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Characterization of a general amino acid permease from *Hebeloma cylindrosporum*

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Abstract **Besides a role in phosphate supply, ectomycorrhizas play a crucial role in nitrogen nutrition of plants. The ectomycorrhizal association between *Hebeloma cylindrosporum* and *Pinus pinaster* serves as a model system accessible to molecular manipulation. *Hebeloma* mycelium is able to take up and use amino acids as the sole nitrogen source. Suppression cloning allowed identification of a *Hebeloma* transporter (HcGAP1) mediating histidine uptake. HcGAP1 mediates secondary active uptake of a wide spectrum of different amino acids. The secondary active transport mechanism together with the expression in hyphae, but not in mycorrhizas, indicate a role in uptake of organic nitrogen from the soil.** © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Amino acid transport; Aspartate uptake; *Hebeloma cylindrosporum*

1. Introduction

Ectomycorrhizal trees dominate boreal and temperate forest ecosystems in which nitrogen is the most important growth limiting nutrient [1,2]. It has been proposed that selection favored symbiosis between tree roots and ectomycorrhizal fungi in growth limiting environments due to fungal improvement of plant access to nutrients [3]. Both the role of mycorrhiza in phosphate nutrition and the molecular basis of phosphate uptake of both symbiotic partners have been well established [4,5]. In contrast, less is known about the molecular basis of the role of ectomycorrhiza in nitrogen nutrition. Most studies in this area have focused on the uptake and assimilation of inorganic nitrogen sources by ectomycorrhizal roots. Importantly, however, most ectomycorrhizal root tips are located in the superficial organic horizons of the forest soil profile [6,7], where organic forms of nitrogen predominate [8,9]. Although the processes of uptake, translocation and transfer of organic nitrogen are understood in broad terms and some mechanisms are inferred by comparison with other organisms such as yeast, there are still considerable gaps in our knowledge of even the simplest symbiotic systems involving the interaction between a single fungus and a single autotrophic host. Mycorrhizal systems have successfully been used to determine rates of translocation [10], but the underlying mechanisms are still not understood. For these reasons it is essential to establish the mechanisms and processes whereby organic nitrogen compounds are mobilized, assimilated and transported.

Net transport of nutrients from the soil solution to the above ground part of the plant is the result of four principal transport components: (i) at the soil–fungus interface; (ii) translocation in hyphae; (iii) export from fungal hyphae and (iv) uptake by the plant root membrane [11]. In order to understand the processes involved in the contribution of mycorrhiza to the nitrogen nutrition of plants, it is necessary to study fungal nitrogen nutrition at the physiological and molecular level. Amino acid transporters have been characterized in detail physiologically in animals, plants and yeasts (for review see [12–15]). On the basis of physiological studies, the existence of a large number of transporters has been postulated differing in substrate spectrum, and tissue specificity, and transport mechanism, i.e. the ions used in cotransport. In *Saccharomyces cerevisiae* it is clear that amino acids are accumulated by a set of 22 secondary active influx systems [13,15,16]. An effective way for isolating transporters is suppression cloning in yeast mutants, e.g. a wide spectrum of plant transporters for nitrogenous compounds has been identified [17,18]. Isolation of transporter genes will allow a better understanding of the mycorrhizal symbiosis by manipulation of fungal metabolic and transport processes [19].

As a first step towards analysis and manipulation of mycorrhizal organic nitrogen transport, nutrition and uptake of amino acids by the model fungus *Hebeloma cylindrosporum*, a gene coding for an amino acid transporter was isolated by functional complementation of a yeast amino acid uptake mutant. *HcGAP1* encodes a general amino acid permease mediating secondary active uptake of amino acids into hyphae. The transporter gene is expressed in hyphae and down-regulated in the mycorrhizal association, indicating that it plays a role in the uptake of amino acids from the soil for fungal nutrition.

2. Materials and methods

2.1. Strains

The *H. cylindrosporum* monokaryotic strain (h1) was obtained from the in vitro fruiting dikaryon HC1 [20]. Mycelia were grown on cellophane-covered medium with glucose as C source and addition of amino acids or ammonium as N source. The *Escherichia coli* strain used was XL1-Blue. Classical procedures for manipulating *E. coli* have been described previously [21]. The yeast strains used were the histidine uptake deficient mutant *JT16* (Matα hip1-614 his4-401 can1 ino1 ura3-52; [22]) and a mutant deficient for multiple amino acid

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uptake systems *22Δ8AA* (Matα gap1-1 put4-1 uga4-1 Δcan1 Δapl1 Δlyp1 Δhip1 Δdip5 ura3-1; [23]). Mycorrhiza formation was induced with *Pinus pinaster* (Ait.) as the plant partner [24] on medium containing 0.5 g/l glucose and 1 mM nitrogen in the form of ammonium or amino acids. Control plants were grown without inoculum. *Pinus* needles, *Hebeloma* mycelia, mycorrhizal and non-mycorrhizal short roots were harvested, pooled, frozen in liquid nitrogen and stored at −80°C.

2.2. Utilization of amino acids as N source by H. cylindrosporum

The ability of the *H. cylindrosporum* strain h1 to utilize amino acids as N source was assessed by growth experiments. Petri dishes were inoculated with one agar plug (0.8 cm) cut from the margin of actively growing cultures maintained on modified Melin–Norkrans (MMN) medium, from which malt extract was omitted, with ammonium as N source [25]. Each dish contained MMN medium supplemented with a single amino acid (2 mM) as sole N source and covered by cellophane. There were five replicates for each treatment. Cultures were incubated in the dark at 20°C for 21 days prior to harvest. Cultures with ammonium as sole N source were used as a control. Cultures were incubated in the dark at 20°C for 21 days prior to harvest. Starvation was obtained by cultivating the mycelia on MMN with ammonium for one week and then transferring it to MMN without ammonium for 2 weeks. After harvesting the mycelium was freeze-dried and dry weight was determined.

2.3. Yeast growth, transformation and selection

The yeast strain *JT16* was transformed with an expression library derived from *H. cylindrosporum* mycelia. Sixty-four transformants were selected directly on solid SC medium supplemented with 6 mM histidine. Colonies able to grow were tested for growth in liquid medium with 6mM histidine. Plasmid DNA was isolated and reintroduced into the mutant strain *JT16*. The cDNA clone GAP1 was able to restore the growth of the mutant on selective conditions.

The yeast strain *22Δ8AA* was transformed with the same expression library. Twelve transformants were selected directly on solid BA medium supplemented with 1 mM proline. Colonies able to grow were tested for growth in liquid medium with 1 mM proline. Plasmid DNA was isolated and reintroduced into the mutant strain *22Δ8AA*. The cDNA clone *HcGAP1* was also identified in this screen.

To test the substrate specificity the yeast strain *22Δ8AA* was transformed with the cDNA clone. Selection was carried out on nitrogen-free medium supplemented with aspartate as the sole nitrogen source. The empty vector pDR196 serve as a negative control.

2.4. RNA gel-blot analysis

Total RNA was isolated from *Pinus* needles, *Hebeloma* mycelia, mycorrhizal and non-mycorrhizal short roots and 20 μg total RNA was separated on 1.5% formaldehyde agarose gels [21]. Hybridization was performed at 68°C in 0.25 M sodium phosphate pH 7.2, 7% sodium dodecylsulfate (SDS), 1 mM EDTA and 1% bovine serum albumin (BSA) for 16 h using the cDNA fragments of *HcGAP1* as a probe. Filters were washed twice with 2×SSC and 0.1% SDS at 68°C.

2.5. Transport measurements

In the *H. cylindrosporum* mycelia uptake experiments, discs of fungal mycelium were cut from the actively growing edge of 10-day-old colonies using a 15-mm-diameter cork borer. The discs were floated for 5 min on a solution containing 1 ml nitrogen and glucose-free MMN (pH 4.2) at 25°C supplemented with [^{14}C]-aspartate, specific activity 7.66 GBq/mmol (Amersham, Braunschweig, Germany). Incubation time varied from 1 to 20 min. At the end of the uptake period the discs were washed with 0.1 mM CaSO4 and solubilized with 80% Soluene 350 (Packard) overnight. The uptake of carbon-14 was determined by liquid scintillation spectrometry.

For *S. cerevisiae* uptake studies, yeast cells were grown to logarithmic phase. Cells were harvested at an OD_{600} of 0.5, washed twice in water, and resuspended in buffer A (0.6 M sorbitol, 50 mM potassium phosphate, at the desired pH) to a final OD_{600} of 5. Prior to the uptake measurements, the cells were supplemented with 100 mM glucose and incubated for 5 min at 30°C. To start the reaction, 100 μl of this cell suspension was added to 100 μl of the same buffer containing at least 18.5 kBq [^{14}C]-aspartate, specific activity 7.66 GBq/mmol (Amersham) and unlabeled amino acid to the concentrations used in the experiments. Sample aliquots of 45 μl were removed after 15, 60, 120, and 240 s, transferred to 4 ml of ice-cold buffer A, filtered on glass fiber filters, and washed twice with 4 ml of buffer A. The uptake of carbon-14 was determined by liquid scintillation spectrometry. Competition for aspartate uptake was performed by adding a five-fold molar excess of the respective competitors to 150 μM aspartate.

For analysis of pH dependence, incubations were performed in 100 mM potassium phosphate buffer adjusted to the different pH values, 100 mM glucose, and 150 μM ^{14}C-aspartate. Influence of plasma membrane energization on the uptake rate of ^{14}C-aspartate was analyzed by incubating the yeast cells for 5 min in the presence of 100 mM glucose (control), without glucose, or with glucose and 0.1 mM 2,4-dinitrophenol (DNP), 0.1 mM diethylstilbestrol (DES), 0.1 mM carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), or 0.1 mM vanadate. Transport measurements were repeated independently and represent the mean of at least three experiments.

3. Results and discussion

3.1. Utilization of amino acids as N source by H. cylindrosporum

To study the ability of *H. cylindrosporum* to use organic nitrogen it was grown on different amino acids and on ammonium as a control (Fig. 1). On glutamine and asparagine *Hebeloma* mycelia grew better than on the ammonium control. Growth measured as dry weight of hyphae was comparable to that on ammonium for glutamine, aspartate, alanine

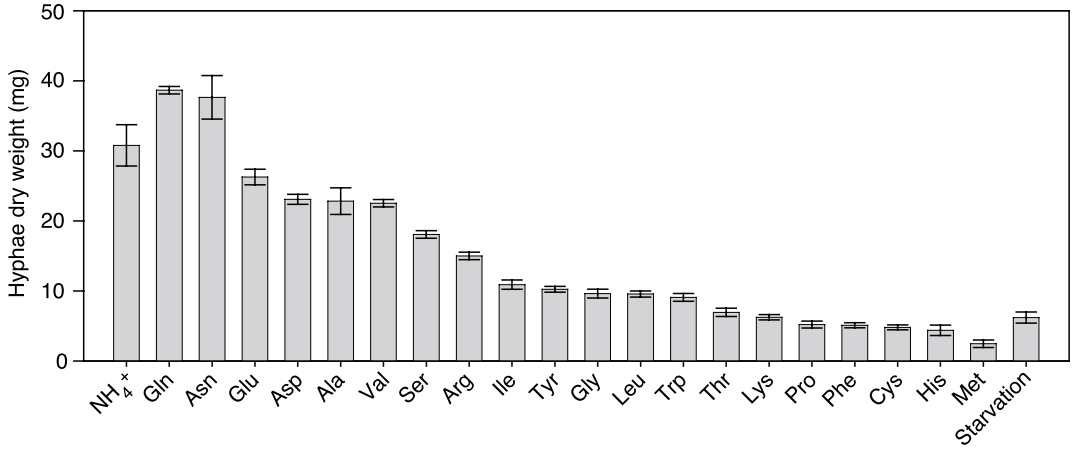

Fig. 1. Growth of *H. cylindrosporum* on different amino N sources and on ammonium. Cultures were incubated in the dark at 20°C for 21 days on 2 mM nitrogen source. Values represent the mean of five independent experiments ± SD.

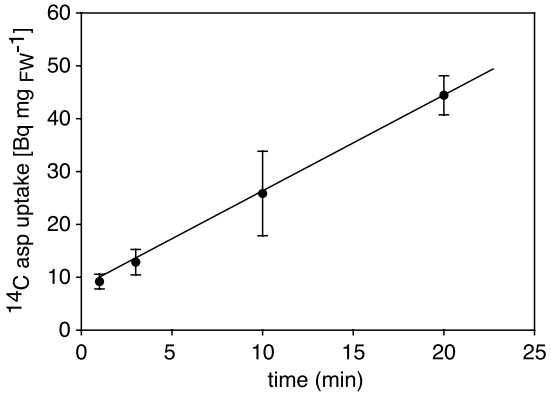

Fig. 2. ^{14}C-aspartate uptake by the ectomycorrhizal fungus *H. cylindrosporum*. Discs of fungal mycelium were cut from the actively growing edge of 10-day-old colonies using a 15-mm-diameter cork borer. The discs were floated for 5 min on a solution containing 1 ml nitrogen and glucose-free MMN at 25°C, supplemented with [^{14}C]-aspartate. Values represent the mean of three independent experiments ± S.D.

and valine. Reduced growth was observed on serine, arginine, isoleucine, tyrosine, glycine, leucine and tryptophan. On threonine, lysine, proline, phenylalanine, cysteine, histidine and methionine the observed growth was not better, in some cases even lower as compared to growth on nitrogen starvation. It is important to note that some amino acids, such as glutamine, glutamate and alanine, which appear to predominate in soil solution [26], are readily assimilated by the *H. cylindrosporum* H1 mycelia, demonstrating the ability of *H. cylindrosporum* to use organic nitrogen sources as it has been shown for other mycorrhizal fungi [10]. The involvement of amino acid transport and its role as primary source of N for the ectomycorrhizal fungus *H. cylindrosporum* and thus for the plant partner which it colonizes was confirmed by the linear uptake for at least 20 min of ^{14}C-asp by the mycelia (Fig. 2) when using an aspartate concentration of 2 μM corresponding to amino acid concentrations related to concentrations observed in soils [27].

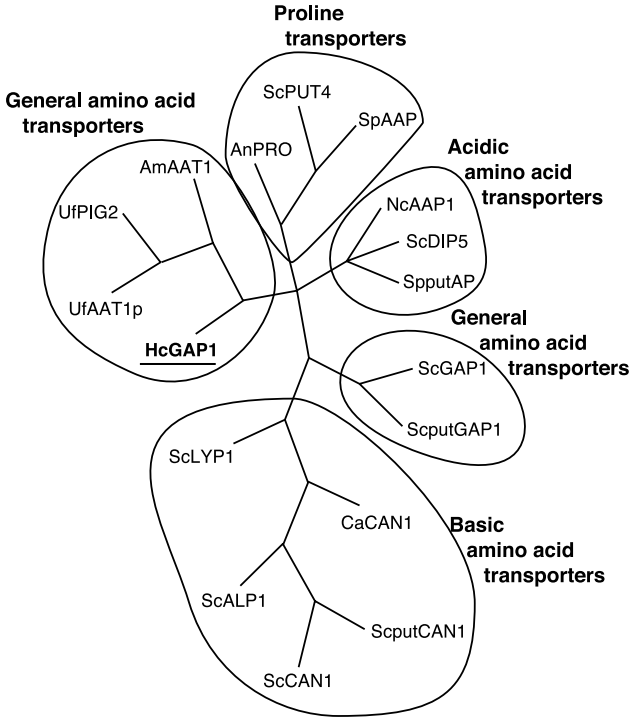

Fig. 3. Phylogenetic analyses of a multiple alignment of the deduced protein sequence of HcGAP1 and other fungal amino acid permeases (Am = *Amanita muscaria*; An = *Aspergillus nidulans*; Ca = *Candida albicans*; Hc = *Hebeloma cylindrosporum*; Nc = *Neurospora crassa*; Sc = Saccharomyces cerevisiae; Sp = *Schyzosaccharomyces pombe*; Uf = *Uromyces fabae*). Maximum parsimony analyses were performed using PAUP 4b10 with all DNA characters unweighted and gaps scored as missing characters. Heuristic tree searches were executed using 100 random sequence additions and the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. The complete alignment was based on 657 sites; 549 were phylogenetically informative.

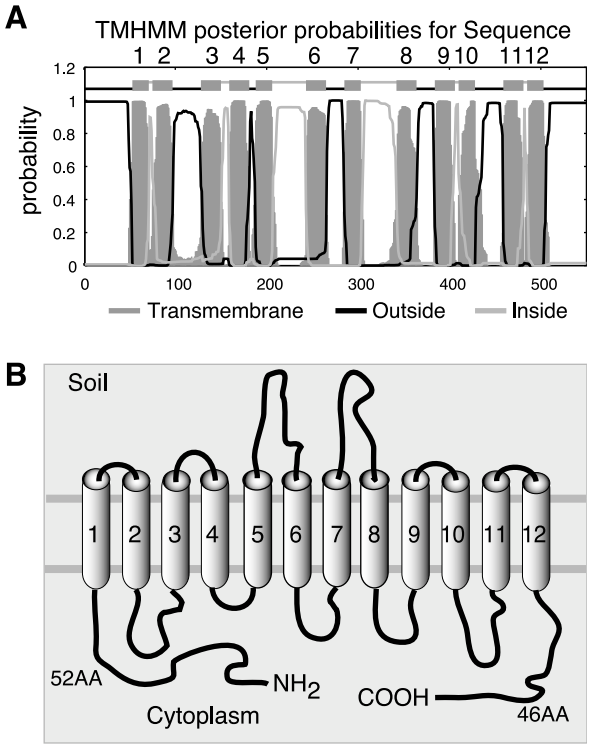

Fig. 4. Topology of the HcGAP1 protein. A: Transmembrane domain prediction was done by using the TMHMM algorithm. B: Schematic model of the HcGAP1 protein.

3.2. Cloning and sequence analysis of HcGAP1

The apparent ability of *H. cylindrosporum* to take up amino acids led us to investigate the molecular basis of amino acid transporters potentially mediating mycelial uptake and transfer of amino acids. For this purpose a yeast mutant deficient in histidine uptake was transformed with a cDNA expression library from *Hebeloma* under control of a yeast promoter [15]. Sixty-four transformants were grown on selective media. The yeast strain *JT16* was retransformed with DNA extracted from the 64 transformants to eliminate false positives. Three clones allowed regrowth of transformed *JT16* on 6 mM histidine. From these three clones a cDNA with strong homology to other fungal amino acid transporter genes was identified and was named *H. cylindrosporum* general amino acid permease 1 (*HcGAP1*). The *HcGAP1* cDNA (Genbank AF521906) has a length of 1784 bp and encodes a 594-amino acid protein with a calculated molecular mass of 65.7 kDa.

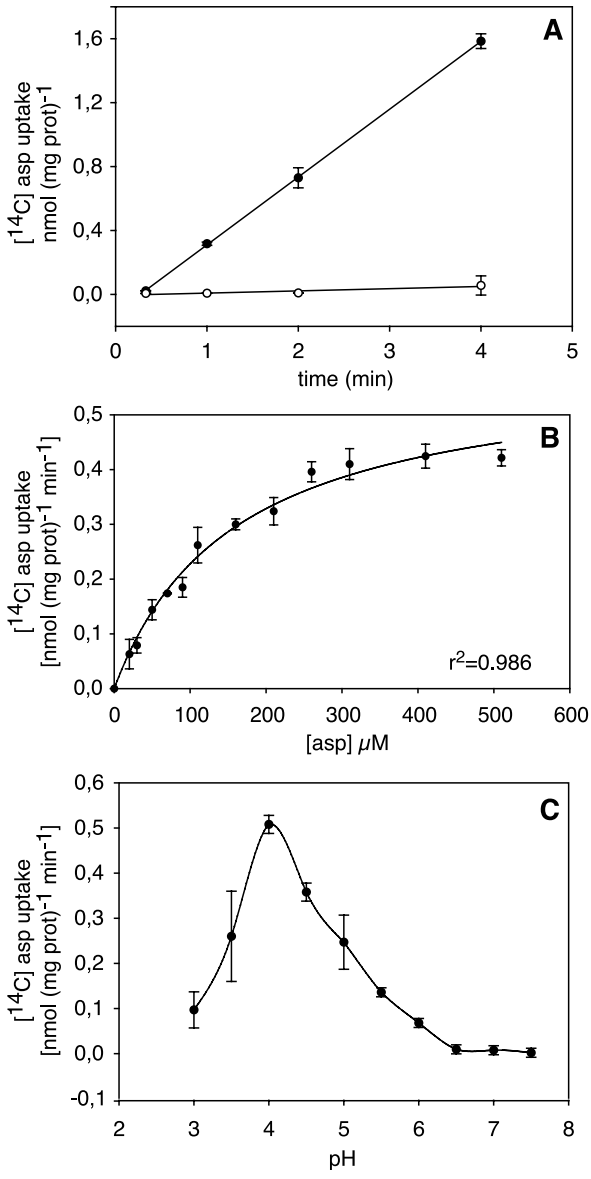

Fig. 5. Uptake of ^{14}C-aspartate by the yeast mutant *22Δ8AA*. A: Time dependence of aspartate uptake. Mutants were transformed with the empty vector pDR196 (white circles) or with pDR196 expressing *HcGAP1* (black circles). Yeast cells were assayed for ^{14}C-aspartate uptake at 150 μM and pH 4.5. B: HcGAP1-mediated aspartate uptake at different substrate concentrations. Experiments were performed at pH 4.5. C: pH dependence. Yeast expressing HcBAP1 in pDR196 measured at different pH values and 100-μM substrate concentration. Values represent the mean of three independent experiments ±S.D.

HcGAP1 sequence includes the amino acid permease conserved domain (RPS-BLAST 2.2.1 (Aug. 1 2001)). The best homology for the deduced HcGAP1 protein sequence was obtained with an amino acid permease of *Uromyces fabae* [28] with an identity of 39% and similarity of 55%. Phylogenetic analyses by maximum of parsimony confirmed the strong homology between *HcGAP1* and *Uromyces fabae* and *Amanita muscaria* [29] amino acid permeases (Fig. 3). The fungal origin of the cDNA is strongly supported by the fact that all homologies revealed by the BLAST searches are homologies to fungal genes. The cDNA also showed homology to the APC family in yeast mediating H^+-coupled amino acid uptake [30]. Hydropathy analyses of HcGAP1 with the TMHMM algorithm [31] predict twelve putative transmembrane domains (Fig. 4). The amino-terminus is approximately the same length as that of the yeast GAP1. The carboxy terminus, however, is just around the half of the yeast GAP1. Both C and N termini are predicted to protrude into the intracellular space.

The same gene was identified three times when using a suppression cloning system for proline uptake deficiency (yeast strain *22Δ8AA*), indicating that HcGAP1 encodes a broad specificity amino acid transporter. This is further supported by growth analysis of the multiple knockout strain *22Δ8AA* expressing HcGAP1 under selective conditions using arginine, aspartate, glutamate and proline as sole N-sources (data not shown).

3.3. Kinetics of aspartate uptake by HcGAP1 in yeast mutant deficient in amino acid uptake systems

To determine the transport properties of HcGAP1 directly, radiotracer uptake studies were performed using ^{14}C-labelled aspartate, as growth of *22Δ8AA* expressing HcGAP1 was best on aspartate as single nitrogen source (data not shown). Yeast cells expressing HcGAP1 showed more than 100-fold increased uptake rates of ^{14}C-aspartate as compared with cells transformed with pFL61 vector alone (Fig. 5A). Under standard assay conditions, ^{14}C-aspartate uptake was linear for at least 4 min. The uptake rate was concentration dependent and displayed saturation kinetics (Fig. 5B). The K_m value for the transport for aspartate of 150 μM is in the range of amino acid concentrations found in the soil [27], making it probable that in the soil HcGAP1 is involved in amino acid uptake for fungal nutrient acquisition. HcGAP1 activity was strictly pH-dependent with an optimum at approx. pH 4 (Fig. 5C), consistent with the pH optimum described for the uptake of glutamate and glutamine by mycelia of the ectomycorrhizal fungus *Paxillus involutus* [32]. ^{14}C-aspartate uptake depended on the presence of glucose and was sensitive to the protonophores 2,4 DNP and CCCP and the plasma membrane H^+-ATPase inhibitors DES and vanadate, indicating that energization is required for transport (Fig. 6). The strong depen-

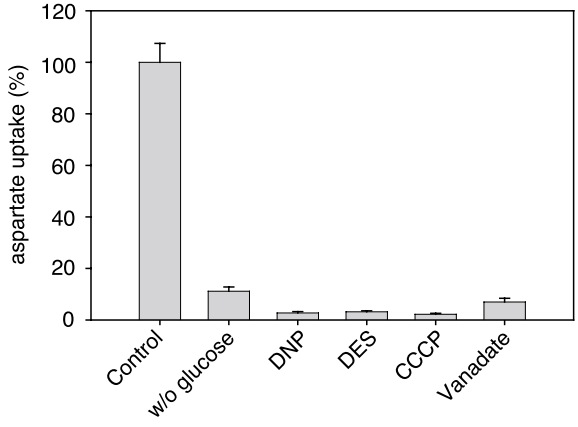

Fig. 6. Influence of plasma membrane energization on the uptake rate of ^{14}C-aspartate in the yeast mutant *22Δ8AA* expressing *HcGAP1*. Yeast cells were preincubated for 5 min in the presence of 100 mM glucose (control), without glucose, or with glucose and 0.1 mM DNP, or 0.1 mM DES, or 0.1 mM CCCP, or 0.1 mM vanadate. Values represent the mean of three independent experiments ±S.D.

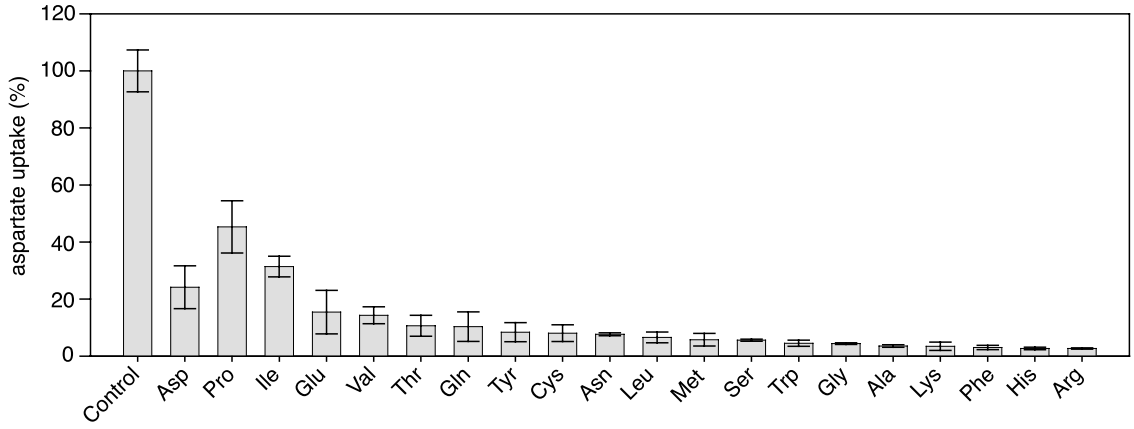

Fig. 7. Substrate specificity of *HcGAP1*. Inhibition of 150 μM ^{14}C-aspartate uptake by a five-fold molar excess of competing amino acids. Data are expressed as percentage of the uptake rate in presence of 150 μM aspartate. Values represent the mean of three independent experiments ± S.D.

dence on the presence of glucose and a proton gradient indicates that HcGAP1-mediated transport is mediated by a secondary active transport mechanism similar to its yeast homologs [16]. The range of amino acids transported by HcGAP1, as well as their transport efficiency, was determined by their competitive effect on the uptake of labeled aspartate (Fig. 7). Most amino acids tested, except proline and isoleucine, competed even more efficiently as compared to aspartate. Thus HcGAP1 is a general amino acid permease with a high affinity, allowing import of a wide spectrum of amino acids from the soil solution into *Hebeloma* mycelia.

3.4. Expression pattern of HcGAP1

To investigate the role of HcGAP1 in the *H. cylindrosporum*/*P. pinaster* ectomycorrhizal association, the expression was analyzed by RNA gel-blot analysis (Fig. 8A). Strong expression of HcGAP1 was detected in mycelia grown on a standard medium. No mRNA was detected in *Pinus* roots and needles, confirming the fungal origin of HcGAP1. No transcripts could be detected in mycorrhiza, where the expression of HcGAP1 to take up amino acids from plant cell would be counterproductive. It has not, however, been shown directly that the expression is also high in extrametrical hyphae. Further experiments are required to determine the pattern of expression along the hyphae in a real interaction in soil. The results may suggest that HcGAP1 plays a role in the uptake of amino acids from the soil for the fungal nutrition and further transfer to the plant partner, but is repressed in the mycorrhizal organ (Fig. 8B)

4. Conclusions

An *Hebeloma* gene coding for a general amino acid permease was cloned by suppression cloning in a yeast mutant deficient in histidine uptake, using a yeast expression cDNA library generated from mycelia of *Hebeloma* grown on an amino acid mixture as nitrogen source. The uptake characteristics allowed description of HcGAP1 as a high-affinity, secondary active proton coupled general amino acid permease. The main function of HcGAP1, as indicated by gene expression, may be the uptake of amino acid from the soil solution for fungal nutrition.

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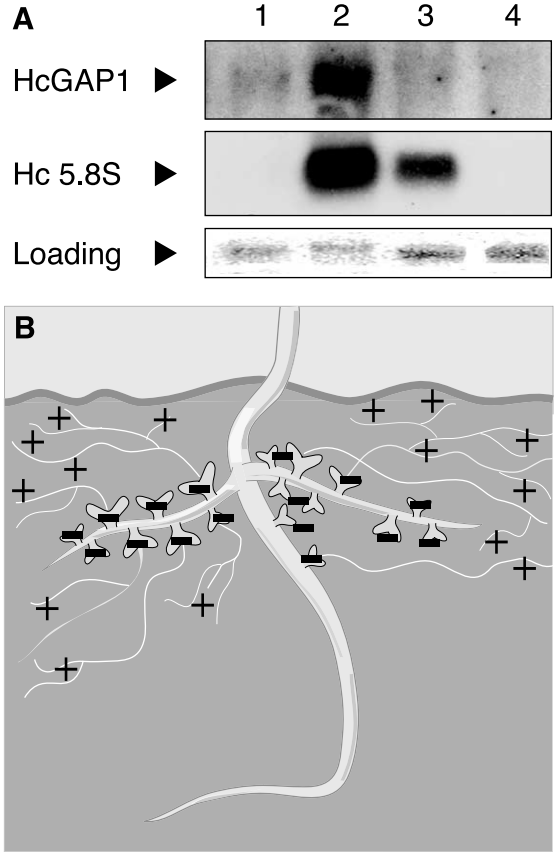

Fig. 8. RNA gel-blot analysis of *HcGAP1* regulation. A: Expression of *HcGAP1* in *P. pinaster* needles (1), *H. cylindrosporum* mycelium in pure culture (2), mycorrhizas (3) and in *P. pinaster* roots (4). *H. cylindrosporum* 5.8S rRNA probe was used as a fungal loading control. B: Scheme of putative HcGAP1 expression along the mycorrhizal association. *HcGAP1* is expressed (+) in the mycelium for fungal nutrition and is repressed (-) in the mycorrhiza itself, where its expression would be counterproductive.

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