Molecular characterization of two ammonium transporters from the ectomycorrhizal fungus *Hebeloma cylindrosporum*

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Abstract Heterologous expression of the yeast triple Mep mutant has enabled the first molecular characterization of AMT/MEP family members in an ectomycorrhizal fungus. External hyphae, which play a key role in nitrogen nutrition of trees, are considered as the absorbing structure of the ectomycorrhizal symbiosis and therefore molecular studies on ammonium transport in hyphae are urgently needed. The kinetic properties of AMT2 and AMT3 from *Hebeloma cylindrosporum* were studied in *Saccharomyces cerevisiae*. Expression of HcAmts in the yeast triple Mep mutant restored ammonium retention within cells. The HcAmts did not complement the ammonium sensing defect phenotype of Mep2Δ cells during pseudohyphal differentiation. Northern blot analysis in *H. cylindrosporum* showed that the *HcAMTs* were up-regulated upon nitrogen deprivation and down-regulated by ammonium.

Key words: Ammonium transport; Methylamine; Nitrogen regulation; *Hebeloma cylindrosporum*; *Saccharomyces cerevisiae*

1. Introduction

Over 95% of plant species belong to families that characteristically form symbiotic associations with mycorrhizal fungi, which can dramatically enhance plant growth [1]. Ectomycorrhizal symbiosis in temperate forests are the dominant type of association between roots of most long-living woody perennials, trees and members of the Basidiomycota and Ascomycota. As many as 6000 fungal species may form ectomycorrhizal associations in which the fungus forms a mantle of cells around the root tips and mycelium radiating out from mycorrhizal roots. External hyphae are considered as the absorbing structure of the ectomycorrhizal symbiosis, which play a key role in nitrogen nutrition of trees. Expanding mycorrhizal mycelium that grows outward from the mantle into the surrounding soil is a very efficient nitrogen scavenger owing to (i) its capacity to explore a larger soil volume than do roots alone [1], (ii) its ability to provide access to the nitrogenous reserves contained in organic horizons [2] and (iii) its greater capacity for uptake of nitrogenous compounds [3,4]. Moreover, ammonium is the major source of mineral nitrogen in forest soils [5], and consequently ammonium absorption by the extraradical mycelium plays a crucial role for nitrogen transfer in ectomycorrhizal symbiosis. Based on both ^{15}N labelling and mass balance data, it was recently demonstrated that hyphal NH_4^+ acquisition contributes 45% of total plant N uptake under N deficiency [6]. The whole process of ammonium assimilation is the result of three metabolic activities – transport, assimilation and transfer to the plant. The pathways of ammonium assimilation in ectomycorrhizal fungi have been well investigated, and previous works have revealed that the fungus plays a major role in nutrition of the host tree [7]. Although the process of ammonium uptake is often considered as a rate-limiting step in its acquisition [8], it has received relatively little attention. The kinetics and energetics of ammonium/methylamine (MA) transport in the ectomycorrhizal fungus *Paxillus involutus* have been characterized and it has been shown that complex regulatory processes are involved when nitrogen nutrition changes [3]. However, the study was hampered by the lack of molecular characterization of the transport systems involved. Ammonium transporters were first cloned from yeast (*MEP1*) and higher plant (*AMT1*) [9,10]. In order to gain further insights into ammonium assimilation, we present in this report the first molecular characterization and expression analysis of two ammonium transporters in the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*.

2. Materials and methods

2.1. Organisms and culture media

The *H. cylindrosporum* monokaryotic strain (h1) was obtained from the in vitro fruiting dikaryon HC1 [11]. It was grown on cellophane-covered agar medium containing (mg/l): $CaCl_2$ (50), $MgSO_4$ (150), KH_2PO_4 (250), NaH_2PO_4 (4.5), Na_2HPO_4 (160), $(NH_4)_2SO_4$ (250) and thiamine hydrochloride (0.04). Carbon was supplied as 2.5 g glucose/l. The *Escherichia coli* strains used were DH5α and JM109. Classical procedures for manipulating *E. coli* have been described previously [12].

The *Saccharomyces cerevisiae* strains used in this study were all isogenic with the wild-type Σ1278b Matα [13]. Cells were grown in a minimal buffered (pH 6.1) medium with 3% glucose [14]. Nitrogen sources used were proline (0.1%), arginine (0.1%), $(NH_4)_2SO_4$ (10 mM) unless other concentrations are specified.

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Abbreviations: MA, methylammonium/methylamine; YNB−N, yeast nitrogen base without amino acids and ammonium sulfate

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2.2. RNA isolation

Fungal colonies were fixed in liquid nitrogen. Total RNA isolation was performed with the RNeasy Plant Mini kit (Qiagen) from approximately 100 mg of frozen mycelia. According to the manufacturer's recommendations, a buffer containing guanidium hydrochloride was used instead of a buffer containing guanidium isothiocyanate to avoid solidification of samples due to secondary metabolites in mycelia of filamentous fungi. An average of 800 ng total RNA per mg frozen material was isolated and stored in DEPC-treated water at −70°C until further use.

2.3. Northern blot analysis

Samples of total RNAs (20 μg) were separated on 1.5% w/v formaldehyde agarose gels, transferred to positive nylon membrane (Appligene-Oncor) by capillary elution according to standard procedures [12] and fixed by UV irradiation for 4 min. Membranes were then prehybridized for 2 h at 37°C as described previously [15]. After a prehybridization step, the radioactive probe [α-^{32}P]dCTP-labelled cDNA *HcAMT2* or *HcAMT3* was denatured and added to the prehybridization buffer. After 24 h, membranes were subjected to three washes in 2× SSC/0.5% SDS at room temperature for 30 min, followed by one wash in 1× SSC/0.1% SDS at 65°C for 40 min. After drying, membranes were exposed to X-ray films.

2.4. cDNA library construction

A cDNA library was prepared from 2 μg of total RNA, isolated from *H. cylindrosporum* grown for 48 h without N, using the SMART PCR cDNA library construction kit (Clontech). cDNA was cloned into λTriplEx2 and phage DNA was packaged using the Gigapack Gold III Plus (Stratagene) and plated using *E. coli* strain BL25 (Clontech).

2.5. Cloning of HcAMT cDNAs

A consensus sequence cDNA was generated by aligning published sequences from *S. cerevisiae* (MEP1, 2 and 3). Two degenerated primers were designed from highly conserved regions, A1 (5′-GGTACGAATTCCARTGGTAYTTYTGGGG-3′) and A2 (5′-GGTACCTGCAGCAKCCANCCRAACCA-3′ R = A+G, Y = C+T, K = G+T, N = A+G+C+T). PCR was performed on the cDNA library using the Advantage cDNA PCR system (Clontech). A 466-bp fragment with 70% homology to the *S. cerevisiae* MEP2 protein, was obtained and used to design the following primers, A3 (5′-CGGCATGAAAGGCGTCCTCGAAGAGCCT-3′) and A4 (5′-TCAGTGCCGTATCCGCGTCGTTTTCCGA-3′) in order to perform 5′ and 3′ RACE reactions using the SMART RACE cDNA kit (Clontech) according to the manufacturer's instruction. The first strand cDNA was synthesized from 1 μg total RNA (Qiagen) from *H. cylindrosporum* using the SuperScript II Reverse Transcriptase (Gibco BRL), a RNaseH$^-$ derivative of Moloney murine leukemia virus, according to the manufacturer's protocol. PCR products were subcloned into pGEM-T Easy vector (Promega) and sequenced. *HcAMT3* clone was isolated from a cDNA *H. cylindrosporum* expression library mycelium, constructed by Dr. F. Gaymard (INRA Montpellier) and supplied by R. Lambilliotte (INRA Montpellier).

2.6. Functional expression in yeast

To generate full length *HcAMT2* cDNA, primers were designed from the 5′ end of the 5′ RACE product sequence and the 3′ end of the 3′ RACE product sequence. The full length cDNA was cloned in pDR195 [16] *Not*I linearized vector, using the following primers: A7 (*Not*I) 5′-CCCCCCCCGCGGCCGCATGGTGAATGTCACCTAC-3′; A8 (*Not*I) 5′-CCCCCCCCGCGGCCGCCTAGGCACTTGAGCGTTC-3′. *HcAMT3* initially cloned into the *Not*I linearized pFL61 vector, was excised using *Not*I restriction enzyme and subcloned into *Not*I linearized vector pDR195. The two resulting plasmids were named pDR195-*HcAMT2* and pDR195-*HcAMT3*. The resulting cDNA, *HcAMT2* and *HcAMT3* were sequenced, and the sequences submitted to the GenBank nucleotide database under accession numbers AF395542 and AF395543, respectively.

The yeast strain 31019b (*MATa ura3 mep1Δ mep2Δ::LEU2 mep3Δ::KanMX2*) [17] was transformed with pDR195-*HcAMT2* or pDR195-*HcAMT3* as described previously [18].

2.7. Pseudohyphal growth

Diploid strains were obtained by crossing between 31044d (*MATα ura3 mep1Δ mep2Δ::LEU2 mep3Δ::KanMX2*) and 31019b transformed with pDR195-*HcAMT2* or pDR195-*HcAMT3* plasmid. Pseudohyphal growth tests were performed as described previously [19]. Transformants were tested on yeast nitrogen base without amino acids and ammonium sulfate (YNB−N) medium containing 100 μM NH_4^+, and grown for 4 days at 29°C.

2.8. [^{14}C]MA uptake

Initial rates of [^{14}C]MA (Amersham) uptake were measured as previously described [9].

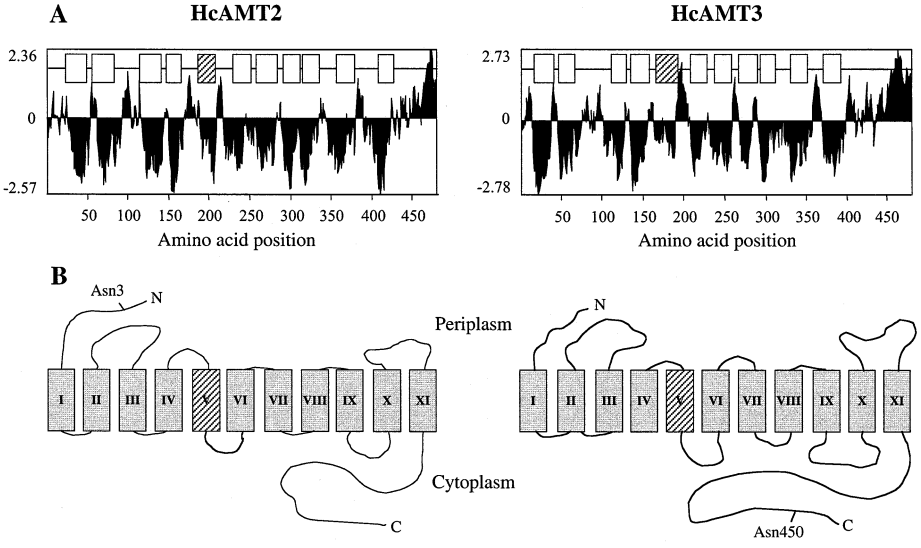

Fig. 1. Topology of the HcAMT2 and HcAMT3 proteins. A: Hydropathy profile of the HcAMT proteins. Hydropathy analyses were performed using the Kyte and Doolittle algorithm [22]. The upper boxes represent the relative positions of the membrane-spanning segments predicted by the algorithm TMpred, HMMTOP and TMHMM [23–25]. The hatched boxes correspond to the ammonium-signature-containing TM segments. B: Schematic model of the two HcAMT proteins. The predicted glycosylation sites (Asn3 for HcAMT2, Asn450 for HcAMT3) are indicated. The hatched boxes correspond to ammonium-signature-containing TM segments.

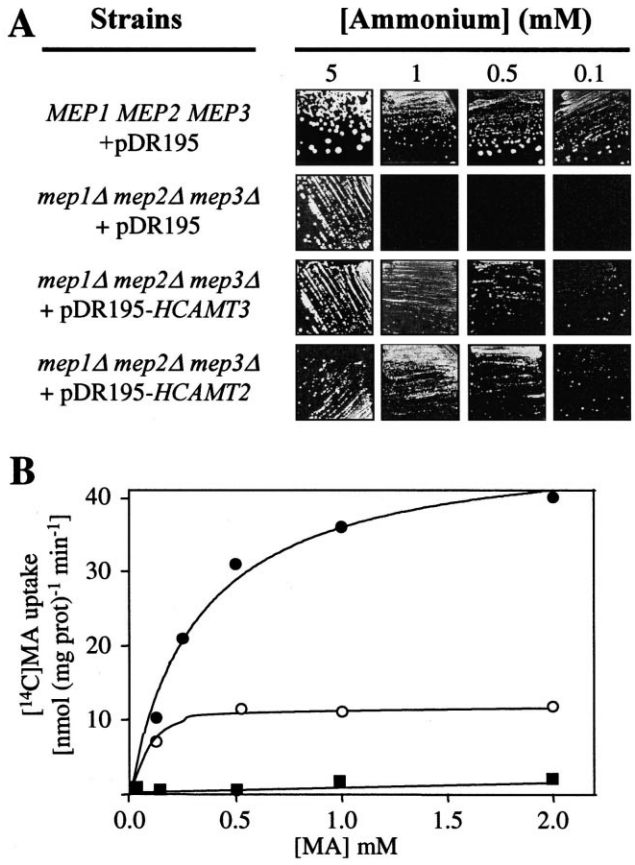

Fig. 2. Yeast complementation of the mutant defective in ammonium uptake (strain 31019b). A: Growth test on minimal medium supplemented with various NH_4^+ concentrations as a sole nitrogen source. All strains were incubated 3 days at 29°C. B: Concentration-dependent uptake of MA. The time course of MA uptake was calculated from measurements of accumulated radioactive labelling in cells grown on minimal proline medium following addition of 0.003–2 mM of ^{14}C-labelled MA. ● *mep1Δ mep2Δ mep3Δ*+pDR195-*HcAMT3*, ○ *mep1Δ mep2Δ mep3Δ*+pDR195-*HcAMT2*, ■ *mep1Δ mep2Δ mep3Δ*+pDR195.

3. Results and discussion

3.1. Cloning and sequence analysis of HcAMT2 and HcAMT3

The *HcAMT2* and *HcAMT3* cDNA encode a 470-amino acid protein (50.4 kDa) and a 480-amino acid protein (52.5 kDa), respectively. The sequences are named HcAMT2 and HcAMT3 by analogy to their high homology with MEP2 (43% homology) and MEP3 (50% homology) from *S. cerevisiae*, respectively. HcAMT2 presents 42% identity with HcAMT3 and the most highly conserved regions are the transmembrane (TM) helices. The helix V includes the ammonium transporter signature D[FYWS]AG[GSC]. {2} [IV]. {3} [SAG]{2}.{2} [SAG] [LIVMF]. {3} [LIVMFYWA] {2}. [GK].R{2} (Fig. 1). The sequences differ mainly at their C-termini. These results are in agreement with previous works indicating that current members of the MEP/AMT family (including bacteria, yeast, plant and animals) vary in length from 400 to 500 amino acids, although some members have extended C-terminal regions with their lengths up to about 600 amino acids. They have been predicted to be integral membrane proteins of around 40–60 kDa containing between 10 and 12 TM helices [20,21]. The hydropathy profiles of HcAMT2 and HcAMT3 generated with the Kyte and Doolittle algorithm [22], consist of 11 hydrophobic domains of sufficient length to be considered as potential membrane-spanning domains (Fig. 1A). Several other algorithms, including TMpred [23], HMMTOP [24], TMHMM [25], predicted that the central hydrophobic regions contain 11 TM segments (Fig. 1). Moreover, the TMHMM algorithm [25] predicted an extracellular N-terminus and an intracellular C-terminus tail according to the 'positive inside rule'. Based on these results and according to the topological experimental model proposed in *S. cerevisiae* [26] and *Arabidopsis thaliana* [20], we conclude that the HcAMT2 and HcAMT3 proteins consist of a central hydrophobic core made of 11 TM segments, with a long loop comprising many charged residues separating the two TM domains II and III (Fig. 1B). However, bacteria, such as *E. coli*, have an additional 12th N-terminal TM helix and a 'six plus six' topology has been established [20]. HcAMT2 contained a putative N-glycosylation site in the N extension of the protein at residue Asn3 (Fig. 1B). A glycosylation site has been experimentally identified at residue Asn4 of the *S. cerevisiae* MEP2 protein [26]. Interestingly, only MEP2 from *S. cerevisiae* contained a N-glycosylation site. This analogy between a yeast (MEP2) and a basidiomycete fungus (HcAMT2) might indicate a conserved functional significance for this N-glycosylation site.

3.2. Kinetics of MA and ammonium uptake by HcAMT2 and HcAMT3 expressed in yeast mutant deficient in ammonium uptake

The function of HcAMTs in ammonium transport was further characterized by yeast mutant complementation, as previously described for ammonium transporters from plants and animals [10,27–32]. The yeast strain 31019b, *mep1Δmep2Δ mep3Δ*, was unable to grow on media containing less than 1 mM ammonium as sole nitrogen source [17]. Transformation of this triple mutant with the yeast expression vector pDR195-*HcAMT2* or pDR195-*HcAMT3*, under the control of the constitutive yeast ATPase promoter [16], resulted in the complementation of the growth defect in the presence of less than 1 mM ammonium as sole nitrogen source (Fig. 2A). In contrast, transformation of 31019b with pDR195 did not enable the strain to grow on media containing less than 1 mM ammonium (Fig. 2A). Thus, *HcAMT2* and *HcAMT3* cDNA encode functional NH_4^+ transporters. Kinetic parameters were determined using [^{14}C]MA as a tracer measured for a short time uptake (2 min) in the transformed yeast strain 31019b. In a range of 0–2 mM [^{14}C]MA, when the component measured in cells containing the vector alone was subtracted, a saturable mediated uptake was obtained, which conformed to simple Michaelis–Menten kinetic (Fig. 2B), consistent with carrier-mediated transport. Values for Michaelis–Menten parameters (V_m, maximum uptake rate) and (K_m, apparent half-saturation constant), for MA uptake by the HcAMTs transporter expressed in yeast are given in Table 1. These results show that HcAMT3 has a higher capacity, but a lower affinity than HcAMT2 for MA. Interestingly, these results are similar to those obtained in *S. cerevisiae*, where MEP2 (homologous to HcAMT2 in sequence and topology) present a lower capacity but a higher affinity than MEP3 (homologous to HcAMT3) [17]. Previous work with mycorrhizal fungi reported K_m values in the range 110–180 μM when using MA as a substrate [3]. However such data could be the result of multiple transporter expression. In *H. cylindrosporum*, as well as in other

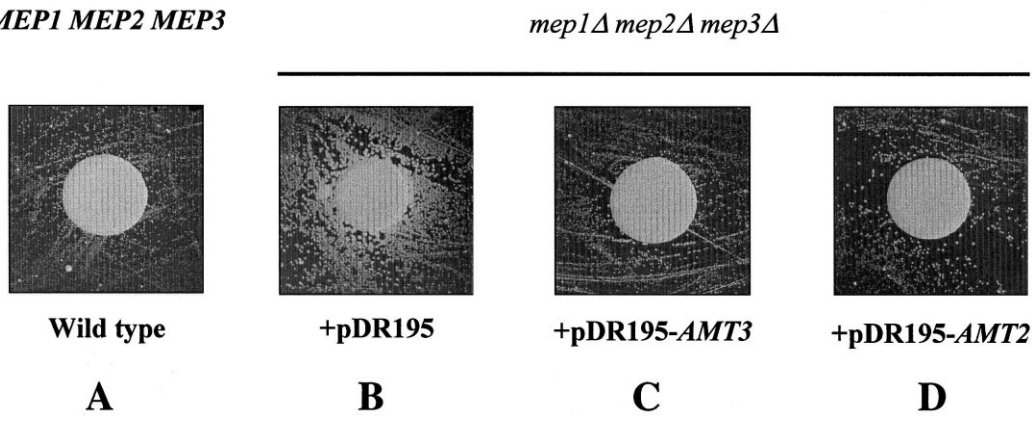

Fig. 3. Feeding of a strain lacking arginine permeases by a mutant lacking ammonium permeases. The receptor strain, 21983c (Matα *gap1-1 can1-1 ura3*) [17], was plated on arginine (0.05%) minimal medium to a low population density so as to give distinct colonies upon growing. Donor strain 23344c (Matα *ura3*) (Grenson, unpublished data) (A), 31019b (*mep1Δ mep2Δ mep3*+pDR195) (B), 31019b (*mep1Δ mep2Δ mep3Δ*+pDR195-*HCAMT3*) (C), 31019b (*mep1Δ mep2Δ mep3Δ*+pDR195-*HCAMT2*) (D) were dropped on the plate at a high population density.

organisms [17,28,30,33], the multiple AMT transporters with complementary affinities, probably allow the fungus to maintain a steady ammonium uptake, over a wide range of concentrations. Indeed, in forest soils, the quality and quantity of nitrogen sources can vary considerably [34,35].

Because MA affinity does not necessarily reflect ammonium affinity, competition experiments were carried out with ammonium. Dixon plots of the inhibition of MA uptake by ammonium in triple MepΔ mutant cells transformed with pDR195-*HcAMT2* or pDR195-*HcAMT3* showed that ammonium competitively inhibited [^{14}C]MA uptake activity (not shown). The much lower values found for HcAMT2 (Table 1) confirm that HcAMT2 is a very high affinity transporter, like its homolog MEP2, whereas HcAMT3 has much lower affinity for ammonium. The kinetic parameters were determined at pH 6.1 (standard medium) and pH 8.5 (Table 1). No significant differences were found between values obtained with standard methods (see Section 2) or those obtained as described in Table 1 with filtration and washing steps. K_m and V_m values were considerably affected by raising the pH from 6.1 to 8.5 (Table 1). This is fully consistent with our previous results, indicating that MA uptake is negatively affected by high pH values in ectomycorrhizal fungi [3]. The uptake systems of ectomycorrhizal fungi therefore seem to be adapted to the acidic pH of forest soil where formation of the protonated form of ammonium is favored. The pK_a values for MA (10.65) and ammonium (9.25) suggest an increase of the uncharged molecule by a factor of 300 upon increasing the pH from 6.1 to 8.5. In *Corynebacterium glutamicum*, the K_m value for MA was not significantly affected by increasing the pH from 6.1 to 8.5 [36]. The authors therefore suggested that (methyl)ammonium but not (methyl)amine was transported. A similar conclusion cannot be drawn from our data.

Table 1
Kinetic parameters and pH effects for uptake of MA by *H. cylindrosporum* ammonium transporters expressed in yeast mutant 31019b

Kinetic parameters	HcAmt2		HcAmt3	
pH	6.1	8.5	6.1	8.5
K_m (μM)	58 (54)	30	260 (290)	10
V_m (nmol/min/mg protein)	11 (11)	2	60 (71)	40
K_i (μM)	0.10–0.17	nd	12–14	nd

Values were estimated from Eadie–Hofstee plots; kinetic parameters derived from the Lineweaver–Burk plots are given in parentheses. Initial rates of [^{14}C]MA (Amersham) uptake were measured as described previously [9]. For the inhibition assays, ammonium was added simultaneously with [^{14}C]MA. For pH-dependent uptake, cells growing on proline medium were filtered, washed and re-suspended in proline medium at the appropriate pH. nd, not determined.

3.3. Expression of HcAMTs in triple Mep mutant restores ammonium retention within cells

Ammonium transporter MEP proteins were also shown to play a role in ammonium retention in *S. cerevisiae*; this was demonstrated by carrying out feeding experiments involving excretion phenomena [17]. We tested the ability of different transformed-strain cells to feed an arginine permease-less strain [*gap1-1 can1-1*] in arginine-containing media (0.05%) as the sole nitrogen source. When wild-type donor cells were

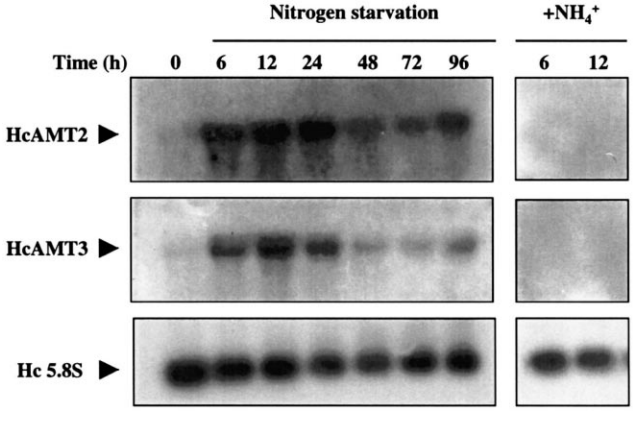

Fig. 4. Northern analysis of *HcAMT* transcripts. Fungal colonies were grown on cellophane-covered agar medium for 10 days and transferred to a nitrogen-deficient liquid for medium for various lengths of time. Some colonies were further transferred to a 2 mM ammonium-containing medium. Total RNA was extracted from 100 mg of mycelium and 20 μg/lane was separated on 1.5% agarose–formaldehyde gel and hybridized to the [α-^{32}P]dCTP-labelled *HcAMT* cDNAs or 5.8S rRNA probe as a loading control.

used, no feeding of the [*gap1-1 can1-1*] receptor cells was detected after a 3-day incubation period (Fig. 3A). In contrast, the triple *mep* strain transformed with plasmid pDR195 was able to feed the cells (Fig. 3B), indicating that it excreted a nitrogen source into the medium that could be taken up by cells lacking the general amino acid permease Gap1p and the specific arginine permease Can1p. When the donor cells expressed a single HcAMT transporter, growth of the receptor cells was reduced (Fig. 3C,D). These data suggest that during growth on arginine-containing media, transformed cells excrete NH_4^+ ions that are then taken back by the high-affinity permeases HcAMT2 or HcAMT3. These results indicate that the HcAMT have a role in ammonium retention, as already demonstrated for yeast MEPs [17].

3.4. HcAMTs and pseudohyphal growth in S. cerevisiae

It has been established that the MEP proteins can act as a nitrogen sensor and especially MEP2 which is involved in the transduction pathway of pseudohyphal differentiation in *S. cerevisiae* under nitrogen starving conditions [37]. Because of the sequence and topology homologies between MEP2 and HcAMT and because *H. cylindrosporum* is a filamentous fungus, we have tested the hypothesis that the HcAMT protein can act in the transduction pathway of pseudohyphal differentiation. Results indicate that neither HcAMT2 nor HcAMT3 are required for pseudohyphal differentiation (not shown) but further investigations are needed to determine whether or not HcAMT is involved in ammonium sensing.

3.5. Expression pattern of HcAMT2 and HcAMT3

In order to gain further insight at the molecular level concerning regulation of ammonium uptake in ectomycorrhizal fungi [3], we have studied the expression of *HcAMTs* by Northern blot analysis. A single mRNA of about 1.7 kb and 1.8 kb was detected for *HcAMT2* and *HcAMT3*, respectively (Fig. 4). A very low level of mRNA was detected when the fungus grew under optimal nitrogen conditions (Fig. 4). However transcript levels of both *HcAMT2* and *HcAMT3* strongly increased under nitrogen starvation. No transcript could be detected when the fungus was further transferred for 6–12 h to a 2 mM ammonium-containing medium. These results show that *HcAMT2* and *HcAMT3* are up-regulated upon nitrogen deprivation and down-regulated by an ammonium concentration higher than 2 mM. Similar results were found in *P. involutus*, where N starvation triggered a four-fold increase in MA transport after 2 h incubation time in nitrogen-free media [3]. In *H. cylindrosporum*, nitrogen starvation induced a dramatic depletion of intracellular concentration of the major amino acids, glutamine and glutamate, which correlates with an increase in the transcript and activity levels of glutamate dehydrogenase and glutamine synthetase (Rodríguez-Pastrana, in preparation), the two ammonium-assimilating enzymes operating in *H. cylindrosporum* [38].

4. Concluding remarks

The transport of low-molecular-weight nutrients and metabolites through the plasma membrane has been investigated in less than 1% of the estimated number of fungal species. Nevertheless, the assumption that the properties of fungal plasma membranes are somehow related to the ecological role of fungi, highlights the need for exploring this role, the distribution and the structure of transport proteins in ectomycorrhizal fungi and ectomycorrhizas. In the present work, *HcAMT2* and *HcAMT3*, the first members of the AMT protein family in basidiomycetes, were characterized. An immunological approach will be used in our laboratory to elucidate the intriguing questions concerning the translational regulation of this protein family and its cytolocalization in ectomycorrhizas.

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