSGLLRRKNALSMIYLSMMTVAVVSFQWFFWGFSLAFSDGASLFIGDLKYFGMKGVLEE
AMT1Tuber       FYSGLARRKSALSLIWLSMMATAVVSFQWFLWGYSLAFSHTGGRFIGNLDNFGFKGLLGY
GintAMT1        FYSGMARSKNALSLIMLSVLSLAVVSIQWWAIGFSLAFGPGTSGYIGNLNYAFFMGVGTE
                ****: * *.***:*  *: : ****:**:  *:****.   . :**:*.   : *:

AMT1Hebeloma    PSIGSSRIPAILFCIYQLMFAAITAALAVGAIAERGRLGPLLVFIFVWSTIVYDPIANWT
AMT2Hebeloma    PSIGSTRIPAIVFSVYQLMFAAITPMIAVGGFAERAHLGPLLFFVFVWSTLVYDPIACWT
AMT1Tuber       PSIGSEKIPDLLFALYQGMFSAITVALAIGAAAERGRMLPCVVFMFVWATLIYDPIACWT
GintAMT1        PLNGAVKIPGIVFAMYQCMFAAITPALAIGSAAERGRIFPSIVFIFIWSTIVYDPIAYWT
                *  *: :** ::*.:** **:***  :*:*. ***.:: * :.*:*:*:*::***** **

AMT1Hebeloma    WNTNGWSFILGGLDFAGGTPVHISSGTAALAISIFLGKRRGYGTERLAYKPHNTTYVILG
AMT2Hebeloma    WNSKGWSFVHGSYDFAGGTPVHISSGTAALAISIYLGKRRGYGTEVLTYKPHNTTYVILG
AMT1Tuber       WNSSGWSFRMGGLDFAGGTPVHISSGSAALAYSLMLGKRRGHGTHELNYRPHNVTHIVLG
GintAMT1        WNLNGWSAKMGGLDFAGGTPVHISSGAAALAYCLILGKRTSHGTD--EFKPHNIANVVLG
                ** .***   *. **************:**** .: **** .:**.   ::*** : ::**

AMT1Hebeloma    TVFLWFGWFGFNGGSALSANLRAIQACIVTNLAASVGGLTWMLWDYRIERKWSAVGFCSG
AMT2Hebeloma    TIFLWFGWFGFNGGSALAANLRAAQACIVTNLAASVGGLTWMLWDYRIEKKWSAVGFCSG
AMT1Tuber       TVFLWVGWFGFNGGSALSGNMRAVMACVVTNLAASVGGITWCLLDYRLEQKWSTVGFCSG
GintAMT1        TVLLWFGWFGFNGGSALNSTSRAAMAIAVTNLSAAVGGLTWCLLDYRLEKKLSALAFCSG
                *::**.*********** .. **  *  ****:*:***:** * ***:*:* *:::****

AMT1Hebeloma    AVSGLVAITPASGFVGAPAAVAFGVLGGTACNFATQLKFVLGYDDALDIFATHAIGGIVG
AMT2Hebeloma    AIAGLVAITPGSGFVGSPAAVLFGFMAGTVCNFATQLKFFAGYDDSLDIFASHAVGGVVG
AMT1Tuber       VIAGLVAITPGSGYVPSWAAVIYGVVGGAACNYATKLKFVIGVDDALDIFAEHGVGGIVG
GintAMT1        AVAGLVAITPGSGYVGTPAAVAFGFIAGICCNLAVKLKHIFDFDDALDVFAVHGVGGVIG
                .::*******.**:* : *** :*.::*  ** *.:**.. . **:**:** *.:**::*

AMT1Hebeloma    NLLTGLFAQASVAGFDGITEIPGGWLDRHYIQLAHQLADSVAGFAYSFVMTTIILWVMHI
AMT2Hebeloma    NLLTALFAQASVAGFDGFTVIPGGWLDRHYIQLAWHVADSAAGLSYSFVVTTIILWVMHF
AMT1Tuber       NILTAFFAADYIAHLDGSTEINGGWLNGHYKQLGYQVADSVAGFAYSFGGTCIILFIMNL
GintAMT1        NILTAIFAEQKIVALD-ETVLPGGWLNQHWIQMGHQLADSVTGVAYSFVVTYLILFIMDK
                *:**.:**   :. :*  * : ****: *: *:. ::***.:*.:*** * :**::*.

AMT1Hebeloma    IPGLTLRTTEEAEILGVDDAEMGEFAYDYVGIDQEIGHTLDTGLTATGG--GREPDHAKA
AMT2Hebeloma    IPGLRLRVPEETEIIGIDEADMGEFAYDYVGLETELKPHVYVRSTGTAYDSGRN------
AMT1Tuber       IPGLSLRATEEAEILGIDDAELGEFAYDYVELTREVISDDDTVPKMSSE---NN---ASV
GintAMT1        IPGLSLRADPESEAKGLDETELGELAYYHVDRLVAVNTRTGETKPVKEETIPQQ------
                **** **.  *:*  *:*::::**:** :*     :          .     .:

AMT1Hebeloma    VSTSSVEEKSA-
AMT2Hebeloma    ---SELKERSSA
AMT1Tuber       ELPAERAEKSA-
GintAMT1        ---NDANATIV-
                   .
```

**B**

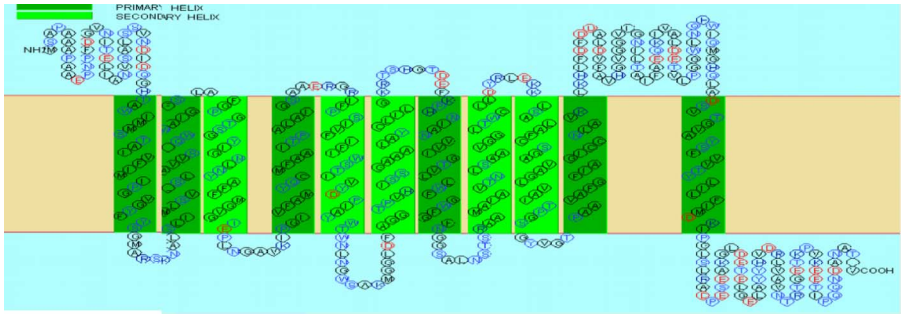


Fig. 1. (A) Alignment of the deduced amino acid sequence of the *AMT1* gene of *Glomus intraradices* with the high-affinity ammonium transporters of the ectomycorrhizal fungi *Tuber borchii* and *Hebeloma cylindrosporum*. (B) Predicted topology of GintAMT1, as defined by SOSUI.

good agreement with predictions made for AMT polypeptides from other fungi. These results suggest that GintAMT1 can be considered as a new member of the AMT/MEP transporter family and the first one identified in an AM fungus. Phylogenetic analysis of the AMT/MEP fungal transporters shows that GintAMT1 is most closely

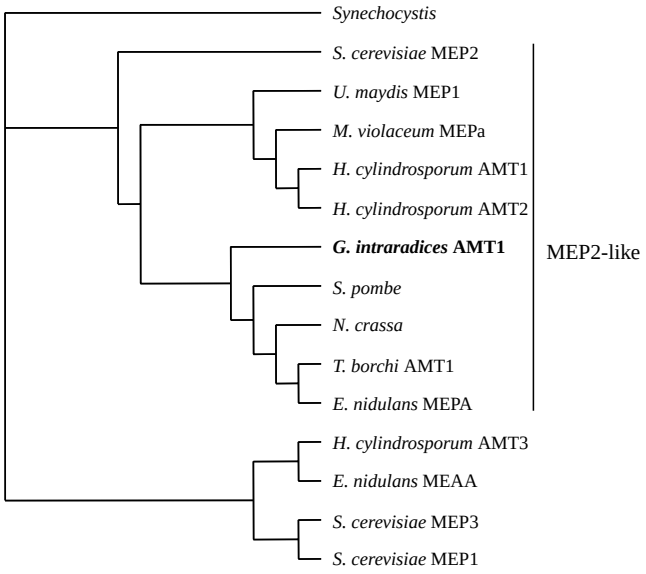


Fig. 2. Neighbour-joining phylogenetic tree of fungal AMT/MEP proteins. Sequences were obtained from the GenBank database with the following accession numbers: *Emericella nidulans* (MEPA: AAL73118, MEAA: AY049706), *Glomus intraradices* (AMT1: AJ880327), *Hebeloma cylindrosporum* (AMT1: AAM21926, AMT2: AAK82416, AMT3: AAK82417), *Microbotryum violaceum* (MEPa: AAD40955), *Neurospora crassa* (CAD21326), *Saccharomyces cerevisiae* (MEP1: P40260, MEP2: P41948, MEP3: P53390), *Schizosaccharomyces pombe* (CAC36934, probable ammonium transporter: T50244), *Synechocystis* sp. (PCC6803), *Tuber borchii* (AMT1: AY050276), and *Ustilago maydis* (MEP1: AF187870). The MEP2-like proteins are indicated.

related to the high-affinity $NH_4^+$ transporter and sensor Mep2-like proteins (Fig. 2).

### 3.2. *GintAMT1* encodes an $NH_4^+$ transporter

To further characterize this protein and to establish whether it has any $NH_4^+$ transporting capacity, the *GintAMT1* cDNA was expressed in the yeast triple *mep* mutant 31019b carrying a deletion of the two high-affinity $NH_4^+$ transporters, *MEP1* and *MEP2*, and of the low-affinity $NH_4^+$ transporter, *MEP3*. This strain is unable to grow in a medium containing less than 5 mM $NH_4^+$ as the sole nitrogen source (Marini et al., 1997). *GintAMT1*-expressing cells were able to grow on 1 mM $NH_4^+$ as the sole N source (Fig. 3), indicating that *GintAMT1* encodes a functional $NH_4^+$ transporter. To quantify ammonium transport by GintAMT1, uptake assays of [$^{14}$C]methylammonium as a substrate analogue were performed in the transformed yeasts. Cells transformed with the empty vector showed a very low level of [$^{14}$C]-methylammonium accumulation and concentration-dependent uptake rates in the *GintAMT1* transformants followed a saturable kinetic (Fig. 4A). The transport kinetic parameters, $K_m$ and $V_{max}$, were determined from Lineweaver and Burk data transformations (Fig. 4B) and were estimated as 26 μM and 4 nmol/min per $10^8$ cells, respectively. Because the affinity for methylammonium does not necessarily reflect the affinity for $NH_4^+$,

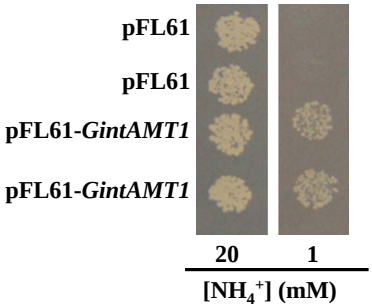


Fig. 3. Yeast complementation of the mutant defective in ammonium uptake by GintAMT1. Growth test on minimal medium containing either 20 mM (left) or 1 mM (right) $NH_4^+$ as sole nitrogen source. Yeast strain 31019b (*mep1Δ mep2Δ*::LEU2 *mep3Δ*::*KanMX2 ura3*) was transformed with the yeast expression vector pFL61 or pFL61 harboring the coding sequence of *GintAMT1*.

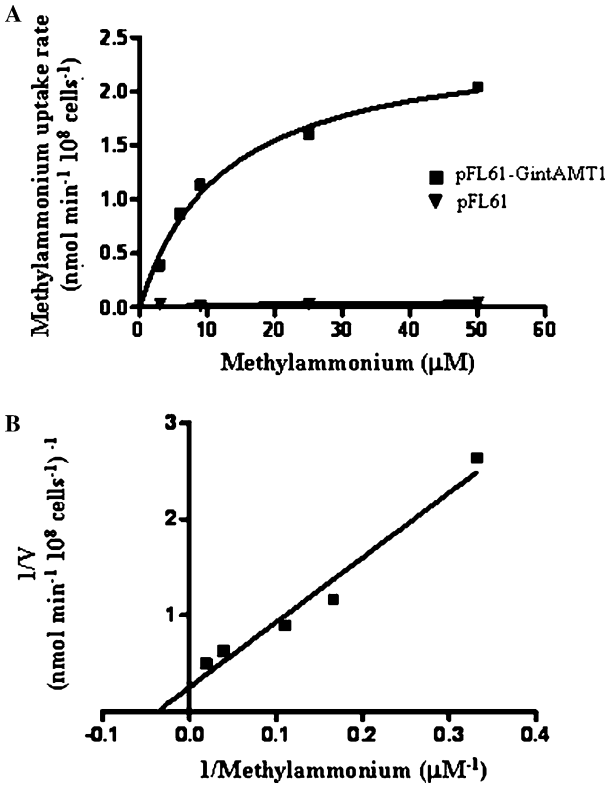


Fig. 4. Kinetic analysis of GintAMT1 in transformed yeasts. (A) Concentration-dependent [$^{14}$C]methylammonium uptake into the yeast strain 31019b transformed with the plasmid pFL61–GintAMT1 (■) or with the empty vector (▼). Cells were incubated under linear uptake conditions in the presence of the indicated concentrations of [$^{14}$C]methylammonium. The datapoints shown correspond to the slope values derived from uptake assays conducted for 0.5, 1, 2, and 4 min at each of the indicated concentrations. (B) Lineweaver–Burk plot of [$^{14}$C]methylammonium uptake into cells transformed with pFL61–GintAMT1. Experiments were conducted three times on two independent transformants. Results shown are from one representative experiment.

competition studies with varying $NH_4^+$ concentrations at 26 μM [$^{14}$C]methylammonium were performed. Ammonium strongly inhibited methylammonium uptake by pFL61–GintAMT1 transformed *mep*Δ cells (data not shown). Moreover, methylammonium accumulation was

nearly completely abolished by the proton gradient uncoupler CCCP (94% inhibition).

### 3.3. GintAMT1 does not induce pseudohyphal differentiation in S. cerevisiae

To test whether the *GintAMT1* protein can act in the transduction pathway of pseudohyphal differentiation, the ability of this protein to restore the pseudohyphal growth defect of the diploid strain ZAM37 (*mep2Δ*) was assayed. Although the expression of *GintAMT1* did not allow the production by the mutant strain of pseudohyphae, the surface of the colonies produced by the *GintAMT1*-expressing cells was rougher than the surface of the colonies produced by cells transformed with the empty vector (Fig. 5).

### 3.4. Regulation of GintAMT1 gene expression

Assessment of $NH_4^+$ regulation of *GintAMT1* gene expression was performed in *G. intraradices* extraradical mycelium developed monoxenically in MC medium containing different $NO_3^-$ concentrations and then exposed to low and high $NH_4^+$ concentrations for 24 and 48 h. The rational behind this experimental set-up was that growth of the extraradical mycelium is considerably altered in media containing $NH_4^+$ as the sole nitrogen source (Bago et al., 2004). When mycelia were grown in media containing 3.6 or 1.8 mM $NO_3^-$, expression of *GintAMT1* remained high but dropped at 0.9 mM $NO_3^-$ (Fig. 6). Addition of either 30 μM or 3 mM $NH_4^+$ to mycelia grown in the presence of the two highest $NO_3^-$ concentrations, provoked a clear inhibition of *GintAMT1* gene expression at the two time points analysed, being this inhibition higher after the addition of 3 mM $NH_4^+$. However, *GintAMT1* transcript levels increased after the addition of 30 μM $NH_4^+$ to mycelia grown in 0.9 mM $NO_3^-$, but decreased after the addition of 3 mM $NH_4^+$.

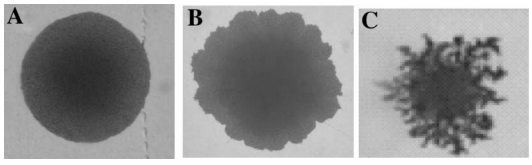


Fig. 5. Pseudohyphal yeast growth tests. Diploid *mep2Δ* (ZAM37) cells defective in pseudohyphal differentiation were transformed with pFL61 (A) or pFL61–GintAMT1 (B) and grown for 4 days at 29 °C on YNB-N medium containing 100 μM $NH_4^+$. The diploid wild-type strain Σ1278b (C) was used as a positive control.

## 4. Discussion

We have identified the first $NH_4^+$ transporter in an AM fungus. The identification has been based on homology to members of the MEP/AMT protein family and on the functional complementation of the yeast triple mutant strain 31019b (*mep1Δ mep2Δ mep3Δ*). MEP/AMT proteins have now been identified and characterized from a range of organisms and a number of similarities and conserved properties have been observed for these important proteins. Members of the MEP/AMT family are predicted to exist as integral membrane proteins containing 10–12 transmembrane helices, and they range in length from 400 to 450 amino acids, though a few have C-terminal extensions, bringing their overall length to as much as 600 amino acids (Thomas et al., 2000). Similarities in predicted amino acid sequence and protein structure between GintAMT1 and other members of the MEP/AMT family suggest that GintAMT1 is an integral membrane protein that mediates $NH_4^+$ transport across the plasma membrane of *G. intraradices*.

The ability of GintAMT1 to support $NH_4^+$ uptake was demonstrated by the observation that this protein was

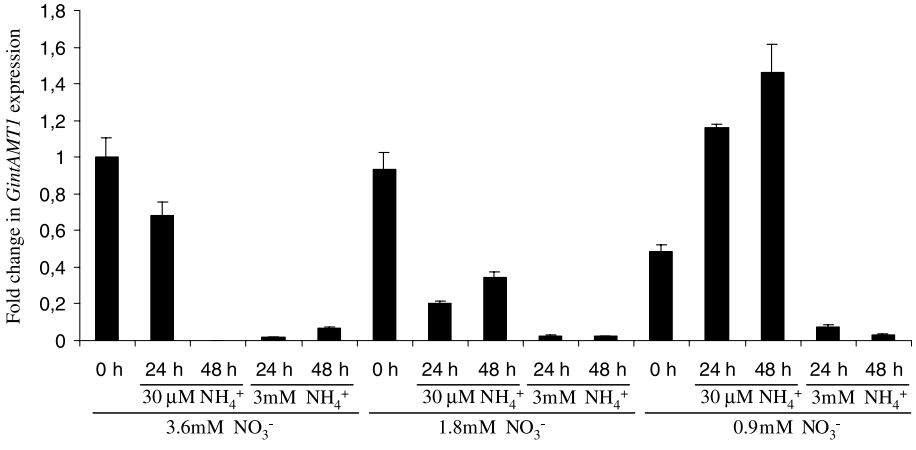


Fig. 6. *GintAMT1* gene expression in the extraradical mycelium of *Glomus intraradices* grown monoxenically. Total RNAs were extracted from extraradical mycelia of *G. intraradices* cultured in MC medium containing either 0.9, 1.8, or 3.6 (control treatment) mM $NO_3^-$ and then exposed for 24 or 48 h to 0, 30 μM, or 3 mM $(NH_4)_2SO_4$. RNAs were reverse transcribed and expression was assayed by quantitative real-time RT-PCR using gene-specific primers for *GintAMT1* and 18S rRNA. The fold change in *GintAMT1* gene expression induced by the nitrogen treatments was calculated using the $2^{-\Delta\Delta C}{}_T$ method. Data represent the means of three replicates from a representative experiment. Error bars represent SD.

able to suppress the severe growth defect on low $NH_4^+$ of the *S. cerevisiae mep1Δ mep2Δ mep3Δ* mutant. Kinetic analysis of the *GintAMT1* gene product in yeast indicates that *GintAMT1* encodes an $NH_4^+$ transporter that behaves as a high-affinity, low-capacity transporter. Inhibition of GintAMT1 transport capacity by CCCP, a compound that collapses the proton motive force established largely by a P-type $H^+$-ATPase across the plasma membrane, indicates that GintAMT1 is dependent on the $H^+$ electrochemical gradient for its activity. Although the mechanism of transport mediated by GintAMT1 remains to be elucidated, an ATP-dependent $NH_4^+$ uniport is the most likely mechanism, as it has been demonstrated for the tomato $NH_4^+$ transporter LeAMT1;1 (Ludewig et al., 2002).

Even though the main physiological function of the MEP proteins is to scavenge $NH_4^+$ from the medium for use as nitrogen source, they are also required for $NH_4^+$ retention inside cells during growth on at least some nitrogen sources other than $NH_4^+$ (Marini et al., 1997). Transcription of *GintAMT1* in mycelia developed in the presence of $NO_3^-$ suggests a role of this transporter in the retrieval of $NH_3$ that has leaked out of the mycelia by diffusion during the amino acid catabolism.

Differential regulation of *GintAMT1* by $NH_4^+$ in mycelia grown in the presence of different nitrogen concentrations indicates that this gene is under the control of the nitrogen status of the fungus. *GintAMT1* up-regulation by 30 μM $NH_4^+$ in mycelia grown in media containing 0.9 mM $NO_3^-$ and down-regulation in mycelia developed in media containing 3.6 or 1.8 mM $NO_3^-$ are in good agreement with the kinetic parameters of a high-affinity $NH_4^+$ transport system. The regulation pattern of *GintAMT1* by N parallels with that of the tomato transporter *LeAMT1;2* (Becker et al., 2002). Up-regulation of *GintAMT1* after the addition of 30 μM $NH_4^+$ to mycelia grown in the presence of 0.9 mM $NO_3^-$ indicates that this transporter is substrate-inducible, as it has been shown for other ion transporters such as LeAMT1;2 (von Wirén et al., 2000). Down-regulation of *GintAMT1* after the addition of $NH_4^+$ to the mycelia grown in the presence of higher N levels suggests that this transporter is regulated by the N status of the fungus. Although the kinetics of $NH_4^+$ uptake by AM fungi have not been yet determined, it is likely that $NH_4^+$ uptake would be suppressed in N-replete mycelia (Morgan and Jackson, 1989) All these data together suggest that both the nitrogen status and the nitrogen availability in the medium control $NH_4^+$ transport at the transcriptional level. In yeast, the high-affinity $NH_4^+$ transporters MEP1 and MEP2 are subject to nitrogen catabolite repression (Magasanik and Kaiser, 2002) and it has been shown that N-regulated transcription is mediated by at least two general positive GATA family transcription factors (Coffman et al., 1996; Mitchell and Magasanik, 1984). Isolation and characterization of the *GintAMT1* promoter as well as of these transcription factors in *G. intraradices* will allow determining the mechanisms involved in the N-regulated transcription of *GintAMT1*.

Our data also suggest that GintAMT1 plays a role in mycelial $NH_4^+$ uptake from the growth medium when it is present at low concentrations. In nature, $NH_4^+$ concentrations in the soil can vary over several orders of magnitude, from micromolar to hundred millimolar (Marschner, 1995). Cells have evolved a large number of transporters that enable them to efficiently import $NH_4^+$ over a wide range of concentrations (Howitt and Udvardi, 2000). Therefore, down-regulation of *GintAMT1* by 3 mM $NH_4^+$ indicates that at high concentrations, $NH_4^+$ uptake is probably occurring via a low-affinity $NH_4^+$ transport system, such as the ones identified in yeast (Marini et al., 1997) and in the ectomycorrhizal fungus *H. cylindrosporum* (Javelle et al., 2001).

The isolation of $NH_4^+$ transporter genes from AM fungi is of particular interest since these genes may play a strategic role in the process of nitrogen acquisition by mycorrhizal roots, which is the predominant situation for plants in natural ecosystems. AM fungi, by exploring soil microhabitats not accessible to the plant, may indeed access nitrogen sources not available to the plant (Smith and Read, 1997). Soil $NH_4^+$ concentrations are often 10–1000 times lower than those of $NO_3^-$, and the low mobility of $NH_4^+$ in soil restricts mass flow and determines that diffusion is the dominant mechanism for $NH_4^+$ transport to the root surface (Marschner, 1995). This leads to the formation of $NH_4^+$ deficient zones around the roots, similar to those developed for phosphorus. In spite of this, $NH_4^+$ nutrition plays an essential role to maintain cellular pH homeostasis and cation/anion balance during nutrient uptake. Moreover, $NH_4^+$ seems to be the preferential form of nitrogen taken up when plants are subjected to nitrogen deficiency (Gazzarrini et al., 1999; Lee and Rudge, 1986) and the main nitrogen source in water-logged or acid soils (Marschner, 1995). Previous studies have reported the capability of AM hyphae to take up $NH_4^+$ and translocate it to the plant (Frey and Schüepp, 1993; Johansen et al., 1992, 1996). The fact that *GintAMT1* is expressed in extraradical mycelia suggests a potential role in covering plant nitrogen demand through the uptake of the slow-diffusing soil $NH_4^+$. The $NH_4^+$ taken up by the AM fungus would be assimilated for its own metabolism (Breuninger et al., 2004; Johansen et al., 1996) or translocated to the plant.

Apart from a pivotal role in nutrient uptake, it has been proposed that AMT proteins may act as $NH_4^+$ sensors to regulate different pathways activated in nitrogen starvation conditions in *S. cerevisiae* (Lorenz and Heitman, 1998), *Ustilago maydis* (Smith et al., 2003), and ectomycorrhizal fungi (Javelle et al., 2003a,b). In *S. cerevisiae*, low nitrogen in the presence of abundant fermentable carbon source leads diploid cells to grow as pseudohyphae (Gimeno et al., 1992) and it has been considered that the $NH_4^+$ transporter MEP2 acts as the sensor for low $NH_4^+$ availability (Lorenz and Heitman, 1998). This developmental pathway has been proposed to be a scavenging mechanism under

nutrient-limiting conditions (Gimeno et al., 1992). In this sense, Bago et al. (2004) reported that extraradical *G. intraradices* hyphae when growing with little nitrogen or no nutrients exhibited a developmental pattern, probably designed to maximize exploration and exploitation of the medium, based on the formation of massive runner hyphae, which radially extend the fungal colony and from which small compact branched absorbing structures develop at irregular intervals. To test whether GintAMT1 could also act as a sensor for low $NH_4^+$ availability, *GintAMT1* was expressed in a diploid *S. cerevisiae* strain lacking MEP2. Even though GintAMT1 was not able to completely suppress the pseudohyphal growth defect of this *S. cerevisiae* strain, expression of *GintAMT1* in the external mycelium as well as the reported high affinity and its phylogenetic position could be consistent with a role as a sensor of the N-limiting conditions. However, further studies need to be carried out to determine whether this gene could play a role in nutrient sensing required for mycelial growth.

In conclusion, data presented in this paper indicate that *GintAMT1* encodes a high-affinity $NH_4^+$ transporter that is expressed in the extraradical mycelium of *G. intraradices*. Based on these data, we hypothesize that GintAMT1 could be mainly responsible for $NH_4^+$ acquisition by the extraradical mycelium from the surrounding environment when it is present at low concentrations, a process potentially very important from an ecological point of view, especially in acid soils.

## Acknowledgments

We thank Dr. Anne-Marie Marini (Institut de Biologie et de Médecine Moléculaire, Université Libre de Bruxelles, Gosselies, Belgium) for providing the *S. cerevisiae* strains used in this study and Dr. José Miguel Barea for critical reading of the manuscript. This research was supported by the EU project Genomyca (QLK5-CT-2000-01319) and by CICyT (project AGL2003-01551), Spain.

## References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Bago, B., Bécard, G., 2002. Bases of the obligate biotrophy of arbuscular mycorrhizal fungi. In: Gianinazzi, S., Schüepp, H., Barea, J.M., Haselwandter, K. (Eds.), Mycorrhizal Technology in Agriculture: From Genes to Bioproducts. Birkhäuser Verlag, Basel, Boston, Berlin, pp. 33–48.

Bago, B., Vierheilig, H., Piché, Y., Azcón-Aguilar, C., 1996. Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol. 133, 273–280.

Bago, B., Cano, C., Azcón-Aguilar, C., Samson, J., Coughlan, A.P., Piché, Y., 2004. Differential morphogenesis of the extraradical mycelium of an arbuscular mycorrhizal fungus grown monoxenically on spatially heterogeneous culture medium. Mycologia 96, 452–462.

Becker, D., Stanke, R., Fendrik, I., Frommer, W.B., Vanderleyden, J., Kaiser, W.M., Hedrich, R., 2002. Expression of the $NH_4^+$-transporter gene LEAMT1;2 is induced in tomato roots upon association with $N_2$-fixing bacteria. Planta 215, 424–429.

Breuninger, M., Trujillo, C.G., Serrano, E., Fischer, R., Requena, N., 2004. Different nitrogen sources modulate activity but not expression of glutamine synthetase in arbuscular mycorrhizal fungi. Fungal Genet. Biol. 41, 542–552.

Coffman, J.A., Rai, R., Cunningham, T., Svetlov, V., Cooper, T.G., 1996. Gat1p, a GATA family protein whose production is sensitive to nitrogen catabolite repression, participates in transcriptional activation of nitrogen-catabolic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 16, 847–858.

Chabot, S., Bécard, G., Piché, Y., 1992. Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84, 315–321.

Felstein, J., 1993. PHYLIP, version 3.5. Department of Genetics, University of Washington, USA.

Ferrol, N., Barea, J.M., Azcón-Aguilar, C., 2002. Mechanisms of nutrient transport across interfaces in arbuscular mycorrhiza. Plant Soil 244, 231–237.

Frey, B., Schüepp, H., 1993. Acquisition of nitrogen by external hyphae of arbuscular mycorrhizal fungi associated with *Zea mays* L. New Phytol. 124, 221–230.

Gazzarrini, S., Lejay, L., Gojon, A., Ninnemann, O., Frommer, W.B., von Wirén, N., 1999. Three functional transporters for constitutive, diurnally regulated and starvation-induced uptake of ammonium into *Arabidopsis* roots. Plant Cell 11, 937–948.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., Fink, G.R., 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. Cell 68, 1077–1090.

Govindarajulu, M., Pfeffer, P.E., Hairu, J., Abubaker, J., Douds, D.D., Allen, J.W., Bücking, H., Lammers, P.J., Shachar-Hill, Y., 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435, 819–823.

Hawkins, H.J., Johansen, A., George, E., 2000. Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226, 275–285.

Hirokawa, T., Boo-Chieng, S., Mitaku, S., 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14, 378–379.

Hodge, A., Campbell, C.D., Fitter, A.H., 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413, 297–299.

Howitt, S.M., Udvardi, M.K., 2000. Structure, function and regulation of ammonium transporters in plants. Biochim. Biophys. Acta 1465, 152–170.

Javelle, A., Rodríguez-Pastrana, B.R., Jacob, C., Botton, B., Brun, A., André, B., Marini, A.M., Chalot, M., 2001. Molecular characterization of two ammonium transporters from the ectomycorrhizal fungus *Hebeloma cylindrosporum*. FEBS Lett. 505, 393–398.

Javelle, A., André, B., Marini, A.M., Chalot, M., 2003a. High-affinity ammonium transporters and nitrogen sensing in mycorrhizas. Trends Microbiol. 11, 53–55.

Javelle, A., Morel, M., Rodríguez-Pastrana, B.R., Botton, B., André, B., Marini, A.M., Brun, A., Chalot, M., 2003b. Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium-metabolizing enzymes (GS, NADP-GDH) in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mol. Microbiol. 47, 411–430.

Johansen, A., Jakobsen, I., Jensen, E.S., 1992. Hyphal transport of $^{15}N$-labelled nitrogen by a vesicular–arbuscular mycorrhizal fungus and its effect on depletion of inorganic soil N. New Phytol. 122, 281–288.

Johansen, A., Jakobsen, I., Jensen, E.S., 1993. External hyphae of vesicular–arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L 3. Hyphal transport of $^{32}P$ and $^{15}N$. New Phytol. 124, 61–68.

Johansen, A., Finlay, R.D., Olsson, P.A., 1996. Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol. 133, 705–712.

Lee, R.B., Rudge, K.A., 1986. Effects of nitrogen deficiency on the absorption of nitrate and ammonium by barley plants. Ann. Bot. 57, 471–486.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25, 402–408.

Lorenz, M.C., Heitman, J., 1998. The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. EMBO J. 17, 1236–1247.

Ludewig, U., Wirén, N., Frommer, W.B., 2002. Uniport of $NH_4^+$ by the roots hair plasma membrane ammonium transporter LeAMT1;1. J. Biol. Chem. 227, 13548–13555.

Magasanik, B., Kaiser, C.A., 2002. Nitrogen regulation in *Saccharomyces cerevisiae*. Gene 290, 1–18.

Marini, A.M., Vissers, S., Urrestarazu, A., André, B., 1994. Cloning and expression of the MEP1 gene encoding an ammonium transporter in *Saccharomyces cerevisiae*. EMBO J. 13, 3456–3463.

Marini, A.M., Soussi-Boudekou, S., Vissers, S., André, B., 1997. A family of ammonium transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 17, 4282–4293.

Marini, A.M., Matassi, G., Raynal, V., André, B., Cartron, J.P., Cherif-Zahar, B., 2000. The human Rhesus-associated RhAG, and protein and a kidney homologue promote ammonium transport in yeast. Nat. Genet. 26, 341–344.

Marschner, H., 1995. Mineral Nutrition in Higher Plants. Academic Press, London.

Marzluf, G.A., 1996. Regulation of nitrogen metabolism in mycelial fungi. In: Brambl Marzluf (Ed.), The Mycota III: Biochemistry and Molecular Biology. Springer-Verlag, Berlin, Heidelberg, pp. 357–368.

Minet, M., Dufour, M.E., Lacroue, F., 1992. Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J. 2, 417–422.

Mitchell, A.P., Magasanik, B., 1984. Regulation of glutamine-repressible gene products by the GLN3 function in *Saccharaomyces cerevisiae*. Mol. Cell. Biol. 4, 2758–2766.

Montanini, B., Moretto, N., Soragni, E., Percudani, R., Ottonello, S., 2002. A high-affinity ammonium transporter from the mycorrhizal ascomycete *Tuber borchii*. Fungal Genet. Biol. 36, 22–34.

Morgan, M.A., Jackson, W.A., 1989. Reciprocal ammonium transport into and out of plant roots. Modification by plant nitrogen status and elevated root ammonium concentration. J. Exp. Bot. 20, 207–214.

Ninnemann, O., Jauniaux, J.C., Frommer, W.B., 1994. Identification of a high affinity $NH_4^+$ transporter from plants. EMBO J. 13, 3464–3471.

Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comp. Appl. Biosci. 12, 357–358.

Schiestl, R.H., Gietz, R.D., 1989. High efficiency transformation of intact cells using single stranded nucleic acids as a carrier. Curr. Genet. 16, 339–346.

Schüßler, A., Schwarzott, D., Walker, C., 2001. A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycol. Res. 105, 1413–1421.

Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis, second ed. Academic Press, London, UK.

Smith, D.G., García-Pedrajas, M.D., Gold, S.E., Perlin, M.H., 2003. Isolation and characterization from pathogenic fungi of genes encoding ammonium permeases and their roles in dimorphism. Mol. Microbiol. 50, 259–275.

St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., Fortin, J.A., 1996. Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol. Res. 100, 328–332.

Thomas, G.H., Jonathan, G.L.M., Merrick, M., 2000. Membrane topology of the Mep/Amt family of ammonium transporters. Mol. Microbiol. 37, 331–334.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tobar, R.M., Azcón, R., Barea, J.M., 1994a. The improvement of plant N acquisition from an ammonium-treated, drought-stressed soil by the fungal symbiont in arbuscular mycorrhizae. Mycorrhiza 4, 105–109.

Tobar, R.M., Azcón, R., Barea, J.M., 1994b. Improved nitrogen uptake and transport from $^{15}N$-labelled nitrate by external hyphae of arbuscular mycorrhiza under water-stressed conditions. New Phytol. 103, 73–81.

Toussaint, J.P., St-Arnaud, M., Charest, C., 2004. Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith and Ri T-DNA roots of *Daucus carota* L. in an *in vitro* compartmented system. Can. J. Microbiol. 50, 251–260.

Villegas, J., Williams, R.D., Nantais, L., Archambault, J., Fortin, J.A., 1996. Effects of N source on pH and nutrient exchange of extrametrical mycelium in a mycorrhizal Ri T-DNA transformed root system. Mycorrhiza 6, 247–251.

von Wirén, N., Lauter, F., Ninnemann, O., Gillisen, B., Walch-Liu, P., Engels, C., Jost, W., Frommer, W.B., 2000. Differential regulation of three functional ammonium transporter genes by nitrogen in root hairs and by light in leaves of tomato. Plant J. 21, 167–175.