Characterization of organic nitrogen transport in the ectomycorrhizal fungus *Hebeloma cylindrosporum*

Dissertation

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Table of Contents

Zusammenfassung

Ektomykorrhizen haben in borealen Nadelwäldern und temperierten Laubwäldern der Nordhemisphäre eine entscheidene Aufgabe in der Versorgung ihrer pflanzlichen Partner mit Stickstoff, der wichtigste wachstumslimitierende Nährstoff. In diesen Waldböden ist Stickstoff hauptsächlich in organischen Verbindungen vorhanden, welche nicht für die Pflanze, aber für den Ektomykorrhizapilz verwertbar sind.

Die mit der Pflanze in Symbiose lebenden Pilze können mit Hilfe von extrazellulären Proteinasen Proteine in diesen Böden spalten. Dies ermöglicht dem Pilz organischen Stickstoff in Form von freien Aminosäuren und Peptiden aufzunehmen, zu assimilieren und schliesslich (zum Teil) an die Pflanze abzugeben. Da der Ektomykorrhizapilz einen wichtigen Beitrag zur Stickstoffversorgung für die mit ihm assoziierten Pflanze leistet, ist es von Bedeutung, die Aufnahmemechanismen von organischen Stickstoff vom Boden in den Pilz (und schliesslich Transport in die Pflanze) und deren Regulation zu verstehen.

Um Transporter zu identifizieren, die an der Aufnahme von organischen Stickstoffverbindungen aus dem Boden in die Pilzhyphen bzw. am Transport vom Pilz in die Pflanze, beteiligt sind wurde eine cDNA Bibliothek vom Myzel des Ektomykorrhizapilzes *Hebeloma cylindrosporum* hergestellt. In dieser Arbeit wurde die Qualität dieser Bibliothek getestet, indem ca. 500 ESTs sequenziert wurden und damit eine Sequenzdatenbank für den Modellorganismus *H. cylindrosporum* hergestellt (www.uni-tuebingen.de/plantphys /hebeloma/index.html).

Von dieser cDNA Bibliothek wurde ein Gen, das für einen Aminosäuretransporter (HcGAP1) kodiert isoliert und charakterisiert. Zusätzlich wurden zwei Peptidtransporter (HcPTR2A, -B) charakterisiert. Die Analyse dieser Transporter durch Aufnahmeexperimenten zeigt, das sie am Import von organischen Stickstoffverbindungen beteiligt sind. Weiterhin konnte durch Expressionsstudien gezeigt werden, dass diese Transporter durch verschiedene Stickstoffquellen reguliert werden.

Da extrazelluläre Proteinasen eine wichtige Rolle für die Verfügbarkeit von organischen Stickstoff für den Pilz bzw die Pflanze spielen, wurde die Proteaseaktivität von *Hebeloma* getestet und ein Gen das möglicherweise für eine Proteinase kodiert isoliert.

Schliesslich wurde eine Methode zur Transformation von *Hebeloma* via *Agrobakterium tumefaciens* etabliert.

Summary

Ectomycorrhizal trees dominate boreal and temperate forest ecosystems in which nitrogen is generally accepted to be the most important growth-limiting nutrient. In these forest soils nitrogen is mainly available as organic compounds which are not accessible to plants but to the ectomycorrhizal fungi.

The fungal partners are able to break down proteins present in these soils by using extracellular proteinase. Thus, they can take up and assimilate organic nitrogen in the form of free amino acids and peptides which can then be transferred to the plant. Since ectomycorrhizal fungi strongly participate in nitrogen nutrition of the plant in these soils, it is necessary to understand uptake of organic nitrogen from the soil by the fungus (its subsequent transport to the plant) and its regulation.

To identify the transporters involved in the uptake of organic nitrogen compounds by the fungal hyphae and their transfer to the plant, it was necessary to develop genomic tools. An oriented expression library was constructed from the mycelia of the ectomycorrhizal fungus *Hebeloma cylindrosporum.*

In this work, the quality of this library was tested by DNA sequencing of ~500 ESTs and a sequence database was generated for the model fungus *H. cylindrosporum* (www.uni-tuebingen.de/plantphys/hebeloma/index.html).

Furthermore the suitability of the library to identify *Hebeloma* genes via their function was demonstrated.

Using *Hebeloma* cDNA libraries a gene encoding for an amino acid transporter (*HcGAP1*) was isolated and characterized. Two peptide transporters (*HcPTR2A, -B*) were also characterized. The characterization of these transporters by uptake experiments shows that they play a role in the import of organic nitrogen compounds into *Hebeloma*. Expression studies demonstrated that these transporters are regulated by different nitrogen sources.

As extracellular proteinases play an important role in organic nitrogen availability, the proteinase activity of *Hebeloma* was characterized and a gene encoding for a putative extracellular proteinase was isolated.

Finally, a method for *Agrobacterium tumefaciens* mediated transformation of *Hebeloma* was successfully established.

Abbreviations:

AA	amino acid
AATA	amino acid toxic analog
AAP	amino acid permease
ABC transporter	ATP binding cassette transporter
ADH	alcohol dehydrogenase
APC superfamily	amino acid polyamine choline superfamily
AS	Acetosyringone
Asp	aspartate
ATF1 superfamily	amino acid transporter superfamily
ATMT	*Agrobacterium tumefaciens* mediated transformation
ATP	adenosine tri-phosphate
BLAST	basic local alignment search tool
BSA	bovine serum albumine
Bq	Becquerel
Bp	base pairs
C	carbon
CCCP	carbonylcyanide m-chlorophenylhydrazone
CAT	cationic amino acid transporters
Cys	cysteine
DEPC	diethyl pyrocarbonate
DES	diethylstillbestrol
DNA	deoxyribonucleic acid
2,4 DNP	2,4 dinitrophenol
EDTA	ethylenediamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EST	expressed sequence tag
FITC	fluorescein-isothiocyanat
GABA	γ - aminobutyric acid
GFP	green fluorescent protein
Gln	glutamine
gpd	glyceraldehyde phosphodehydrogenase
hph	hygromycin B phosphotransferase gene
K	potassium
K_M	Michaelis constant
Leu	leucine
LHT	lysine histidine transporter
MCT	H^+/monocarboxylate transporters
MFS	major facilitator super family
MMN	modified Melin Norkans medium
MDR	multi drug resistance
N	nitrogen
NADP	nicotinamide-adenine-dinucleotide-phosphate
NCBI	National Center for Biotechnology Information
NCR	nitrogen catabolic repression
NH_4^+	ammonium
NO_3^-	nitrate

nt	nucleotide
NTS	neurotransmitter superfamily
OD	optical density
OPT	oligopeptide transporter
P	phosphate
PCR	polymerase chain reaction
PGK	3-phosphoglycerate kinase
PMA	plasma membrane H^+-ATPase
PMF	proton motive force
POT	proton coupled oligopeptide transporter
ProT	proline transporter
PTR	peptide transporter
PVC	polyvinyl chloride
RNA	ribonucleic acid
RT	room temperature
SC	synthetic complete
SDS superfamily	Sodium dicarboxylate symporter
VGT	vesicular glutamate transporter
YMG	yeast extract maltose glucose

Introduction

1 Introduction

1.1 Nitrogen sources available for plants

In the soil nitrogen (N) occurs in various forms such as ammonium, nitrate, amino acid, peptides and other complex nitrogen-containing molecules. The form in which nitrogen is available for the plants is largely dependent on climate, soil type acidification and nitrification processes, vegetation and fertilization. Nitrogen is an essential nutrient for plants and serves as a component of amino acids and consequently proteins, nucleic acids, vitamins and other important compounds such as hormones and chlorophyll. Nitrogen, as the mineral element of which plants require the greatest amount, often limits plant growth. The acquisition and distribution of nitrogenous compounds by plants is a complex network of demand and supply. A diverse array of transporters for these compounds varying in their specificity, affinity, capacity and/or expression pattern play a key role in these processes. Plants are generally thought to acquire nitrogen primarily in the form of inorganic nitrogen, e.g. ammonium and nitrate. Following uptake, inorganic nitrogen is assimilated into organic nitrogen forms. Ammonium assimilation usually takes place in root cells. In comparison, nitrate assimilation can occur either in root cells or in leaves, depending on the nitrogen supply and the plant species. In temperate and boreal forest ecosystems (largest terrestrial biome of the earth) nitrogen is mainly available as organic compounds not easily accessible for plants. These areas exhibit low mineralization rates due to low temperature and/or pH, causing these soils to become depleted of easily available nutrients (Van Cleve & Yarie, 1986; Read, 1991; Kielland, 1994). It has been proposed that selection favored symbiosis between plant roots and ectomycorrhizal fungi in growth limiting environments due to fungal improvement of plant access to nutrients (Read, 1991).

Plants, which live in symbiotic associations with nitrogen fixing bacteria, can use atmospheric nitrogen provided by the bacteroid in the form of ammonium or alanine (reviewed in Udvardi and Day, 1997; Waters *et al*, 1998). Mycorrhizal fungi make a valuable contribution to the nitrogen nutrition of their host by absorbing, assimilating and translocating simple nitrogenous compounds from the soil to the root and by converting complex nitrogen sources to more readily utilizable forms. The exchange

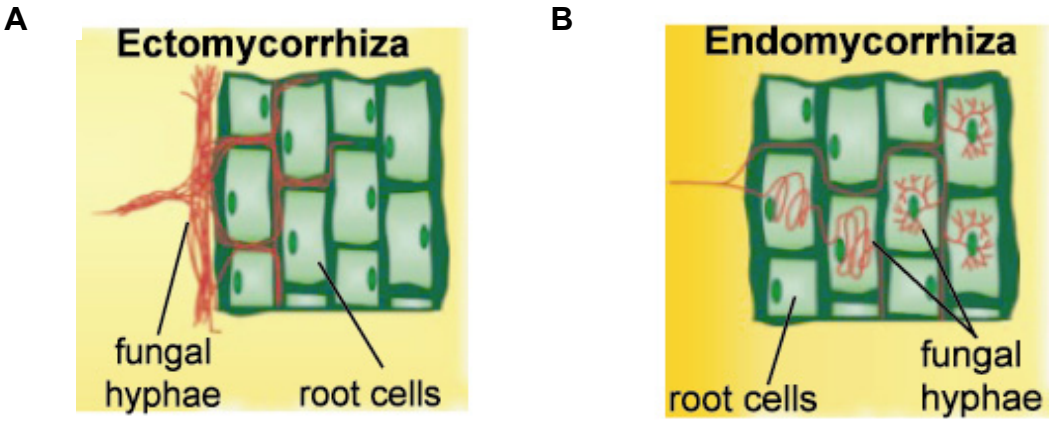
from the fungus to the plant is thought to occur in the form of amino acids (Martin and Botton, 1993).

1.2 What are mycorrhizas ?

1.2.1 Introduction

Mycorrhizas are symbiotic associations that form between the roots of most plant species and fungi. These symbioses are characterized by bi-directional movement of nutrient where carbon (C) flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and soil. In infertile soils, nutrient taken up by the mycorrhizal fungi can lead to improved plant growth and reproduction. As a result, mycorrhizal plants are often more competitive and better able to tolerate environmental stresses (pathogens attack, drought…) than are nonmycorrhizal plants.

At least seven different types of mycorrhizal associations have been recognized (vesicular-arbuscular mycorrhizas, ectomycorrhizas, orchid mycorrhizas, ericoid mycorrhizas, ectendo-, arbutoid and monotropoid mycorrhizas) (Brundrett, 1996), involving different groups of fungi and host plants and distinct morphology patterns (Tab 1.1). The most studied mycorrhizal associations are the vesicular-arbuscular mycorrhizas (Fig 1.1.A) (or endomycorrhizas), where fungi produce arbuscules, hyphae, and vesicles within root cortex cells, and ectomycorrhizas (Fig. 1.1.B) (described later in more details).



(from Mykorrhiza-CD, Fester *et al.*)

Figure 1.1: Looking at the mycorrhizal association. **A.** Scheme of the interaction between the fungus and the root cell in ectomycorrhiza association; the fungal hyphae form a mantle around roots and a Hartig net between the root cells. **B**. Scheme of the endomycorrhiza association; the hyphae penetrate the root cells and form arbuscules and vesicules.

The Orchidaceae form mycorrhizas with basidiomycetes of various affinities (orchid mycorrhizas) where the fungi produce coils of hyphae within roots (or stems) of the plants. Some fungi forming orchid mycorrhiza are saprophytes or parasites of other plants and transfer organic C and mineral nutrient to the orchids. Ericoid mycorrhizas involve hyphal coils in outer cells of the narrow “hair roots” of plants. Ectendo-, arbutoid and monotropoid mycorrhiza associations are similar to ectomycorrhizal associations, but have specialized anatomical features. In the ectendomycorrhizas, the mantel may be reduced or absent, the Hartig net is usually well developed, but the hyphae penetrate into the cells plant. The same species of fungus may form ectomycorrhiza with one plant species and ectendomycorrhiza with another. In Arbutoid mycorrhizas, mantle, external hyphae and a developed Hartig net are present. In addition there is an extensive intracellular development of hyphal coils in the plant cells. The monotropoid mycorrhizas are somewhat similar to the arbutoid and ectendomycorrhizas and in addition they have a haustorium-like structure which penetrate the epidermal cells and goes through a complicated developmental pattern as the plant grows and flowers.

	Endo-mycorrhiza	Ecto-mycorrhiza	Ectendo-mycorrhiza	Arbutoid	Monotropoid	Ericoid	Orchid
Fungi							
septate	- (+)	+ -	+ -	+	+	+	+
Intracellular colonization	+	-	+ -	+	+	+	+
Fungal mantle	-	+ (-)	+ (-)	+	+	-	-
Hartig net	-	+	+	+	+	-	-
Vesicles	+ -	-	-	-	-	-	-
Host plant	Vascular plant	Gymno- & Angiosperm		Ericales	Mono-tropaceae	Ericales	Orchidaceae
Chlorophyllous plant	+	+	+	+ -	-	+	+ -
Fungi taxa	Glomeromyceta	Most Basid-, but some Asco- and Glometo				Asco -(Basid-)	Basid-

The fungal taxa are abbreviated from Glomeromyceta, Basidiomycetes and Ascomycetes. (after Brundrett *et al.*, 1996)
+ = present, - = absent, (+) = sometimes present, (-) = sometimes absent, +- = present or absent

Table 1.1 : The characteristics of the important mycorrhizal types

Fossil records and molecular clock dating suggest that all extant land plants have arisen from an ancestral arbuscular mycorrhizal condition. Arbuscular mycorrhizas evolved concurrently with the first colonization of land by plants some 450-500 million years ago and persist in most extant plant taxa. Ectomycorrhizas (about 200 million years ago) and ericoid mycorrhizas (about 100 million years ago) evolved subsequently as the organic matter content of some ancient soils increased and sclerophyllous vegetation arose as a response to nutrient-poor soils respectively. Mycorrhizal associations appear to be the result of relatively diffuse coevolutionary processes. While early events in the evolution of mycorrhizal symbioses map have involved reciprocal genetic changes in ancestral plants and free-living fungi, available evidence points largely to ongoing parallel evolution of the partners in response to environmental change (Axelrod, 1986) (Cairney, 2000).

1.2.2 Ectomycorrhiza

In the North-Hemisphere forest soil, more than 95% of the root tips of boreal forest trees form an ectomycorrhiza symbiosis (Fransson *et al.*, 2000). The importance of the mycorrhizal fungi for the performance of the trees is also highlighted by the fact that around 30% of the carbon assimilated by the tree has been estimated to be allocated to the fungal symbionts (Smith & Read, 1997). Ectomycorrhizal fungi receive simple carbohydrates directly from their host trees, and in return the fungi supply the trees with nutrients and water (Smith & Read, 1997).

The ectomycorrhiza roots are characterized by the presence of a mantle of fungal tissue which encloses the root. The hyphae grow between the epidermal and cortical cells and form the Hartig net. The apoplast of the hyphae mantle forms a impermeable layer and thus must be a barrier to nutrient movement between soil and root. The presence of the impermeable layer means that all solutes reach the root cells via the fungal symplast of the mantle, first by translocation in the external mycelium and subsequently by efflux to the interfacial apoplast in the Hartig net region. Conversely, solutes from the root cells effluxing to the apoplast must pass to the fungal symplast and cannot “leak” to the soil via the apoplast of the fungal mantle. Thus, the impermeable layers offer an opportunity control conditions and solute concentrations in the cortical apoplast and Hartig net region, where transport between the ectomycorrhizal symbionts must occur (Smith and Read, 1997).

Intercellular penetration induces profound morphogenetic change in the mycorrhizal hyphae. The fungal cells form a labyrinthine structure, which is the product of repeated and prolific hyphal branching at the root cell surface. These prolific branching of the fungus as it encircles the epidermal or cortical cells leads to a structure of immensely enlarged surface area. The hyphal walls are so closely associated with those of the plant that the two appear to be fused in a joint structure which has been termed “the contact zone” (Smith & Read, 1997). The similarities between the elaborate structures produced by the proliferation of the hyphae in the Hartig net, and those seen in transfer cells which increase the surface area for exchange of solutes in many physiologically active plant tissues, has been recognized (Kottke & Oberwinkler, 1987).

The bi-directional movement nutrient and metabolite is the essential features of functional mycorrhizas (Smith & Read, 1997). The fungal symbionts absorb nutrients available in inorganic/organic form in soil (water, minerals, cations) and translocate them (or their metabolite) to the symbiotic roots through the extensive vegetative mycelium. Organic C derived from photosynthesis is also transferred from the plant to the fungus, followed by translocation to the growing margins of the extraradical mycelium and to developing spores and fruit bodies (Smith & Read, 1997).

1.3 Transport across cell membranes

The uptake of nitrogen by the fungus and later the exchange of carbohydrates and nitrogen with the plant, requires a number of membrane transport steps along the translocation pathway. Biological membranes are gradient selective regarding transport of molecule, which is often permitted in only one direction. The explanation for such a selective permeability is the presence of transmembrane transport proteins, which mediate the transfer of specific molecules, including ions, across biological membranes.

Due to a limited number of transporters in the membrane, such transport is saturated when the substrate is present in high concentration. This kind of transport can be described by the Michaelis-Menten formula, in which the K_M value represents the substrate concentration for which half of the transporters are bound to a specific substrate. The substrate specificity can be studied by competitive inhibition. All

membrane transport proteins are integrated membrane proteins and are classified in the three major groups: channels, ATP-driven pumps and carriers transport.

Channels act mainly as selective pores for passive flux of ions and water molecules with or without transient binding of the substrate. The size and the charge density at the surface of a pore govern the selectivity of the channel. As long as the pore is open, water and ions diffuse spontaneously across membranes down a concentration or electric potential gradient in an energetically favorable process. The rate of the transport by channels can be very high and approach the limits posed by diffusion.

Carrier-mediated transport includes binding of the solute on one side of the compartment, conformational changes of the carrier protein leading to exposure of the substrate to the other side of the membrane and finally the release of the substrate. The transport rates for carrier are much lower than the channels. Carrier-mediated transport can be coupled to driving ions- typically H+ or Na+ - or uncoupled. Uncoupled carries catalyze transport down its electrochemical gradient (or simple concentration for uncharged solutes). Coupled carriers utilize the gradient of a coupling ion to generate a transmembrane solute gradient. The driving ion can be transported in the same direction (symporter) or in the opposite direction (antiporter).

Active transport can be differentiated into primary active and secondary active energy. Primary active transport is directly coupled to a metabolic source, such as ATP hydrolysis. These carriers are called pumps, e.g. the H+-ATPase which pump H+-ions across membranes out of the cytoplasm into cell wall space or vacuole, generating a membrane potential and a pH gradient. The energy which is stored in these electrochemical gradients, the proton motive force (PMF), is used to drive transport of other solutes by secondary active transport catalyzed by coupled carriers.

1.3.1 Organic nitrogen transport

1.3.1.1 Amino acid transport

In yeast, animals and plants, amino acids play fundamental roles in a multitude of functions including protein synthesis, hormone metabolism, nerve transmission, regulation of cell growth, production of metabolic energy, synthesis of nucleobases, nitrogen metabolism and biosynthesis of urea. In those organisms, amino acids

transport was therefore well characterized and can be divided into five superfamilies (Wipf *et al.*, 2002a).

1.3.1.1.1 Amino acid-Polyamine-Choline transporter superfamily (APC)

Most of the APC family members were described for yeast where the amino acid transport is understood in detail (Fischer *et al.*, 1998). In *Saccharomyces cerevisiae*, 24 transporters involved in amino acid uptake, have been characterized. All contain 12 putative membrane spanning domains, most of them are not highly specific and can even transport a wide spectrum of substrate (Regenberg *et al.*, 1998). In addition, some members of the APC family, like SSY1, has been shown to be involved in the sensing of extracellular amino acid and to control the amino acid uptake.

APC homologs are also found in animals and plants. Nevertheless the phylogenetic analysis clearly shows that APC members group in three clusters reflecting the three kingdoms (yeast, plant and animal) (Wipf *et al.*, 2002a) (Fig.1.2).

APC transporters from animals and plants into two subgroups were categorized into two subgroups. The cationic amino acid transporters subfamily (CATs), which have 14 putative transmembrane domains, are found in both animals and plants. Mammalian CATs are represented by transporter mediating Na^+-independent uptake of cationic amino acids, (Closs *et al.*, 1993) and AtCAT1, a homolog of those transporters, has been found in plants (Frommer *et al.*, 1995). The second subfamily, including the yeast APC transporters, comprises of proteins characterized by 12 putative transmembrane domains.

In yeast, plant and animals, the amino acid transporters of the APC family are involved in the uptake of different substrates and can be coupled to Na^+ or H^+. They might be important for selective accumulation of specific amino acids, redistribution and to keep intracellular concentrations constant.

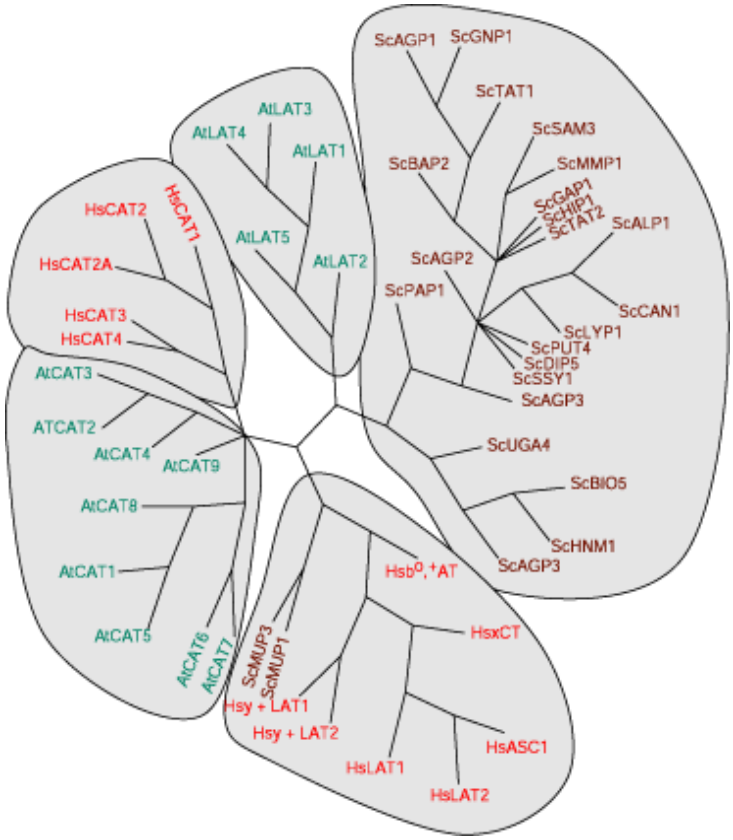


Figure 1.2: Phylogenetic tree of the amino acid-polyamine-choline (APC) superfamily (SLC7). Maximum parsimony analyses were performed using PAUP 4.0b4a informative(http://paup.csit.fsu.edu/index.html). The APC superfamily can be divided into five different clusters (shadowed areas).
Abbreviations: At, *Arabidopsis thaliana*; Hs, *Homo sapiens;* Sc, *Saccharomyces cerevisiae.*
(Wipf *et al*., 2002a)

1.3.1.1.2 Sodium-dicarboxylate symporter superfamily (SDS)

Members of the SDS family were described mainly for animals, but homologs exist in flies, worm and bacteria. SDS transporters mediate glutamate and aspartate uptake by cotransport of Na^+ and counter-transport of K^+, with no apparent dependence on chloride gradients [GLAST (Storck *et al.*, 1992); GLT-1 (Pines *et al.*, 1992), EAAC1 (Kanai & Hediger, 1992)]. Members of this superfamily are found mainly in brain tissue and are characterized by 10 putative membrane domains with C- and N-termini in the cytosol. Since the SDS transporters are involved in amino acid transport at synapses it may not be surprising that no related proteins were found in yeast and plant genomes, whereas homologs exist in other animals like flies and worms and in also bacteria.

1.3.1.1.3 Neurotransmitter superfamily (NTS)

Neurotransmitter transporters represent the third large superfamily of transporters (Wipf *et al*, 2002a). NTS members have 12 putative transmembrane helices and transport γ-aminobutyric acid (GABA), proline, glycine and betaine (Nelson, 1998). Like the SDS members, carriers belonging to this family are mainly found in brain but they couple amino acid uptake to electrogenic cotransport of Na^+ and Cl^-, allowing uptake of neurotransmitter molecules against a concentration gradient. Similar to the SDS family, NTS homologs do not seem to be represented in yeast and plant genomes.

1.3.1.1.4 Amino acid transporter superfamily 1 (ATF1)

In contrast to the other superfamilies, members of the ATF1 family were first described in plants, and structurally related proteins have only recently been identified in yeast and animals (Fischer *et al.*, 1998). The superfamily contains plant-specific subbranches, and branches structurally more closely related to yeast and human transporters. Members of this superfamily are predicted to have a structure with 9-11 putative membrane spanning domains with cytosolic N- and extracellular C-termini (Chang & Bush, 1997).

In plants the ATF1 superfamily can be divided into five subfamilies : the lysine histidine transporter (LHTs), the proline transporters (ProTs), the AUX1-related proteins and a new branch comprising of vacuolar and vesicular amino acid carrier from yeast and animals, and amino acid permease (AAPs).

The best characterized members of the ATF1 superfamily are the *Arabidopsis* AAPs (amino acid permeases from 1 to 8), which mediate proton-coupled uptake of a wide spectrum of amino acids (Fischer *et al.*, 2002). All characterized members of AAP family have overlapping spectra of transported substrates. Interestingly, each member of AAP family shows a distinct expression pattern, indicating non-redundant roles for each AAP in plant.

The mammalian ATF1 homologs are most closely related to yet non-characterized plant genes, but functionally resemble plant transporters for cellular uptake of amino acids. The coupling mechanisms are different, animal transporters couple amino acid uptake to the sodium gradient whereas plant transporters couple amino acid uptake to the proton gradient.

1.3.1.1.5 Amino acid transporters within the Major Facilitator Super-family (MFS)

The MFS family can be subdivided in two subfamilies : (i) the H^+/monocarboxylate transporters (MCT), which have been described in animals and mediate uptake of aromatic amino acids (Kim *et al.*, 2001). Homologs of MCTs have also been found in yeast (ii) the vesicular glutamate transporters (VGT), which are closely related to inorganic phosphate transporters, with homologs in yeast (allantoate transporters) and in Arabidopsis but their function is still unknown.

Beside these five families, an other group of amino acid transporters, designated as organellar transporters, has to be mentioned. The mitochondrial transporter family and the plastidic transpoters belong to this group.

1.3.2 Peptide transporters

Peptide transport is a widely observed physiological phenomenon. Gene encoding peptide transporters have been cloned and characterized from Bacteria (Smid *et al.*, 1989), fungi (Perry *et al.*, 1994), plants (Rentsch *et al*., 1995), vertebrates (Fei *et al.*, 1994) and invertebrates (Fei *et al.*, 1998). Peptide transporters are categorized into three families (Tab 1.2; Stacey *et al.*, 2002) : the ATP binding cassette family (ABC transporters; Higgins, 1992), the oligopeptide transporter (OPT; Lubkowitz *et al*., 1997; Hauser *et al*., 2001) family and the peptide transporter (PTR) or proton-coupled oligopeptide transporter (POT) family (transporting di- and tripeptides but also including nitrate transporters, Paulsen & Skurray, 1994; Steiner *et al*., 1995).

Family	Substrates	Reported occurrence	Energy source
ABC family	Amphipathic ions (including peptides)	Bacteria, fungi, plants and animals	ATP hydrolysis
PTR family	Di- and tripeptides (also nitrate and histidine)	Bacteria, fungi, plants and animals	Proton gradient
OPT family	Tetra- and pentapeptides	Fungi and plants	Probably proton gradient

Stacey *et al*, 2002

Table1.2: Peptide transporters families

1.3.2.1 ABC family

The ABC superfamily is the largest protein family known (Stacey *et al*., 2002), with members in prokaryotes, eukaryotes and archaea. Most of the proteins are transporter, which export or import with a wide spectrum of substance like sugar, anorganic anion, polysaccharide, protein or peptide.

ABC-transporter from prokaryotes are formed of two membrane proteins, each have six transmembrane domain, and two ATP-binding protein consisting of a conserved Walker A motif (Gx4GK[S/T]) and a Walker B motif ([R/K]x3Gx3L[hydrophobic]3) (where x represents any amino acid; Detmers *et al.*, 2001). In the Eukaryotes, both function are combine together in one protein, which 12 to 14 transmembrane domain. The ABC-transporter use energy from the ATP hydrolysis for the transport. Plant member of the ABC-family belong to the MRP-subfamily (multi drug resistance proteins) (Sánchez-Fernández *et al.,* 2001).

1.3.2.2 OPT family

This family was recently described. The members have like the ABC - or the PTR - family, 12 to 14 putative transmembrane domains but no sequence similarity was shown. So far the OPT family has only been described from the yeasts *Candida albicans* (*Ca*Opt1p; Lubkowitz *et al*., 1997), *Schizosaccharomyces pombe* (Isp4p; Lubkowitz *et al*., 1998) and *Saccharomyces cerevisiae* (Opt1p and Opt2p; Hauser *et al*., 2000). By database search nine genes from *Arabidopsis*, similar to the OPT family, were identified (Koh *et al*., 2002). Seven of the genes showed specific expression in various tissues. Using leucine containing tetra- and pentapeptides five genes could complement the leucine auxotrophy of a *S. cerevisiae* mutant, suggesting uptake of these substrates. Like the PTR transporters, members of the OPT family probably use the PMF to energize transport.

1.3.2.3 PTR or POT family

PTR transporters have been shown to transport a wide range of nitrogen-containing substrates, including amino acids, peptides and nitrate (Williams & Miller, 2001) and were identified in prokaryotes and eukaryotes. Data showed pointed out that

members of this family may cotransport peptides and proton, and most likely di- and tri- peptides (Rentsch *et al*., 1995).

Analysis of the PTR members structure suggested they include approximatively 12 transmembrane domains and a unique signature FING motif (FYxxINxGSL) in the fourth or fifth hydrophobic segment (Steiner, 1995). Yeung et al. (1998) showed that FING motif is necessary for peptide transport. Most of the data about peptide uptake are available from *Saccharomyces cerevisiae*. In yeast, the transport of dipeptides and tripeptides has been well characterized physiocally and genetically (Becker and Naider, 1995) and involves 3 genes *ptr1*, *ptr2* and *ptr3* (Island *et al.*,1991; Perry *et al*, 1994; Barnes *et al.*, 1998). Only *ScPTR2* has been shown to have transport properties, *ScPTR1* and *ScPTR3* seems to regulate peptide transport. Other members of the PTR-family were described in *Lactococcus lactis* (DtpT, Harting *et al.*,1994) and *Candida albicans* (*Ca*PTR2, Basrai *et al*., 1995).

In higher plants, peptide transport has been reported to occur in several species (Higgins & Payne, 1982), i.e.: in *A. thaliana* (*AtPTR2*; Steiner *et al*, 1995), in *Hordeum vulgare* (*HvPTR1*; West *et al.,* 1998), in *Vicia faba* (Delrot *et al.,* 2001) in the carnivorous plant *Nepenthes alata* (Schulze *et al.*, 1997), and they are expressed in different tissues, in plasma membrane of the scutellar epithelium (*Hordeum vulgare)*, leaf tissue (*Vicia faba*) and phloem tissue (*Nepenthes alata)*.

The diversity of peptide transporters as a result of, for example, different patterns of tissue expression and cellular location, indicates that they probably play a variety of roles in plant metabolism. Moreover, in *Arabidopsis*, antisense silencing of *AtPTR2* (Song *et al.*, 1996) resulted in severe defects in seed and embryo development. Taken together, this suggests that peptide transporters might have other functions besides nutrient acquisition. In fact, Peptide transport systems might be involved in the movement of hormone–peptide conjugates across the plant cellular membrane (Salisbury & Ross, 1992). It was also suggested that they might play a role in the recognition mechanism of pathogens or symbionts, like mycorrhizal fungi. Microorganisms secrete modified peptide, which allows the plants to recognize pathogen from symbiotic partner (Gross, 1991)

1.4 Nitrogen nutrition in ectomycorrhizal fungi

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.

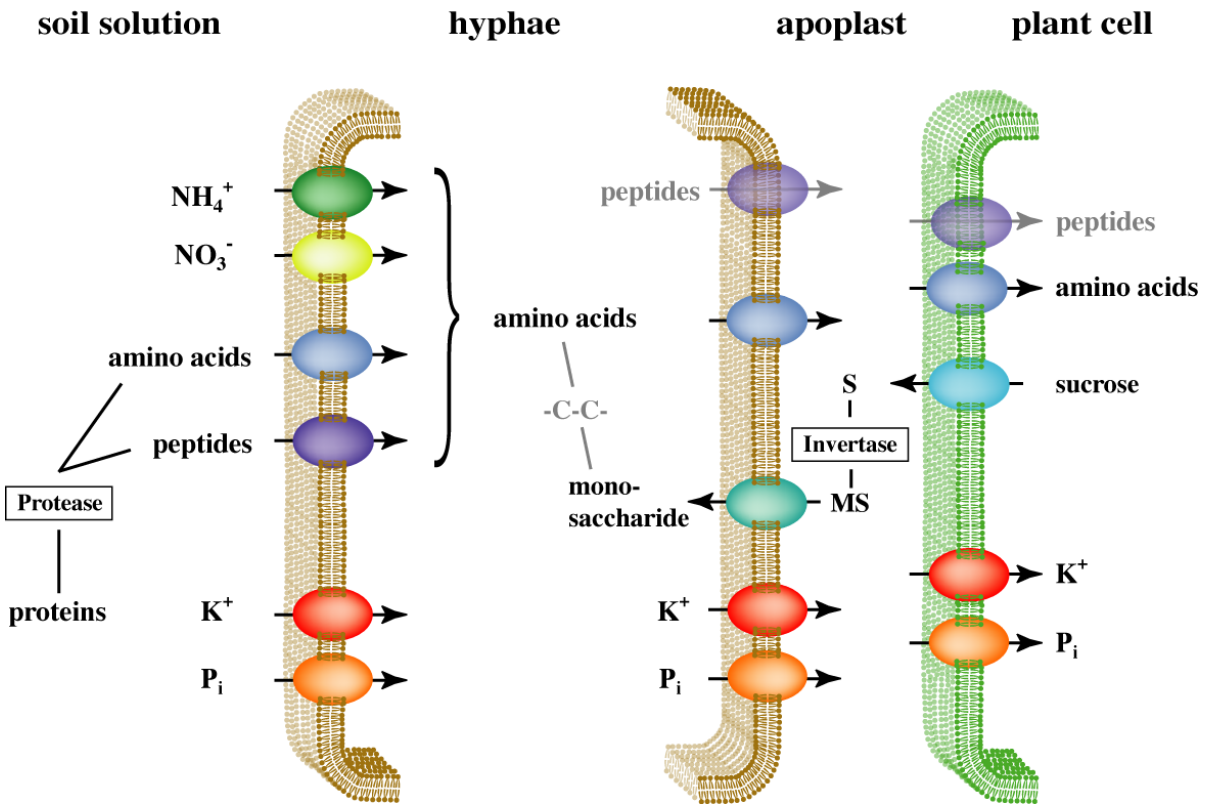
In fungi, data are mostly available for ascomycetes : *Saccharomyces cerevisiae, Neurospora crassa,* and *Aspergillus nidulans*. All studied fungi, ascomycetes or basidiomycetes, can use different organic or inorganic N sources. Better growth is obtained on ammonium, glutamine or glutamate. The presence of this N “primary” source leads to the repression of enzyme involved in the utilization of “secondary” N sources (urea, nitrate, proteins…) which are more complex or need more energy. Some fungi, like *Hebeloma* (Plassard *et al.*, 1994), and some yeasts (*Hanseluna*, *Rhodotorula*…) can use nitrate but *Saccharomyces cerevisiae* not (Smith & Read, 1997)

Moreover, several fungi, by producing extracellular proteinase, are able to use proteins as nitrogen sources and assimilate organic nitrogen in the form of free amino acids and peptides. Most of the work concerning extracellular proteinases was done in ascomycetes fungi , *Neurospora crassa* and *Aspergillus nidulans* (Jenning, 1989). However, it was also shown that some mycorrhizal fungi, *Hymenoscyphus ericae*, *Hebeloma crustuliniforme*, *Amanita muscaria*, *Amanita rubescens*, *Cenococcum geophilum*, *Paxillus involutus*, *Rhizopogon roseolus*, can use proteins as nitrogen source (Finlay *et al.*, 1992; El Badaoui & Botton, 1989). Fungi can take up all amino acids and some better than others. In most of the soil, glutamate, alanine and glutamine are the predominant amino acids and can be used by mycorrhizal fungi (Smith & Read, 1997).

In ectomycorrhizal fungi, all nitrogen sources are metabolized in glutamate, alanine or glutamine, exported to the Hartig net and through plant amino acid importer transferred to the plant (Fig 1.3) (Chalot *et al.*, 1991).

However, Chalot et al. (1996), could show the presence of amino acid transport systems in the ectomycorrhizal fungus *Paxillus involutus*; a high affinity transport system with a wide substrate spectrum and a low affinity system, which also has a

wide substrate spectrum. Until now, only few nitrogen transporter genes have been described in ectomycorrhizal fungi : amino acid- (*AmAAP1*; Nehls *et al.,* 1999), ammonium- (Javelle *et al*., 2003) and nitrate- transporter (Jargeat *et al*., 2003) genes



1.5 Nitrogen metabolism regulation

Many fungi have the ability to metabolize a broad range of compounds, which, in turn, demand a sophisticated array of regulatory mechanisms in order to achieve the appropriate genetic and physiological responses. An example of this is nitrogen metabolism in which hundreds of genes are coordinately regulated to utilize available nitrogen sources optimally. In *Aspergillus nidulans*, *Neurospora crassa* and *Saccharomyces cerevisiae*, the principal regulatory genes involved encode GATA transcription factors (Coffman *et al.*, 1997; Fu & Marzluf, 1990a; Kudla *et al.*, 1990; Minehart & Magasanik, 1991).

The GATA-binding family of transcription factors constitutes a subgroup of DNA-binding proteins whose members both bind a consensus 5´-HGATAR-3´motif (where H can be $^{A}/_{C}$ or T and R can be $^{A}/_{G}$). They are characterized by a highly conserved DNA-binding motif comprising a Cys(4) zinc finger followed by a basic domain (Omichinski *et al.*, 1993). They are found in plants, fungi and metazoans. In metazoans, they are involved in differentiation of a number of cell lines ranging from the erythroid line of vertebrates to the endoderm line of *Caenorhabditis elegans* (Pandolfi *et al.*, 1995; Pevny *et al.*, 1991; Tsai *et al.*, 1994). In fungi, GATA factors are involved in processes as diverse as regulation of nitrogen metabolism (Fu & Marzluf, 1990a; Kudla *et al.*, 1990), repression of siderophore biosynthesis and transcriptional activation of light-induced processes (Ballario *et al.*, 1996; Linden *et al.*, 1997). Generally speaking, there appears to be evolutionary conservation with regard to function.

The first of these genes to be characterized genetically was *areA* from *A. nidulans* (Arst & Cove, 1973; Caddick *et al.*, 1994; Wiame *et al.*, 1985). In the absence of the primary nitrogen sources, $NH4^+$ and glutamine, the *areA* product acts in concert with other transcription factors to facilitate the expression of the many structural genes required for the metabolism of other nitrogen sources. Loss-of-function mutations in *areA* (designated *areA*r) prevent utilization of most nitrogen sources apart from $NH4^+$ and Gln. Additionally, AreA has a direct or indirect role in regulating NADP-linked glutamate dehydrogenase (Christensen *et al.*, 1998), the key enzyme in nitrogen assimilation.

In *Neurospora crassa*, a homolog of *area* gene, *nit-2*, was identified and characterized (Fu & Marzluf, 1990b).

Nitrogen regulation of gene expression in *Saccharomyces cerevisiae* has been shown to be mediated by four GATA homologous proteins : Gln3p, Nil1p/Gat1p, Uga43p/ Dal80p and Gzf3p/Nil2p/Deh1p (Coffman *et al.*, 1997; Coornaert *et al.*, 1992; Cunningham & Cooper, 1991; Minehart & Magasanik, 1991; Soussi-Boudekou *et al.*, 1997; Szyroki *et al.*, 2001).

In fungi, GATA factors are involved in a physiological process designated nitrogen catabolic repression (NCR) (Wiame *et al.*, 1985). For instance, in yeast, NCR occurs at the level of transcriptional activation of genes encoding the permease and

catabolic enzyme systems needed to degrade poor nitrogen sources (e.g., allantoin, proline, and GABA). When readily used nitrogen sources (e.g., asparagine, glutamine or ammonia) are available, NCR-sensitive gene are expressed only at low levels (Cunningham *et al.*, 1996). Upon depletion of these repressive nitrogen sources, NCR is relieved and transcription of NCR-sensitive genes increases; (Cooper & Sumrada, 1983; Cunningham *et al.*, 1994). NCR-sensitive transcription is observed for a wide variety of genes like *DAL5, CAN1, GAP1, UGA1, UGA4, PUT1, PUT2,* … (Cunningham *et al*., 1996).

1.6 Biological model

As the external mycelium of all mycorrhizal types plays a key role in uptake of nutrient by plants, to understand organic nitrogen transport in ectomycorrhiza we need to investigate this kind of transport in the fungal partner. There are a number of ectomycorrhizal associations that have been widely used over the past few years for physiological studies on nitrogen metabolism. But for most of them genetic studies are not possible, due to the lack of information like the number of chromosomes, genome sequence, transformation technique.

However, Debaud *et al*. (1997) showed that homokaryotic *Hebeloma cylindrosporum* strains are appropriated for genetic studies. A dikaryotic strain is also available for mycorrhizal synthesis. *Hebeloma* is one of the few ectomycorrhizal fungi for which the different stages of the life cycle including fruit body production (Fig. 1.4) have been characterized and obtained in axenic culture under laboratory conditions (Debaud & Gay, 1987). Moreover, *H.cylindrosporum* can be transformed (protoplast transformation, Marmeisse *et al.*, 1992; *Agrobacterium* mediated transformation; Pardo *et al.*, 2002 and Combier *et al*., 2003) and the transformation of the plant partner *Pinus pinaster* is currently set up in the INRA Bordeaux (France) (Frigerio *et al.*, unpublished).

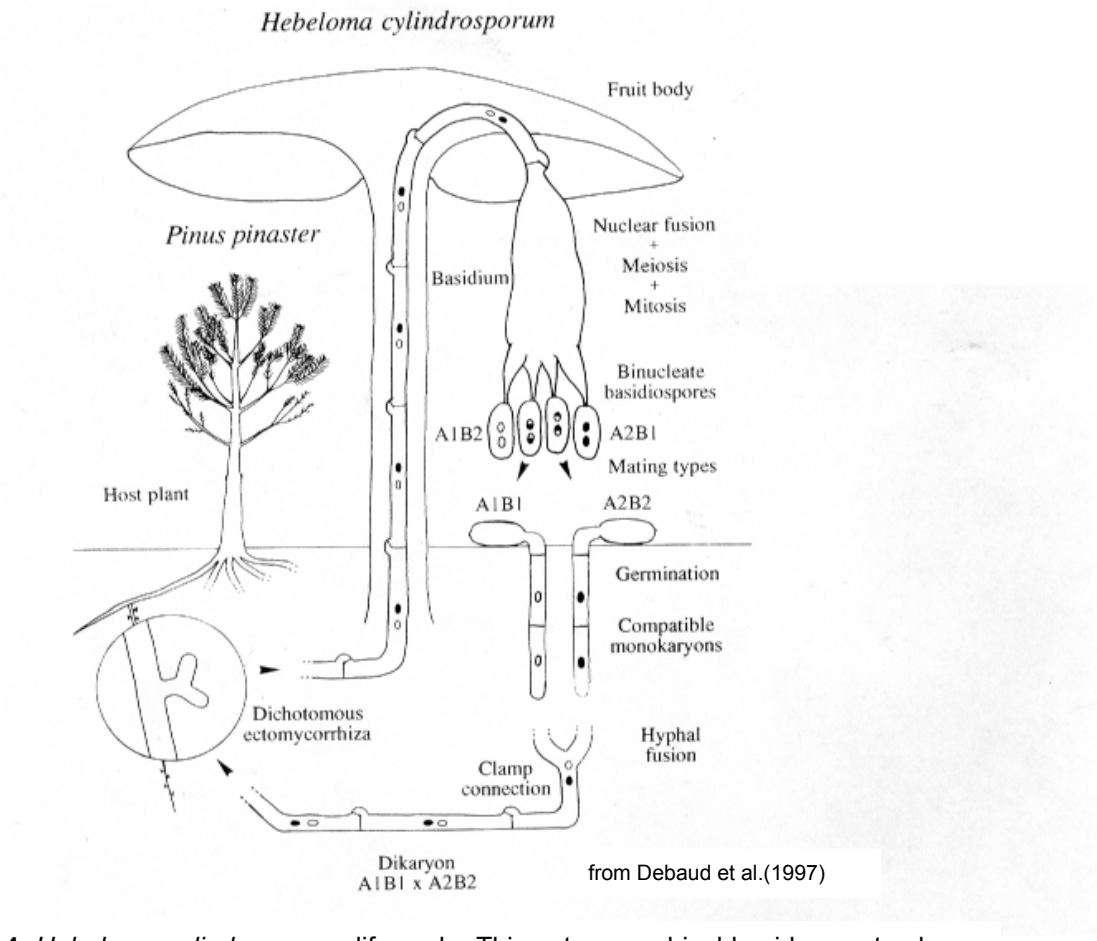


Figure 1.4: *Hebeloma cylindrosporum* life cycle. This ectomycorrhizal basidomycete shows a typical tetrapolar mating system. Spore germination gives rise to monokaryotic mycelia belonging to four mating types. Crossing between two compatible monokaryons results in dikaryons formation. The dikaryotic mycelium forms ectomycorrhizas on *Pinus pinaster* root systems, which allows the formation of sporulating basidiocarps.

Recently, a cDNA library from *Hebeloma* mycelia was constructed (Wipf *et al.*, 2003). Two features characterize this cDNA library; (i) it was prepared from homokaryotic mycelia subjected to different nitrogen nutrition conditions, in order to increase the chance of identifying genes involved in N acquisition by mycorrhiza (ii) the cDNA library was cloned in the yeast expression vector pDR196 (Wipf *et al.*, 2003) allowing assignment of functions to sequences by functional complementation of yeast mutant.
A second cDNA library prepared from K+- and Pi- starved dikaryotic mycelia, has been constructed in the yeast expression plasmid pFL61; and has been used to identified two peptide transporters from *Hebeloma* HcPTR2A and HcPTR2B (Wipf *et al.,* 2003; Rikirsch, 2002; Lambilliotte, unpublished data). The two putative peptide transporters, HcPTR2A and HcPTR2B, present 11 transmembrane spanning domains. HcPTR2A has a N-terminus in the cytoplasm and a C-terminus outside, and HcPTR2B

has both N- and C- termini in the cytoplasm. PTR signature, specific of the PTR transporter, was found in HcPTR2A and B.

Phylogenetic analysis of PTR-Family members from animals, plants, yeast and bacteria underlined the fungal origin of the genes (Fig 1.5). The two HcPTR2 form a distinct group, closely related to t he transporters from lower fungi.

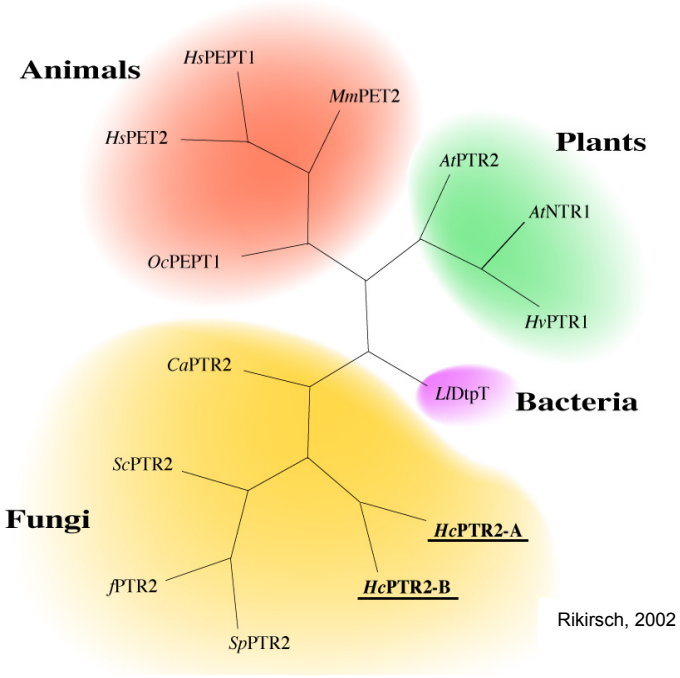


Figure 1.5: Phylogenetic tree generated by using the PAUP 4.0b10 package (Maximum of parsimony) on an alignment of peptide transporters (Swofford, 1998).
Arabidopsis thaliana (At), *Candida albicans* (Ca), Fungus (f), *Hebeloma cylindrosporum* (Hc), *Homo sapiens* (Hs), *Hordeum vulgare* (Hv), *Lacto-coccus lactis* (Lc), *Mus musculus* (Mm), *Oryctolagus cuniculus* (Oc), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp)

1.7 Aim of this work

Most of the plant in boreal and temperate forest make ectomycorrhiza and in these forest soils nitrogen is mainly available as organic compounds, which are not easily accessible for plants but can be taken up by ectomycorrhizal fungi.

The fungal partners, by using extracellular proteinases, are able to break down proteins, present in these soils, and to assimilate organic nitrogen in the form of free amino acids and peptides. After metabolism in the fungus, amino acids are transfered to the plant and compensate for the low availability of anorganic nitrogen in these ecosystems. Since, in these areas, ectomycorrhizal fungi fully participate to nitrogen nutrition of the plant, it is necessary to understand uptake of organic nitrogen from the soil to the fungus and its regulation. In this work we used the *Hebeloma cylindrosporum / Pinus pinaster* ectomycorrhizal association to investigate organic nitrogen transport in ectomycorrhiza.

Materials & Methods

2 Materials and Methods

2.1 Materials

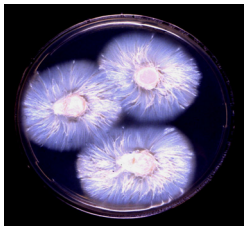
2.1.1 Biological materials

2.1.1.1 *Hebeloma cylindrosporum*

Hebeloma cylindrosporum Romagnesi is an ectomycorrhizal basidiomycete associated with gymnosperm trees in coastal sand dunes.

Two wild-type homokaryotic strains of *H. cylindrosporum,* h1 and h7 (Debaud & Gay, 1987), and dikaryotic strain D2, resulting from the crossing of h1 and h7, were used in this study.





2.1.1.2 *Pinus Pinaster* (Ait.)

Pinus Pinaster is the natural mycorrhizal partner of *H. cylindrosporum.*

Seeds of maritime pine were obtained from Conrad Appel “Samen und Pflanzen” (Darmstadt, Germany). Seeds were surface-sterilized in a 30% (w/w) H_2O_2 solution for 30 min, rinsed with sterile distilled water and air-dried. Germination was carried out on agar plate(14 $g.l^{-1}$) containing 2 $g.l^{-1}$ glucose.


2.1.1.3 Bacterial and Yeast strains

Escherichia coli XL1-Blue MRF (Bullock *et al.*, 1987)
recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F' proAB, lacIqZ∆M15, Tn10 (Tetr)]. Procedures for manipulating *E. coli* have been described previously (Sambrook *et al.*, 1989).

LE 392
Host strain for the lambda phage GEM®-12.

Agrobacterium tumefaciens AGL-1 (Lazo *et al.*, 1991)
AGL0 recA::bla pTiBo542 ∆T Mop+ CbR.

Saccharomyces cerevisiae 23344c (Soussi-Boudekou *et al.*, 1997)
MATα ura3-52.

Saccharomyces cerevisiae 22∆8AA (Hahn *et al.*, 1997)
MATα gap1-1 put4-1 uga4-1 ∆can1 ∆apl1 ∆lyp1 ∆hip1 ∆dip5 ura3-1.
Yeast mutant deficient for multiple amino acid uptake system.

Saccharomyces cerevisiae JT16 (Tanaka & Fink, 1985)
MATα hip1-614 his4-401 can1 ino1 ura3-52.
Yeast mutant deficient in histidine uptake and metabolism.

Saccharomyces cerevisiae LR2 (Rentsch *et al.*, 1995)
MATα, ura3-52, his4-401, hip1-614, ptr2 ∆hisG; ino1, can1.
Yeast mutant deficient in histidine uptake and metabolism, and peptide uptake.

Saccharomyces cerevisae BJ2168 (Jones, 1990)
MATa prc1 prb1 pep4 leu2 trp1 ura3.
Yeast strain deficient in vesicular protease.

2.1.1.4 Vectors

(Maps of the vector are available in the appendices)

pBGghg (Chen *et al.*, 2000)

pGEM-T easy (Promega corporation, USA)

Vectors for yeast transformation

Vector	Promoter	Terminator	Reference
pDR196	PMA Plasma Membrane-ATPase (strong promoter)	ADH3 Alcohol dehydrogenase	(Wipf *et al.*, 2003)
pFL61	PGK 3-phosphoglycerate kinase (weak promoter)	PGK	(Minet *et al.*, 1992)

2.1.1.5 Oligonucleotides

All the used oligonucleotides are listed in the appendices.

2.1.1.6 Genomic DNA library from *Hebeloma cylindrosporum*

The library was provided by Dr. Roland Marmeisse. (University of Lyon, France). It was prepared from the homokaryotic *Hebeloma* strain h1 in the Lambda GEM®-12 vector.

2.1.2 Media

Solid media was prepared by adding 1.5% of agar.

2.1.2.1 *Escherichia coli*

E. coli was grown at 37°C on **L**uria **B**roth (5 $g.l^{-1}$ yeast extract, 10 $g.l^{-1}$ Bacto tryptone, 10 $g.l^{-1}$ NaCl) medium according to Sambrook et al. (1989).

2.1.2.2 Hebeloma cylindrosporum

H. cylindrosporum mycelium was grown at 21°C on YMG (full medium) (Rao & Niederpruem, 1969) or MMN (synthetic medium) (Marx, 1969).

YMG-Medium (**Y**east extract **M**altose **G**lucose):

4 $g.l^{-1}$	yeast extract
4 $g.l^{-1}$	glucose
10 $g.l^{-1}$	malt extract

MMN - Medium (**M**odified **M**elin **N**orkrans)

5 $g.l^{-1}$	KH_2PO_4
2.5 $g.l^{-1}$	$(NH_4)_2HPO_4$
500 $mg.l^{-1}$	$CaCl_2$, $2H_2O$
250 $mg.l^{-1}$	NaCl
1.5 $g.l^{-1}$	$MgSO_4$, 7 H_2O
10 $mg.l^{-1}$	$FeCl_3$
0.01 $mg.l^{-1}$	thiamine
10 $g.l^{-1}$	glucose

for MMN medium without nitrogen source (MMN–N), 7.5 $g.l^{-1}$ KH_2PO_4 was used instead of 5 $g.l^{-1}$ KH_2PO_4 and 2.5 $g.l^{-1}$ $(NH_4)_2HPO_4$.

2.1.2.3 *Saccharomyces cerevisiae*

S. cerevisae was grown at 28°C on full medium **YPD** (Adams et al., 1997) (**Y**east extract **P**epton **D**extrose: yeast extract 10 $g.l^{-1}$, peptone 20 $g.l^{-1}$, glucose 20 $g.l^{-1}$), or **SC**-medium (Adams et al., 1997) [**S**ynthetic **C**omplete-medium : 1,7 $g.l^{-1}$ Yeast Nitrogen Base (without amino acids, without ammonium), 20 $g.l^{-1}$ Glucose, 5 $g.l^{-1}$ $(NH_4)_2SO_4$, 2 $g.l^{-1}$ Drop-Out-Mix)].

Drop-Out-Mix :

0.5 g	adenine	2 g	lysine
2 g	alanine	2 g	methionine
2 g	arginine	0.2 g	para-aminobenzoic acid
2 g	asparagine	2 g	phenylalanine
2 g	aspartic acid	2 g	proline
2 g	cysteine	2 g	serine
2 g	glutamine	2 g	threonine
2 g	glutamic acid	2 g	tryptophane
2 g	glycine	2 g	tyrosine
2 g	isoleucine	2 g	uracile
4 g	leucine	2 g	valine

Yeast synthetic medium (Jacobs *et al.*, 1980) was prepared as follows :

One litre macroelement solution (A) was autoclaved separately and the following supplements added :

100 ml	glucose (30%w/v)
1 ml	micro elements (1000X) (**B**)
1 ml	vitamin solution (1000X) (**C**)
X ml	Nitrogen source (**D**)

A. Macroelement solution

0.7 $g.l^{-1}$	$MgSO_4$, $7H_2O$
1 $g.l^{-1}$	KH_2PO_4
0.4 $g.l^{-1}$	$CaCl_2$,$2H_2O$
0.5 $g.l^{-1}$	NaCl
1 $g.l^{-1}$	K_2SO_4
10.5 $g.l^{-1}$	$C_6H_8O_7$,H_2O
9.03 $g.l^{-1}$	KOH
15 g	oxoid agar

pH 6.1 (adjust with citric acid or KOH)

B. Micro element solution (1000X)

- Stock solutions (100 ml each)

S1	100 mg	H_3Bo_4
S2	100 mg	$CuSO_4$,$5H_20$
S3	100 mg	KI
S4	100 mg	Na_2MoO_4,$2H_2O$
S5	1.4 g	$ZnSO_4$,$7H_20$

All stock solutions are sterilized by filtration

- Micro element solution (1000X) (1 liter)

10 ml	S1	0.4 g	$MnSO_4$,H_2O
1 ml	S2	5 g	$FeCl_3$,$6H_2O$
2 ml	S3	1 g	$C_6H_8O_7$,H_2O
4 ml	S4		
1 ml	S5		

(filtrated and store at 4°C in dark)

C. Vitamin solution (1000X) (100ml)

250 µg	biotine
100 mg	thiamine
1 g	inositol
200 mg	Ca panthoenate
100 mg	pyridoxin

(filtrated and store at 4°C in dark)

D. Nitrogen source

Ammonium or amino acids were added to the desired concentration

2.1.2.4 *Pinus pinaster* / *Hebeloma cylindrosporum* mycorrhiza synthesis

Mycorrhiza synthesis was carried out with the *Hebeloma* strain h1h7 (dikaryotic).

2.1.2.4.1 For northern analysis

Mycorrhizal synthesis was realised on MMN/2. The concentration of MMN medium components was reduced to half and 0.5 $g.l^{-1}$ of glucose was added.

Thirty day-old *Pinus* seedlings were transferred to square Petri dishes (145 cm) containing MMN/2 medium. Two agar plugs were cut from an actively growing fungal culture precultivated for 2 weeks on MMN medium and placed close to the plant root. Plants were then grown for 6 to 10 weeks in a growth chamber at 22° and 16h day / 8h night.

2.1.2.4.2 For the mycorrhiza cDNA library

Fungal stock cultures were grown at 24 °C in the dark, in Petri dishes containing solid N6 medium (see composition below). Mycorrhizal synthesis was carried out in test tubes with seedlings, which had been germinated in aseptically Petri dishes (Plassard *et al.*, 1994). Only the root of a 2 week-old seedling was introduced into the tube containing a piece of Whatman paper (cat. No. 1542.185) and 10 ml of nutritive solution "N0.5" (see composition below). Each test tube was equipped with two Teflon tubes that permitted changing and sampling the solution within the tube. Plants were inoculated in sterile conditions by placing three fungal agar plugs (8 mm in diameter) cut from the edge of an actively growing stock culture in the tube at the time of transfer of the seedling. Plant was grown in a growth cabinet (16h light, 210 $\mu mol.m^{-2}.s^{-1}$) for 3 months. Culture solution from the tubes was renewed every week under aseptic conditions. Mycorrhized plants were transferred on soil in mini rhizoboxes. The soil used is a fersialitic soil collected from southern France (Cazevieille, Hérault). Before use, the soil was air-dried, crushed gently and sieved at 210 µm. Physical and chemical analysis showed that its pH was close to neutrality. In addition, it had a very low level of soluble phosphate (P). The soil was used either without any treatment (NT) or after addition of P (350 $mg.kg^{-1}$ dry soil). Then soil water mixed suspensions were stirred for 72 h and centrifuged. After rinsing, the soil pellet was mixed with water (25% of the soil weight) and put in plastic bags. Non-treated soil and treated soil were mixed with deionised water (1/1, w/w) in plastic bags. The different soils were sterilised twice by autoclaving them for 40 min at 114 °C. Soil samples were then kept sterile, at 4 °C, before use.

Mycorrhized plants were grown in mini rhizoboxes that are presented in Figure 2.1 as described in Casarin et al. (2003). They were made from 2 glass plates (100 x 200 x 2 mm) separated by a PVC band.

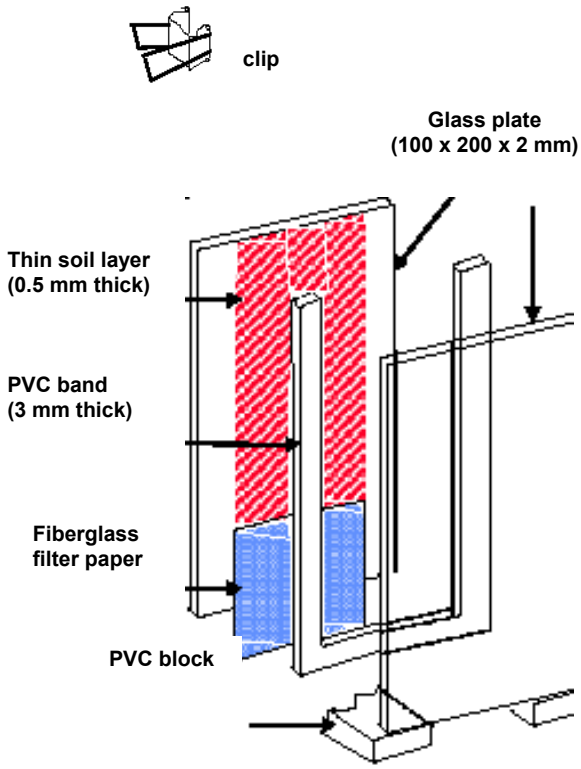


Figure 2.1: Scheme of the rhizobox designed for culture of *Pinus pinaster* plants on a thin layer of soil (Casarin *et al.*, 2003)

The set-up of each mini rhizobox with the plant was carried out in sterile conditions. Every piece of the box were cleaned with a solution containing ethanol (70%, v/v) and sodium hypochloride (5%) in a laminar flow hood. A soil layer (500 µm thick) was spread on one side of a glass plate by using a sterile syringe. A piece of fibreglass filter paper (Schleicher et Schuell GF50, 104280087) (6 cm x12 cm), previously sterilised (120 °C, 20 min, twice at 48 h intervals), was added to the lower part of the soil layer. The plant, with roots previously aseptically grown in tubes, was put on the surface of the soil layer before adding the second glass plate on the top of the roots. The root boxes were then closed with 4 drawing clips fixed on the sides and tape fixed on the top of the box to minimise water evaporation. Finally the root boxes were taken out of the laminar flow hood and placed in a PVC box previously cleaned with the ethanol-sodium hypochloride solution. The PVC boxes with the plants were placed in the growth chamber as previously described. Plants were continuously

supplied with a simplified nutrient solution containing 1 mM KNO3, 0.2 ml.l^{-1} of Morizet and Mingeau microelements solution, pH 6.0. The N6 solution (50 ml per plant), previously autoclaved (120 °C, 20 min), was added to the PVC box. The solution was renewed every week, in non-sterile conditions, in the growth chamber. The duration of the culture was 4 months.

Solution N6 :

6 mM	KNO_3,
4 mM	KCl,
1 mM	NaH_2PO_4,
1 mM	$CaCl_2$,
1 mM	NaCl,
1 mM	$MgSO_4$ $7H_2O$
110 mM	glucose
100 µg. l^{-1}	thiamine-HCl
10 mg. l^{-1}	ferric citrate
0.2 ml. l^{-1}	microelement solution

microelement solution (Morizet & Mingeau, 1976):

18.55 g.l^{-1}	H_3BO_4
8.45 g.l^{-1}	$MnSO_4,H_2O$
2.88 g.l^{-1}	$ZnSO_4,7H_20$
2.5 g.l^{-1}	$CuSO_4,5H_20$
1.4 g.l^{-1}	$NH4(Mo_7O_2)_4,4H2O$

Solution "NO,5" :

0.1 mM	KNO_3
0.1 mM	KH_2PO_4
2 mM	$Ca(NO_3)_2$
0.1 mM	$MgSO_4$
0.5 mg.l^{-1}	Fe-EDTA
50 mg.l^{-1}	thiamine-HCl
0.5 ml.l^{-1}	microelement solution

NP2/2 (1 liter) MES 25mM pH 5,5-6

50 mg	$CaCl_2$
150 mg	$MgSO_4$, $7H_2O$
245.7 mg	KH_2PO_4
4.5 mg	NaH_2PO_4, $2H_2O$
158 mg	Na_2HPO_4, 2 H_2O
1 ml	Biotine 0.4µg/ml
1 ml	Thiamine 40µg/ml
1 ml	Iron citrate 1.2%
2.5 g	Glucose
10 ml	Heller microelements
10 ml	N source $(NH_4)_2SO_4$, $NaNO_3$, NH_4NO_3 (100X)

Autoclave
Add 20 ml steril filtrated MES 50X pH 5.5- 6
If medium w/o MES then pH 6.0

Heller micorelements stock solution (1 liter) (100X)

100 mg	$FeCl_3$, $6H_2O$
100 mg	$ZnSO_4$, $7H_2O$
100 mg	H_3BO_3
10 mg	$MnSO_4$, $4H_2O$
2 mg	$CuSO_4$, $5H_2O$
3 mg	$AlCl_3$
3 mg	$NiCl_2$, $6H_20$
1 mg	KI

Store at –20° C

2.2 Methods

2.2.1 Standard methods

Standard methods such as plasmid preparations, restriction digestion, gel electrophoretic DNA separation, DNA isolation from agarose gels, cloning steps, PCR amplification, Southern blotting, etc... were perfomed as described in Sambrook et al. (1989). The screening of the phage library was performed according to Ausubel et al. (1996). Sequencing of DNA was performed by the chain-terminating method of Sanger et al. (1977) using “Big Dye Terminator Cycle Sequencing Ready Reaction Kit“ (BD-Kit) (Perkin Elmer, Warrington, UK) on ABI PRISM sequencer 310 (Perkin Elmer, Warrington, UK) or by GATC company (Constance, Germany). Sequence comparisons with databases were performed using the Basic Local Alignment Search Tool program at the US NCBIs Gene info network (Altschul *et al.*, 1990); http://www.ncbi.nlm.nih.gov/blast/.

2.2.2 Transformation

2.2.2.1 Bacteria

Transformations of *E. coli* were performed as described by Nishimura et al. (1990).

2.2.2.2 Yeast

Yeast cells were transformed according to a modified method from Dohmen et al. (1991). Competent *Saccharomyces cerevisiae* were prepared by inoculating 200 ml YPD with an yeast overnight culture in the ratio 1:100. Cells were grown at 30°C and harvested at an OD_{600} of 0.6 by a centrifugation at 3000 rpm for 5 min. Cells were then washed with 20 ml solution A (10 mM Bicine-NaOH, pH 8.35, 1 M Sorbitol, 3 % (v/v) Ethylene glycol) centrifuged and resuspended in 2 ml solution A and 200 µl aliquots were frozen at –70°C for later use. For transformation, 1 – 2 µg of plasmid DNA were added to the frozen cells and incubated for 5 min at 37°C with shaking. After the addition of 1 ml solution B (200 mM Bicine-NaOH, 40 % (w/v) PEG 1000, pH 8.35), the cells were mixed by inversion and incubated for 1 hour at 30°C. Following centrifugation at 3000 rpm, the cells were washed with 1ml solution C

(10 mM Bicine-NaOH, 150 mM NaCl, pH 8.35), centrifuged, resuspended in 100 µl solution C and plated on selective media.

2.2.2.3 *Agrobacterium tumefaciens*

Cells were transformed by electroporation (Transporator^{TM} Plus; BTX inc. CA, USA). DNA and electrocompetent cells (40 µL) were mixed in a cold electroporation cuvette and a pulse (1.5 kV, 25 µF) was given to the cells. LB medium (1ml) was quickly added, cells were grown on a shaker at 28°C for 2 H, plated on selective medium and grown at 28°C for 2 - 4 days.

2.2.2.4 *Hebeloma cylindrosporum*

The transforming plasmid, pBGgHg, contains a disarmed T-DNA in which the hygromycin B phosphotransferase gene (*hph*) fused to the *Agaricus bisporus gpd* promoter sequence, was inserted. This modified T-DNA confers hygromycin resistance to the fungi. The pBGgHg plasmid was propagated in the hypervirulent *A. tumefaciens* AGL-1 strain then used for Hebeloma transformation.

Hebeloma was transformed as follows : two week-old mycelia grown on YMG agar medium were roughly macerated using an raiser blade and the hyphal fragments were transferred to YMG liquid medium and grown for one week. Maceration with an Ultra-turrax homogenizer and sub-cultivation in fresh liquid medium was repeated every two day in order to get a fast growing mycelium. Two thalli obtained in 90 mm Petri dishes were finally macerated and resuspended in 50 ml YMG medium. Fifty µl of macerated mycelium were absorbed on a 1 cm in diameter glass microfibre disc (GF/D Whatman, United Kingdom).

A. tumefaciens was grown at 28°C for two days in LB medium supplemented with 50 µg.ml^{-1} carbenicillin and 50 µg.ml^{-1} kanamycin.

Bacterial cell suspensions were subsequently diluted to an OD_{600} of 0.15 in Induction Medium (IM .l^{-1}: 10.5 g K_2HPO_4; 4.5 g KH_2PO_4;1 g $(NH_4)_2SO_4$; 0,5 g Na_3-citrate $2H_2O$; 0.2 g $MgSO_4$ $7H_2O$; 1 mg thiamine-HCl; 2 g glucose; 0.5% glycerol; 40 mM MES pH 5.3; 50 µg.ml^{-1} kanamycin and 50 µg.ml^{-1} carbenicillin (Hanif *et al.*, 2002).The cells were grown for an additional five-hour period.

A. tumefaciens and *H. cylindrosporum* were co-cultivated between as follows : 100 µl of bacterial culture (~1.10^6 bacteria) were added to each glass microfibre disc containing macerated fungal mycelium. Discs were placed on co-cultivation medium (Pardo *et al*.,2002) ($.l^{-1}$: 5g KH_2PO_4; 2,5 g $(NH_4)_2SO_4$; 0,5 g $CaCl_2$ $2H_2O$; 1,5 g $MgSO_4$ $7H_2O$; 0,25 g NaCl; 0,01 mg thiamine-HCl; 2 g glucose; 10 mg FeCl3; 0,5% glycerol; 40 mM MES-KOH pH 5,3) supplemented with 200 µM AS. The plates were incubated at 23°C for 96 hours. After co-cultivation, glass microfibre discs were transferred to YMG medium supplemented with 200 µM claforan (Aventis, Germany) to counterselect *Agrobacterium* cells and 100 $\mu g.ml^{-1}$ hygromycin B to select for fungal transformants. Each transformant was subsequently transferred to YMG agar medium containing 200 $\mu g.ml^{-1}$ hygromycin B.

2.2.3 Uptake assays

(Wipf *et al.*, 2002)

2.2.3.1 Yeast uptake assays

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 (Whatman, GF/C), and washed twice with 4 ml of buffer A. The uptake of carbon-14 was quantified by liquid scintillation spectrometry. Competition for aspartate uptake was performed by adding a five-fold molar excess of the respective competitors to 150 µM ^{14}C-aspartate.

For analysis of pH dependence, incubations were performed in 100mM 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 5min 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.

2.2.3.2 *Hebeloma* uptake assays

In the *H. cylindrosporum* mycelia uptake experiments, discs of fungal mycelium were cut from the actively growing edge of 10 day-old mycelium 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 or with ^{3}H-Leucine-Leucine, 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 quantified by liquid scintillation spectrometry.

2.2.4 RNA isolation

Material (1g) was harvested, immediately frozen in liquid nitrogen and ground with a mortar and pestle in liquid nitrogen. Before thawing, 1ml phenol was added to the material then 2 ml lyse buffer (100 mMTris/HCl, 20 mM EDTA, 100 mM NaCl, 2% (w/v) SDS, 0.1% (v/v) β-mercaptoethanol, pH 9.0) and 1 ml chloroform. Phases were separated by centrifugation at 4500 rpm for 10 min (Heraeus). The aqueous phase was extracted a second time with 2 ml phenol/chloroform. After a subsequent chloroform extraction, RNA was precipitated with 1/10 vol. 3 M NaAc pH 5,2 and 2.5 volumes of ethanol for 1 hour at –20°C. After pelleting at 4500 rpm for 40 min, the RNA was solubilized in DEPC treated H_2O and precipitated with 1/1 vol. 4 M LiCl on ice overnight. Following centrifugation (40 min, 4500rpm, at 4°C) the pellet was washed twice with 80% ethanol, dried, solubilized in DEPC treated H_2O and the DNA was quantified by measuring the OD_{260}.

2.2.5 RNA gel-blot analysis

RNA (20 μg) was separated on 1.5% (w/v) formaldehyde agarose gel (Sambrook *et al.*, 1989). Prehybridization (2 to 3 hours) and hybridization (16h) was performed at

68°C in phosphate - SDS buffer (0.25 M sodium phosphate pH 7.2, 7% sodium dodecylsulfate (SDS), 1 mM EDTA and 1% bovine serum albumin (BSA)).
Probes (100ng) were labeled with α^{32}P-dCTP (Amersham, Braunschweig, Germany) using the Hexalabel DNA Labeling Kit (MBI Fermentas, USA).
Filters were washed twice in 2x SSC and 0.1% SDS at room temperature for 5 min, in 0.2x SSC and 0.1% SDS at 68°C for 10 min, then for 20 min. Filters were exposed to a phosphorimager screen (Molecular Dynamics, California, USA) for 16 hours or to X-ray film (Amersham, Germany) for 7 days.

2.2.6 Genomic DNA isolation from *H. cylindrosporum*

(van Kan *et al.*, 1991)
Hebeloma mycelia were grown on cellophane-covered YMG plate, harvested, frozen in liquid nitrogen and ground. The extraction buffer (NaCl 0,5M, SDS 1 %, Na_2EDTA 10 mM, Tris-HCl 10 mM, pH 7,5) was warmed at 65°C and 2 ml were added to fungal material (1g). After incubation for 10 min at RT, 2 ml phenol (pH 8) was added and phases were separated by centrifugation at 13000 rpm for 15 min. The upper phase was extracted a second time with 2 ml chloroform / isoamylic alcohol (24:1) and centrifuged at 13000 rpm for 15 min.
Between each step, extracts were incubated for 10 min at room temperature (RT).
The supernatant was transferred to a new Eppendorf tube containing 10 µl RNase A (10 $mg.ml^{-1}$) and incubated for 15 min at 37°C. The RNase A was then extracted with 400 µl phenol and the samples were centrifuged at 15000 rpm for 10 min. The DNA was precipitated with 400 µl isopropanol at RT for 15 min. After centrifugation at 13000 rpm for 10 min, the pellets were washed twice with 400 µl 70% ethanol, air-dried and solubilized in 20 µl water.

2.2.7 Proteinase assay

(Leake & Read, 1990)

H. cylindrosporum was grown in 100 ml conical flask each of which contained 20 ml of MMN/2-N. Nitrogen was supplied as $(NH_4)_2SO_4$ (16 $\mu g.ml^{-1}$) or as BSA (0.1 $mg.ml^{-1}$).
Each flask was inoculated with a single 4 mm diameter disc of an 8 day-old mycelium, which was cut from MMN-N agar plate. There were three replicate flasks

for each treatment, and harvests were taken 1, 2, 3, 4, 5, 7, 12, 16 days after inoculation. At each of these harvests fungus was separated from the culture solutions by filtration on glass micro-fiber filters (Whatman GF/A). The mycelium was then oven dried at 80°C for 24 h and weighed.

Proteinase activity in cultures filtrates was measured as follows. The reaction mixture contained 1ml 0.1 M citric acid:Na_2PO_4 buffer (pH 2.2), 1 ml of culture filtrate and 50 μl of 2 $mg.ml^{-1}$ FITC-bovine serum albumin (FITC-BSA). After 3 h in a shaking water bath at 37°C, the reaction was terminated by addition of 1 ml of 10% w/v trichloroacetic acid. To provide a measure of background fluorescence, media from parallel uninoculated control flasks were assayed at each harvest. The sample tubes were centrifuged at 3000 g for 7 min, after which 0.2 ml of the supernatant was mixed with 1 ml of 0.4 M boric acid:NaOH buffer (pH 9.7). Fluorescence was then measured in a BioTek FL600 microplate fluorescence reader. Excitation and emission wavelengths were respectively 485 and 535 nm. Three replicate assays were performed on culture-filtrates from each flask.

Results

3 Results

3.1 EST analysis from the symbiotic basidiomycete *H. cylindrosporum*

The sequences have been deposited in the NCBI database (accession Nos. BU963873–BU964314).

To identify the transporters involved in uptake of solutes (particularly nitrogen) by the fungal hyphae, a *Hebeloma cylindrosporum* cDNA library was prepared. This cDNA library was constructed in a yeast expression vector, which allows the identification of genes by functional complementation in the yeast *Saccharomyces cerevisiae*. However the other goal of this cDNA library preparation was the generation of a sequence database for the model fungus *H. cylindrosporum*. The primary library contains $2x10^5$ clones and the average insert size is 1.1 kb and range from 0.6 to 3.4 kb.

To analyze the quality of the library in more detail, ~500 randomly picked clones were sequenced from the 5′end. Among the sequenced clones, most cDNAs were properly oriented in the vector as 99.5% possessed a start codon close to the PMA promoter and only 0.5% showed a poly(A)+ tract adjacent to the promoter. Thus, the vast majority of the ESTs contain 5′end sequences of cDNAs from the mRNA transcripts.

After editing the sequences, an average length of 300 bp was used for database searches and the distribution of ESTs based upon deduced sequence homology to known or hypothetical proteins was determined (Fig. 3.1).

About 17% of the ESTs returned no homology at all and were not included in further statistical analyses. The lack of homology could suggest that these genes might be expressed only in *H. cylindrosporum*, or that they were rare transcripts that have not been found in previous EST projects.

Seven percent of the ESTs, matched ribosomal proteins, reflecting the difficulty in purifying mRNA from total RNA and the presence of oligo(dA) tracts in ribosomal genes. Seventy-six percent of the ESTs showed a homology to known (14%) or hypothetical proteins (62%).

The low percentage of known homology reflects the fact that although yeast, animal, and plant genomes have been fully sequenced, there is less information available concerning higher fungi. For this reason, there is a statistical bias of the databases against fungal proteins, even if several fungal EST projects have recently been developed (Voiblet *et al.*, 2001), (Polidori *et al.*, 2002), (Soanes *et al.*, 2002).

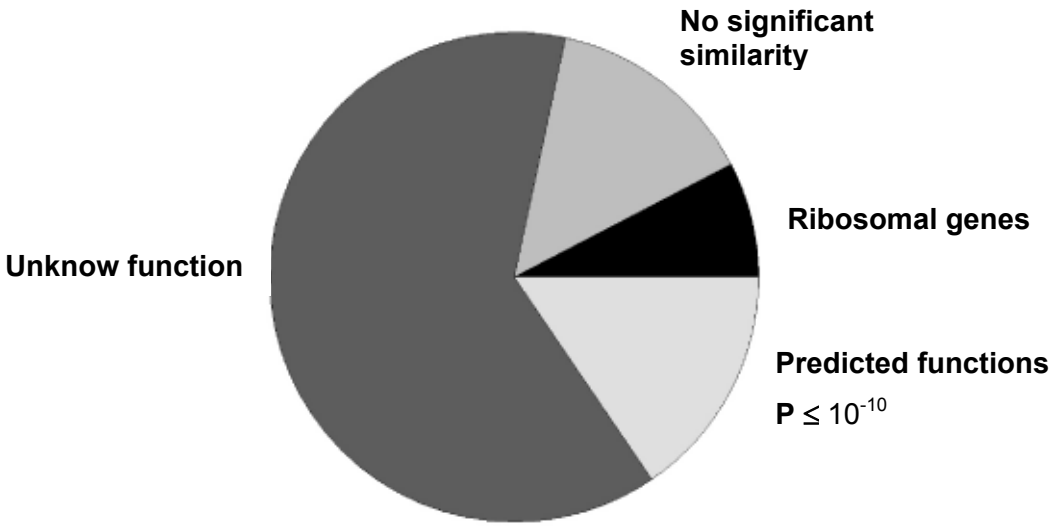


Figure 3.1 : Distribution of ESTs based on deduced amino acid sequence homologies to known or hypothetical proteins.

The high score of ESTs producing homology with unknown proteins illustrates the usefulness of an *Hebeloma* functional cDNA library for genome analysis.

More than half of the ESTs homologous to known proteins were homologous to known characterized fungal genes, including genes of known function, putative open reading frames, and ESTs. This correlates with the purity of the library, because the fungal sequences are under-represented in the databases. This result is similar to the 51% of homology to known characterized plant and fungal genes observed by Voiblet et al. (2001) when analyzing ESTs from a mycorrhizal library. Among the ESTs that showed high homology to known proteins, it is interesting to note the presence of a mycorrhizal fungus gene that codes for *Laccaria* symbiosis-related transcription factor. Thirty percent of the ESTs produced a significant match ($p = 10^{-10}$) and only 20% produced highly significant matches ($p = 10^{-20}$) (Fig. 3.2), which can be compared with the 60% of clones showing no similarity upon the analysis of

Tilia, or truffle, mycorrhizas (Polidori *et al*, 2002). This again illustrates the bias of the DNA sequence databases for fungal genes and especially “higher fungi”, i.e., mycorrhizal fungi genes. The ESTs exhibited a GC content of between 44 and 56%, most of them having a GC content of 52–54%, which could be correlated with GC contents of fungal genes (40–54%), which are higher than in plant (31–43%), but lower than in rhizobacteria (55–63%) (Hraber & Weller, 2001) . The *Hebeloma* genes with predicted functions show very low or no homology to yeast genes (*P* scores ranging from 3×10^{-30} to no significant homology), which highlights the divergence between both genomes during their evolution.

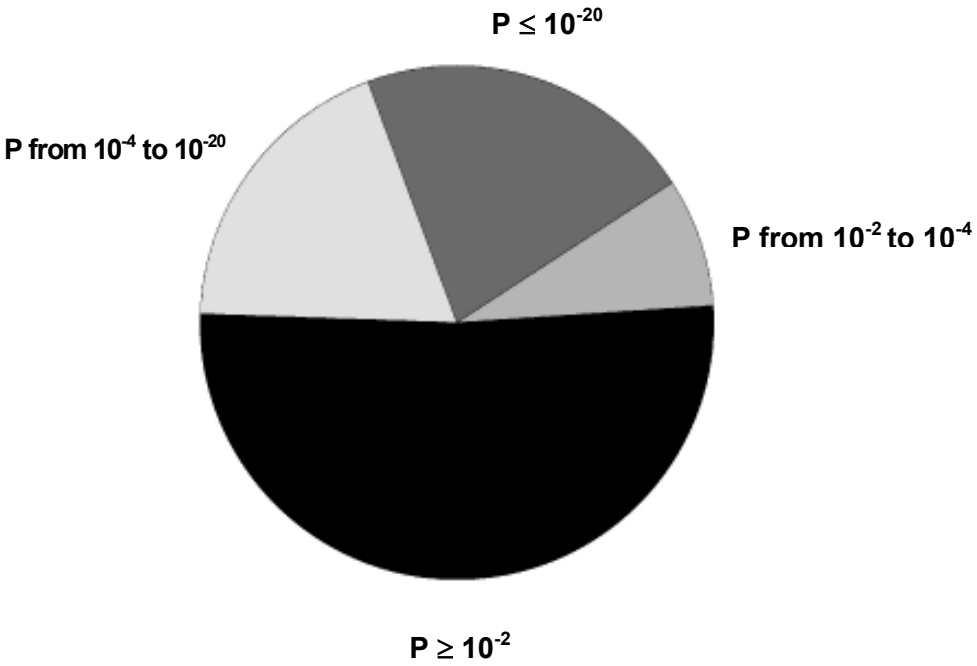


Figure 3.2 : Frequency distribution of ESTs from the library according to P values returned by BLASTx searches.

3.2 Suitability of the cDNA library for suppression cloning

To test the suitability of the cDNA library for suppression cloning and to identify genes that might be used as auxotrophic markers for transformation, the *S. cerevisiae* JT16 deficient in histidine biosynthesis was transformed with the *Hebeloma* cDNA library. Transformants were selected for growth on 6 mM histidine, the concentration at which JT16 is an auxotroph. This allowed selection of a histidine synthesis gene (*HIS4*). The yeast *HIS4* is a large gene (2400 bp) and contains three domains encoding a trifunctional enzyme catalyzing the 2nd (phosphoribosyl-ATP pyrophosphohydrolase), 3rd (phosphoribosyl-AMP cyclohydrolase), and 10th (histidinol dehydrogenase) steps in histidine biosynthesis. *Hebeloma HIS4* (AY135023) is also homologous along the whole coding region, which, when taken together with the functional complementation of the deficiency, strongly suggests that *Hebeloma* also harbors all three functions fused into one polypeptide of 91 kDa. Thus, the cDNA library is efficient for cloning even large fungal cDNAs by complementation of yeast mutants.

In parallel, the screening of the library also allowed the selection of a putative histidine transporter gene, which was later shown to be a *Hebeloma cylindrosporum* general amino acid transporter gene (*HcGAP1*) (Wipf *et al.*, 2002a).

3.3 Organic nitrogen transport in *Hebeloma cylindrosporum*

To study the ability of *H. cylindrosporum* to use organic nitrogen, uptake experiments with ^{14}C-labeled aspartate (asp) and ^{3}H-labeled Leucine-Leucine (Leu-Leu) were performed in *Hebeloma cylindrosporum*. Discs of fungal mycelium were cut from the actively growing edge of 10 day-old colonies and were floated for 5 min on a solution containing 1 ml nitrogen and glucose-free MMN at 25°C, supplemented with ^{14}C-asp (Fig. 3.3.A) or ^{3}H-Leu-Leu (Fig. 3.3.B).

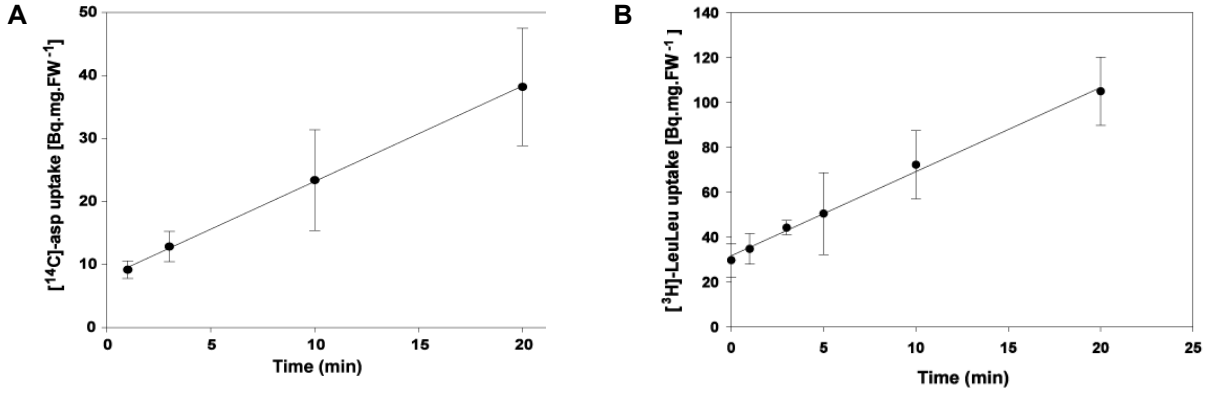


Figure 3.3 : Organic nitrogen uptake by the ectomycorrhizal fungus *H. cylindrosporum*. Discs of fungal mycelium were cut from the actively growing edge of 10 day-old colonies using a 15-mm-diameter cork borer. The discs were floated for 5 min on a solution containing 1 ml nitrogen and glucose-free MMN at 25°C, supplemented with 2 μM of ^{14}C-asp (**A**) or ^{3}H-Leu-Leu (**B**). Uptake by the fungus was measured at different times. Values represent the mean of three independent experiments ± S.D..

The uptake experiments show that *Hebeloma* can take up, in a linear way, ^{14}C-aspartate or ^{3}H-Leu-Leu, for at least 20 min. The concentration of radiolabeled compounds of 2 μM correspond approximatively to the concentration of amino acid and peptide present in the soil (Scheller, 1996). Taken together this confirms the involvement of amino acid and peptide transport and their role as primary source of N for the ectomycorrhizal fungus *H. cylindrosporum* and thus for the plant partner which it colonizes. Therefore transport systems involved in amino acid and peptide uptake in *Hebeloma* were investigated.

3.3.1 Characterization and regulation of an *H. cylindrosporum* general amino acid permease

The apparent ability of *H. cylindrosporum* to take up amino acids (Wipf *et al.*, 2002b) 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 the control of a yeast promoter (Wipf *et al.*, 2002b). 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.

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* (Hahn *et al.*, 1997) 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* (Nehls *et al.*, 1999) amino acid permeases (Fig. 3.4). 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 (André, 1995).

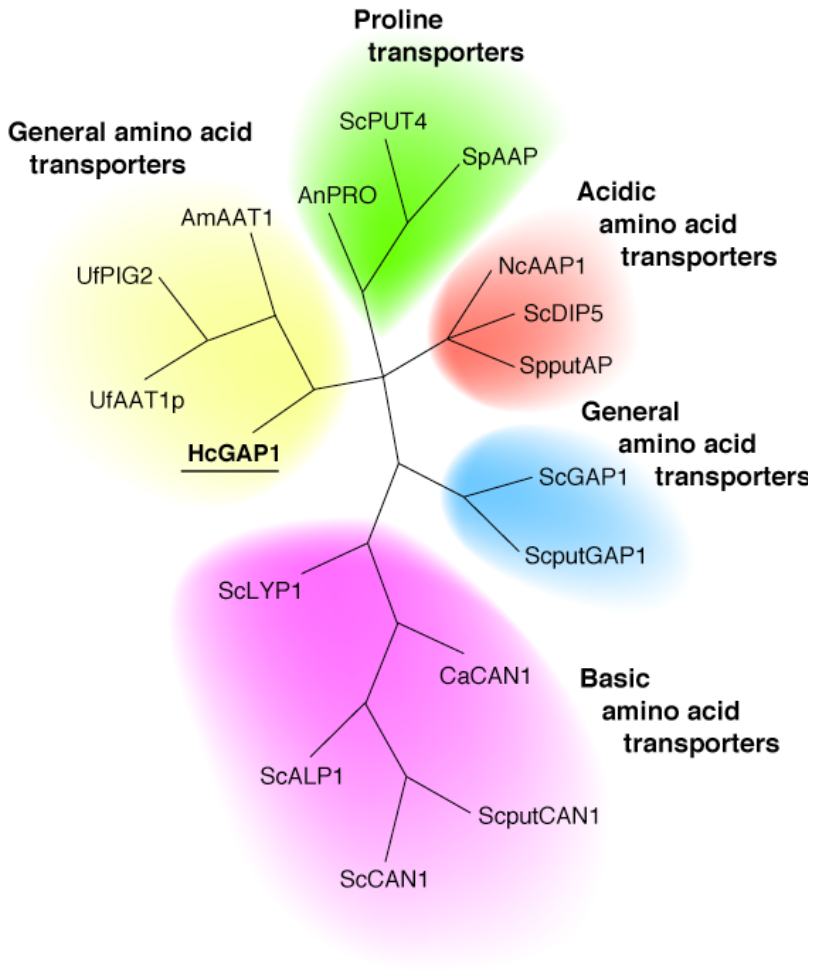


Figure 3.3 : Phylogenetic analyses of a multiple alignment of the deduced protein sequence of HcGAP1 and other fungal amino acid permeases Tree generated by using the PAUP 4.0b10 package (Maximum of parsimony) (Swofford, 1998) (Am=*Amanita muscaria*; An= *Asper-gillus nidulans*; Ca=*Candida albicans*; Hc =*Hebeloma cylindro-sporum;* Nc = *Neurospora crassa*; Sc=*Saccharomyces cerevisiae*; Sp=*Schyzosaccharo-myces pombe*; Uf=*Uromyces fabae)*.

Hydropathy analyses of *HcGAP1* with the TMHMM algorithm (Sonnhammer *et al.*, 1998) predict twelve putative transmembrane domains (Fig. 3.5). 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.

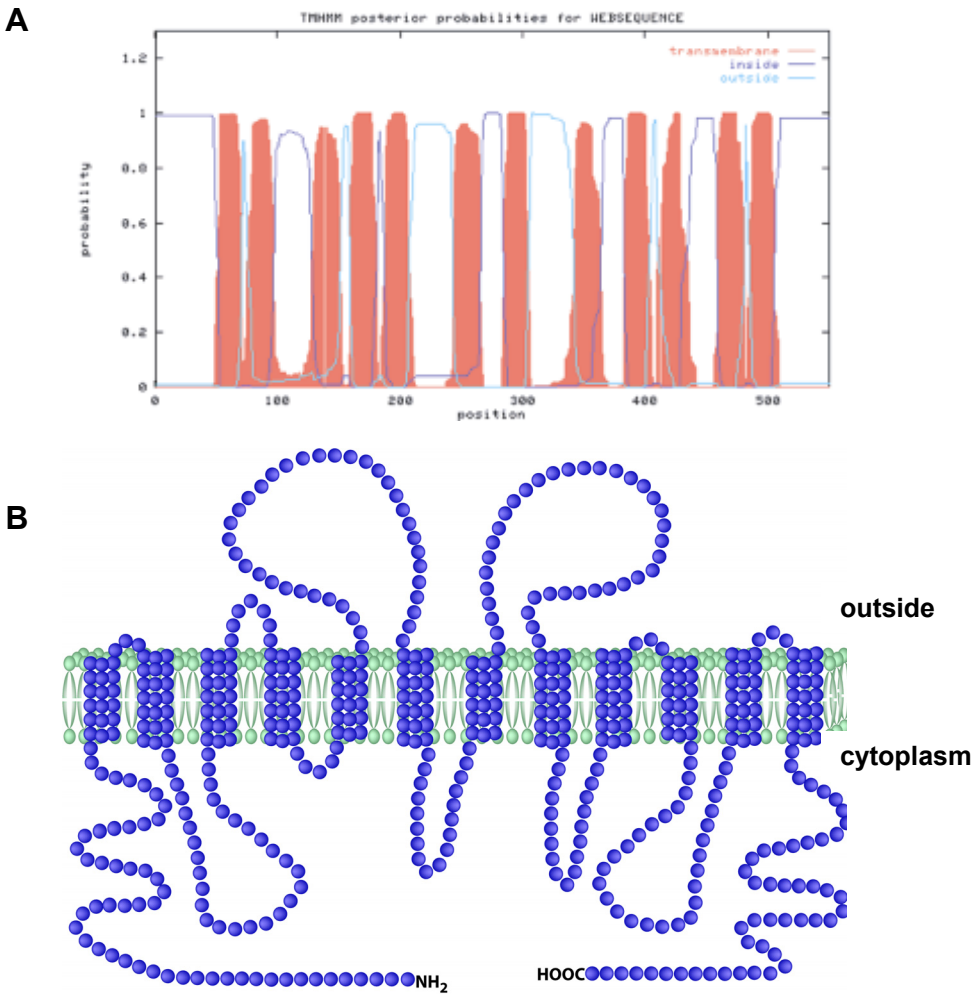


Figure 3.5 : Topology of the HcGAP1 protein. **A**: Transmembrane domain prediction was done by using the TMHMM algorithm. **B**: Schematic model of the HcGAP1 protein.

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 transporters. This is further supported by growth analysis of the multiple knockout strain 22Δ8AA expressing *HcGAP1* under selective conditions using arginine, aspartate, glutamate, citrulline, GABA and proline as sole N-sources (Fig. 3.6).

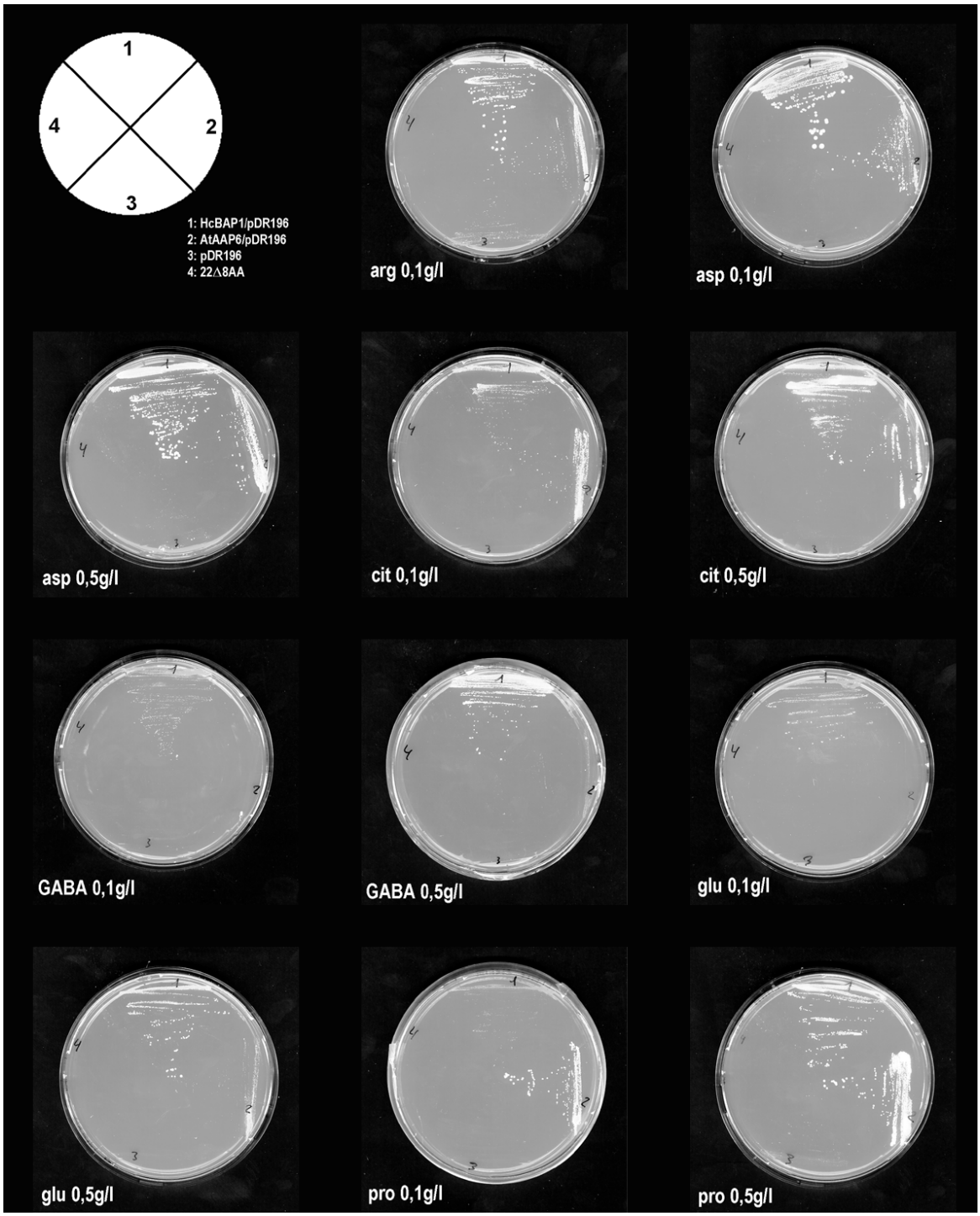


Figure 3.6 : Growth test of 22Δ8AA cells transformed with different plasmids on minimal medium containing different amino acids at different concentrations. (1) HcGAP1/pDR196, (2) AtAAP6/pDR196, (3) pDR196 and (4) 22Δ8AA.

3.3.1.1 Functional characterization of HcGAP1 : 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-labeled aspartate, as growth of 22Δ8AA expressing *HcGAP1* was best on aspartate as single nitrogen source. Yeast cells expressing *HcGAP1* showed more than 100-fold increased uptake rates of ^{14}C-aspartate as compared with cells transformed with pDR196 vector alone (Fig. 3.7.A). 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. 3.7.B). The Km value for the transport for aspartate was 150 μM, indicating that this amino acid permease is a high affinity amino acid transport system. HcGAP1 activity was strictly pH dependent with an optimum at approx. pH 4 (Fig. 3.7.C).

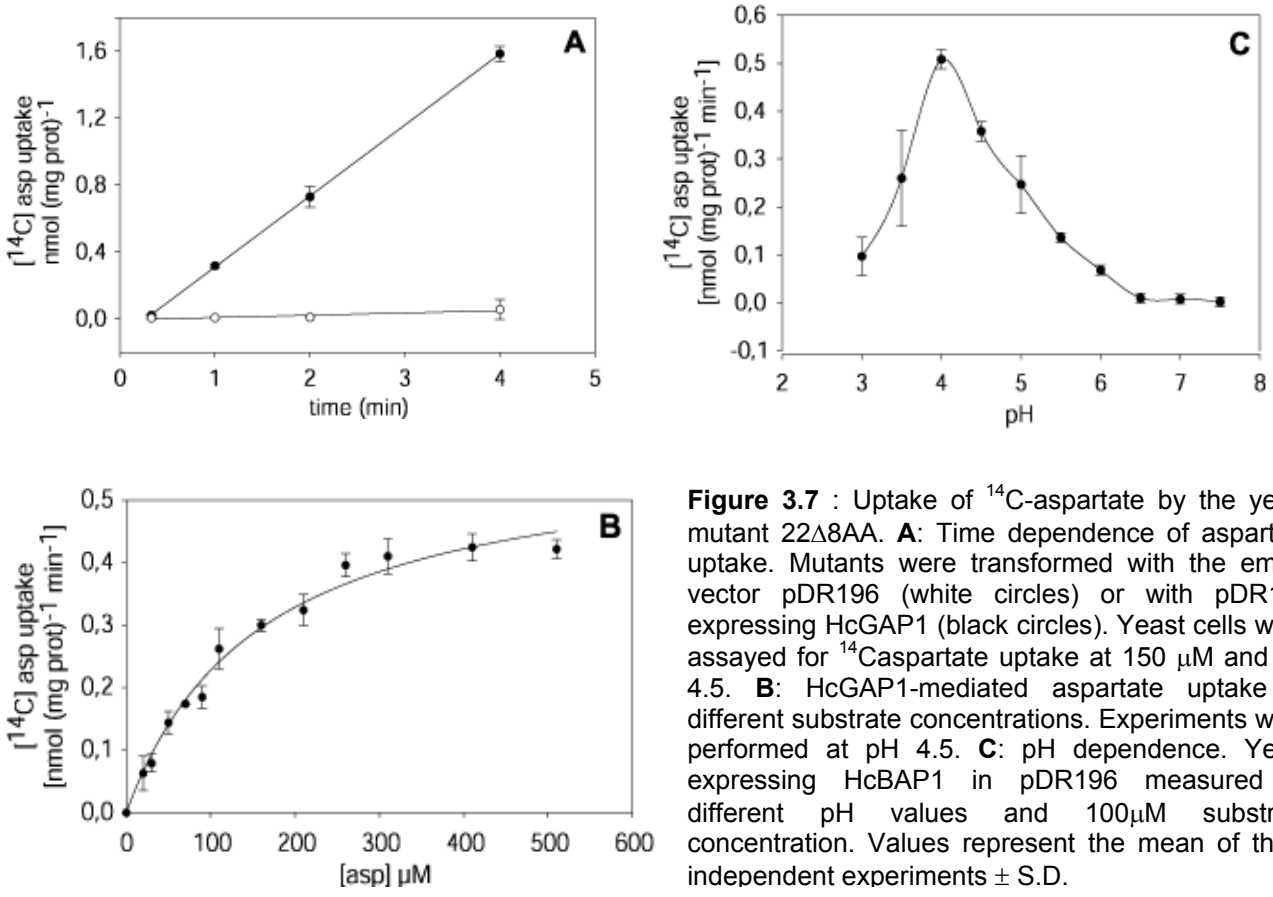


Figure 3.7 : Uptake of ^{14}C-aspartate by the yeast mutant 22Δ8AA. **A**: Time dependence of aspartate uptake. Mutants were transformed with the empty vector pDR196 (white circles) or with pDR196 expressing HcGAP1 (black circles). Yeast cells were assayed for ^{14}Caspartate uptake at 150 μM and pH 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 100μM substrate concentration. Values represent the mean of three independent experiments ± S.D.

^{14}C-aspartate uptake depended on the presence of glucose and was sensitive to the protonophores 2,4 -dinitrophenol (DNP), and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and to the plasma membrane H^+ -ATPase inhibitors, diethylstilbestrol (DES) and vanadate, indicating that energization is required for transport (Fig. 3.8.A). The strong dependence on the presence of glucose and on the proton gradient indicates that HcGAP1-mediated transport is mediated by a secondary active transport mechanism similar to its yeast homologs (Opekarova *et al.*, 1993). 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. 3.8.B.) 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.

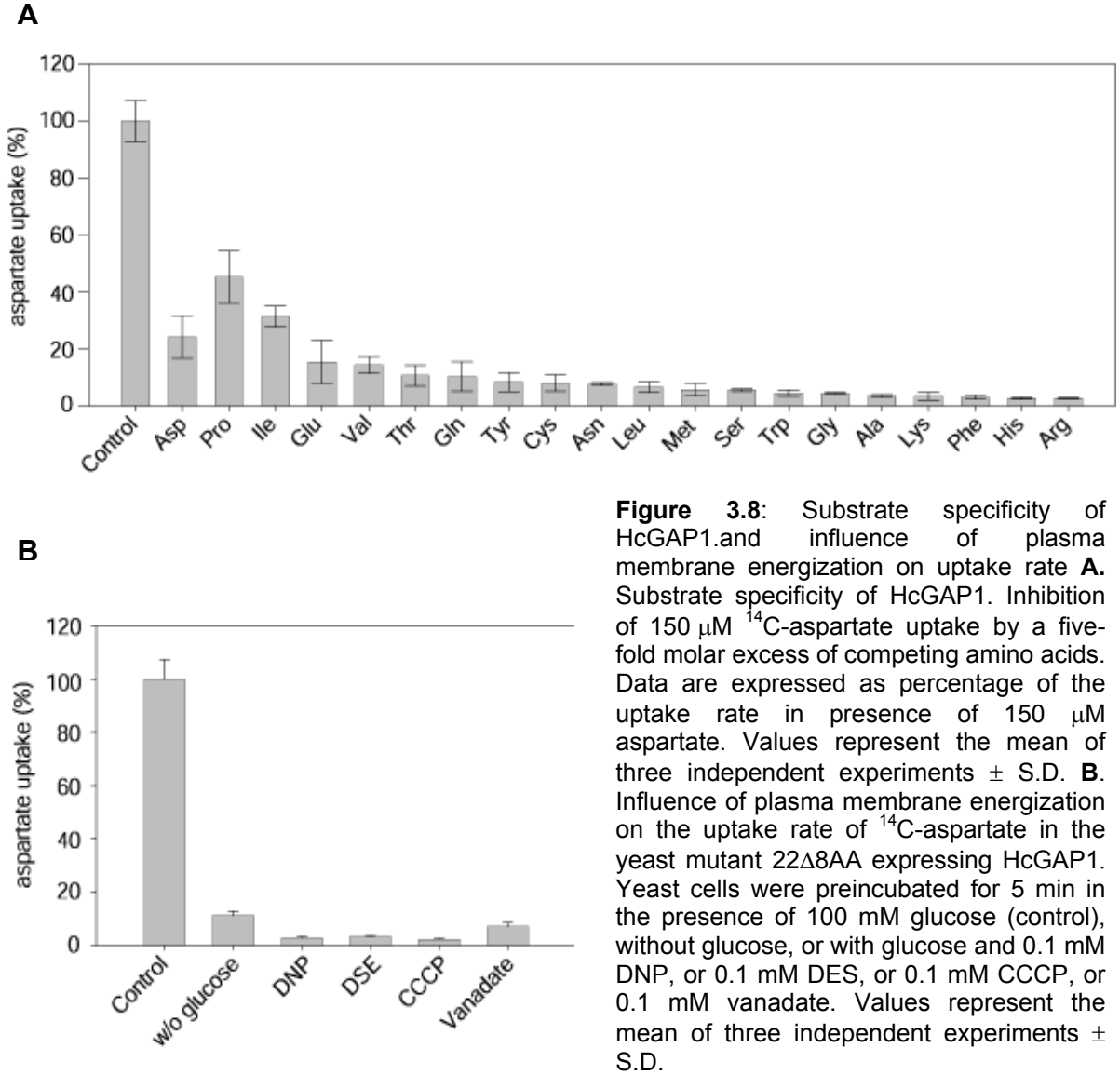


Figure 3.8: Substrate specificity of HcGAP1.and influence of plasma membrane energization on uptake rate **A.** Substrate specificity of HcGAP1. Inhibition of 150 μM ^{14}C-aspartate uptake by a five-fold molar excess of competing amino acids. Data are expressed as percentage of the uptake rate in presence of 150 μM aspartate. Values represent the mean of three independent experiments ± S.D. **B**. 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.

3.3.1.2 Regulation of *HcGAP1*

3.3.1.2.1 Expression pattern of HcGAP1

To investigate the regulation of *HcGAP1* at the transcriptional level, its expression was analyzed by RNA gel-blot analysis.

At first, *HcGAP1* expression was studied in different tissues. The non-mycorrhized plants were grown for 3 weeks as described before. The mycorrhized short roots were collected from 12 week-old mycorrhized plants. Total RNA was isolated from *Pinus* needles, *Hebeloma* mycelia, mycorrhized and non-mycorrhized short roots and loaded on a gel (Fig.3.9.A).

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 extramatrical hyphae. 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.3.9.B).

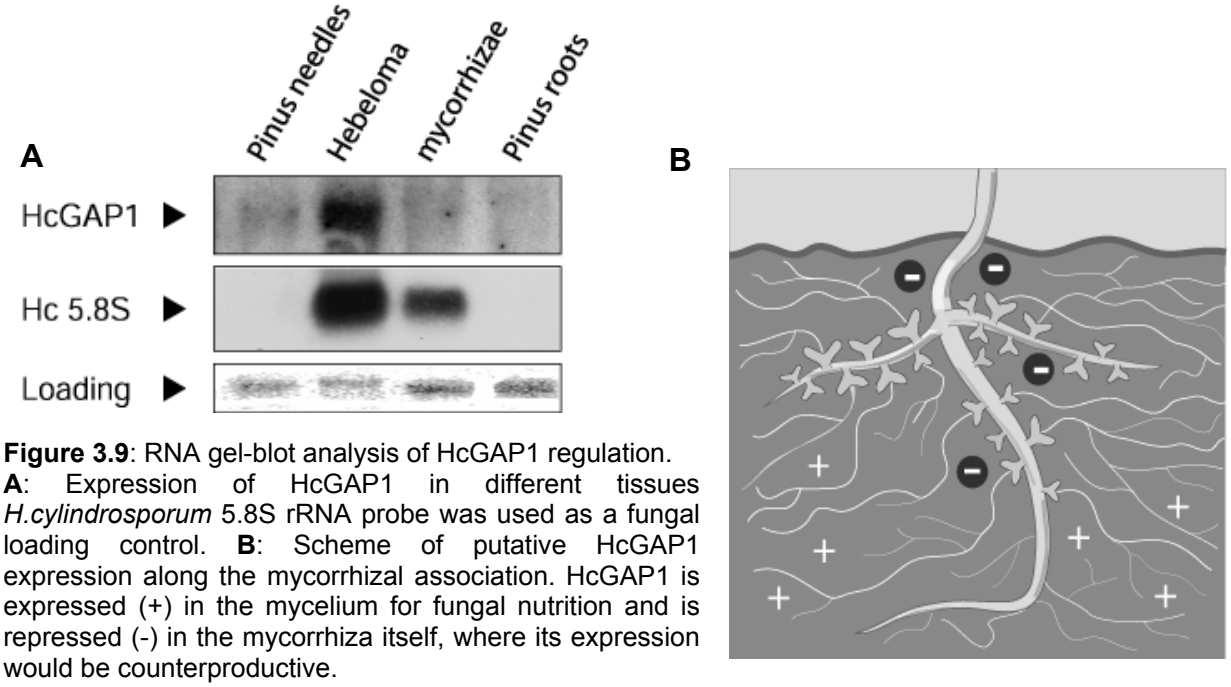


Figure 3.9: RNA gel-blot analysis of HcGAP1 regulation. **A**: Expression of HcGAP1 in different tissues *H.cylindrosporum* 5.8S rRNA probe was used as a fungal loading control. **B**: Scheme of putative HcGAP1 expression along the mycorrhizal association. HcGAP1 is expressed (+) in the mycelium for fungal nutrition and is repressed (-) in the mycorrhiza itself, where its expression would be counterproductive.

To further analyze the regulation of *HcGAP1*, in the mycelium, HcGAP1 expression was investigated under different N conditions (Fig.3.10).

HcGAP1 is strongly expressed in mycelia grown on nitrate as nitrogen source and on nitrogen starved medium. The mRNA quantity seems to increase with time in mycelia supplied with NO_3^-. On complete media, glutamine, glutamine-glutamine di-peptide or ammonium (for 6h and 12h) only weak transcripts could be detected. When mycelia were grown on ammonium as nitrogen source, strong expression of *HcGAP1* could be seen after 24h. These results showed that transcription of *HcGAP1* depends on nitrogen source and led us to think that, *in vivo, HcGAP1* expression should be regulated by the availability of nitrogen in the soil.

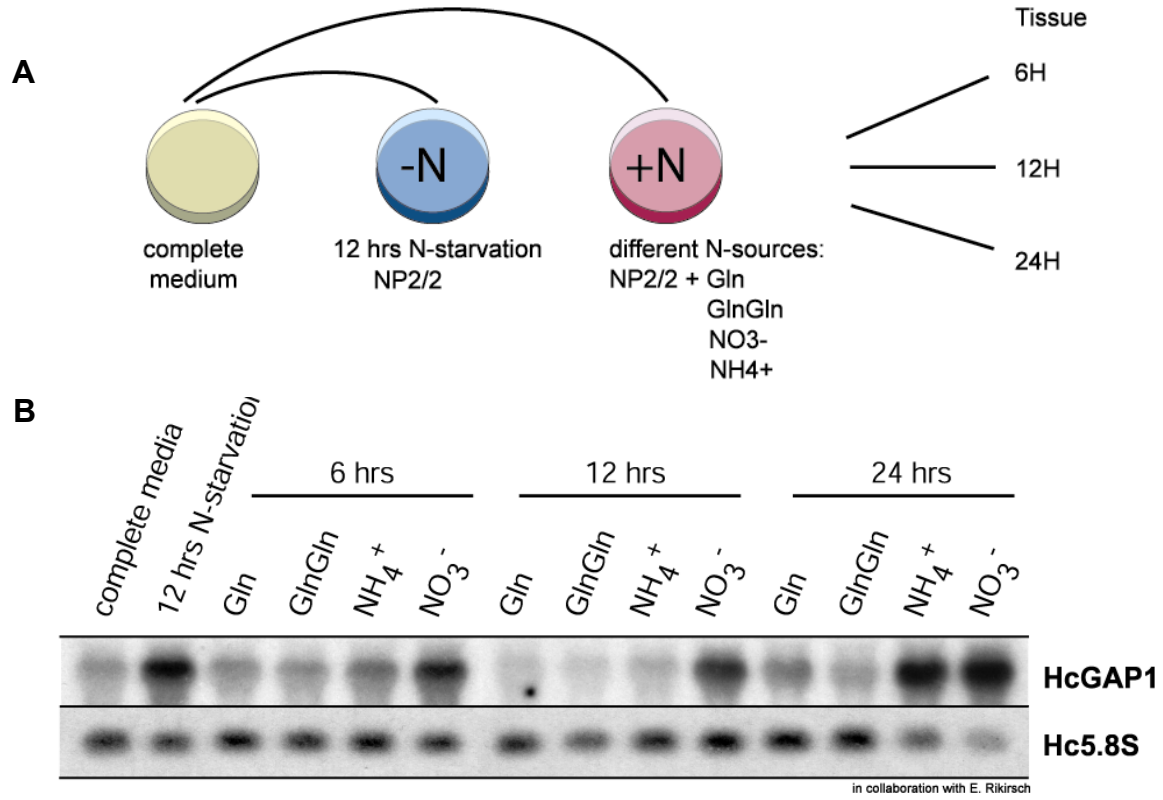


Figure 3.10: Regulation of *HcGAP1* expression by nitrogen. **A**. Scheme of the experiment. A 0.5 cm fungal agar plug was placed on cellophane-covered YMG plates and grown for 2 weeks. The cellophanes, with the mycelium, were placed on plate containing liquid NP2/2 medium without nitrogen source for 12h then transferred to NP2/2 medium containing 1 mM glutamine (Gln), glutamine-glutamine (Gln-Gln), NO_3^- or NH_4^+, for 6h, 12h, or 24h. After each time, the mycelia were frozen, total RNAs were extracted, transferred to nylon membrane and hybridized with ^{32}P-*HcGAP1* cDNA. **B**. Expression level of *HcGAP1* according to the nitrogen source in the medium. *H.cylindrosporum* 5.8S rRNA probe was used as a loading control.

3.3.1.2.2 *Isolation of the HcGAP1* promoter

To understand how *HcGAP1* is regulated, it was necessary to isolate the promoter. A genomic DNA library from *Hebeloma* strain h1 was screened according to Ausubel et al. (1996). The library, constructed in ą λGEM-12 vector, was provided by Roland Marmeisse (Univ. of Lyon, France). The screen was carried out on 250 000 phages that represented approximately 50 times *Hebeloma* genome, as each phage contained 15 to 20 kb inserts and the size of *H. cylindrosporum* genome is estimated at 20 Mb (Roland Marmeisse, personal communication).
By hybridization of a ^{32}P-labelled *HcGAP1* probe (complete), 15 clones were identified. In order to have a higher chance to get a clone containing *HcGAP1* promoter, the membranes were then hybridized with a ^{32}P-labelled probe corresponding to the 5´ end of *HcGAP1* (523 first nt). Four λ-phages (A, E, I, L), out of six positive clones were identified and used to obtain λ-phage DNA by plate lysates.

To amplify the *HcGAP1* promoter, a PCR was realized on the four DNA phages using a primer specific of *HcGAP1* gene (5´- *HcGAP1)* (Fig 3.11) and the SP6 and T7 primers specific of the ąGEM-12 vector. A 2,5 kb fragment was obtained with the primer pair SP6/5´-HcGAP1 on the clone E. In order to get only the promoter sequence, a nested primer, promHcrev–50 was designed (Fig 3.11) and this primer, together with SP6 primer, were used to carry out a PCR on the clone E. A 2,1 kb fragment was obtained and cloned in pGEM-T vector. The primers promHcGAP1+1281 and promHcGAP1+724 (Fig 3.11) were designed to fully sequence the 2,1 kb fragment (Fig. 3.12).

Figure 3.11: Scheme of *HcGAP1* promoter sequencing strategy

```
   1  ATTGGGCCGA CGTCGCATGC TCCCGGCCGC CATGGCCGCG GGATTATTTA
       TAACCCGGCT GCAGCGTACG AGGGCCGGCG GTACCGGCGC CCTAATAAAT

       GGTGACACTA TAGAAGAGCT CGCGGCCGCG GATCCCGGGA ATTCTCGATC
       CCACTGTGAT ATCTTCTCGA GCGCCGGCGC CTAGGGCCCT TAAGAGCTAG

 101   CAACACTGTT GAAGTACCCA GCGGAAAATC CACCAGTAAA GGCGCCATGT
       GTTGTGACAA CTTCATGGGT CGCCTTTTAG GTGGTCATTT CCGCGGTACA

       AATCTCCGTC GACCCTTCTC ATCTCGTACC TGCAGTTTTG AGGAGATTTA
       TTAGAGGCAG CTGGGAAGAG TAGAGCATGG ACGTCAAAAC TCCTCTAAAT

 201   AACGAGACAT TTTCGAAAAA GAAAACCGGG AAACCGGACC TCTTGTTTCC
       TTGCTCTGTA AAAGCTTTTT CTTTTGGCCC TTTGGCCTGG AGAACAAAGG

       AGAGTGGAAC AAACTCCCCT GTGTCTTTTG ATTTTTCCAG AGGGGGCAAA
       TCTCACCTTG TTTGAGGGGA CACAGAAAAC TAAAAAGGTC TCCCCCGTTT

 301   GGGGTGCCAA TCTACATGTC AATTATCAGC ATTCTCTCTC TCATAATCAA
       CCCCACGGTT AGATGTACAG TTAATAGTCG TAAGAGAGAG AGTATTAGTT

       AGGAATCAAC GGGCCCACCA AACAGAAGTT TTCGTTCGCC TTTCGGCTAG
       TCCTTAGTTG CCCGGGTGGT TTGTCTTCAA AAGCAAGCGG AAAGCCGATC

 401   TCGTATCCAC CCCGAGGTCT CCAAGCTTGC GTTTAAGTCG GCTTGTCATT
       AGCATAGGTG GGGCTCCAGA GGTTCGAACG CAAATTCAGC CGAACAGTAA

       TTGAGCTCCA GCCGTGTTCA ACGGTTCAAC CTTGTTGGTT CATAACAAAG
       AACTCGAGGT CGGCACAAGT TGCCAAGTTG GAACAACCAA GTATTGTTTC

 501   TCAGGCTTTG CGTGCTTGTA TCAAAGAAAT CCAGGTAGGT GATAGTCAAG
       AGTCCGAAAC GCACGAACAT AGTTTCTTTA GGTCCATCCA CTATCAGTTC

       GATCAAAGGG TAGCATCACT GTCAAATACT TAGTGTCAGA TTCATCAGAC
       CTAGTTTCCC ATCGTAGTGA CAGTTTATGA ATCACAGTCT AAGTAGTCTG

 601   CATGGCACGT GCAACTTATG AGGCGGCCCG GTGAACCGAA TTTGGGCGTC
       GTACCGTGCA CGTTGAATAC TCCGCCGGGC CACTTGGCTT AAACCCGCAG

       TAATTAAAAC TTGGAAGTAA ATATACATGA AGGACACGCG TTTAAAATCC
       ATTAATTTTG AACCTTCATT TATATGTACT TCCTGTGCGC AAATTTTAGG

 701   ATCCTATTAC CTGCAAGACC TGTACCGATG GGTTTCTTTA TTCGTTACCA
       TAGGATAATG GACGTTCTGG ACATGGCTAC CCAAAGAAAT AAGCAATGGT

       ATGACTCGGA AATAATTTTG AACTCATCAT TAAGTACTTA CTTATTTCTG
       TACTGAGCCT TTATTAAAAC TTGAGTAGTA ATTCATGAAT GAATAAAGAC

 801   ACGACTGACG GACCACCCAA CTTGTGACTC TGACTTGTTG TATATTAGGG
       TGCTGACTGC CTGGTGGGTT GAACACTGAG ACTGAACAAC ATATAATCCC
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      ACATAACCAC GGTCTGTTCC ATGAACACAC CTCGTATCTA GATTTCAGAA
      TGTATTGGTG CCAGACAAGG TACTTGTGTG GAGCATAGAT CTAAAGTCTT

  901 TAAGGTTGGG TATGGAACGT GTACCTTGTC AAGTTTAAGG CCACTGTAGT
      ATTCCAACCC ATACCTTGCA CATGGAACAG TTCAAATTCC GGTGACATCA

      TCCCTGAGTT AGAAAAGGAA AACAAATTAG TGTCAGAAAA TCAGAAAATC
      AGGGACTCAA TCTTTTCCTT TTGTTTAATC ACAGTCTTTT AGTCTTTTAG

 1001 AGCGAAGAAA TAAAAATAAA CTGCGACTTA CTACATTTTA ATCCTTCATA
      TCGCTTCTTT ATTTTTATTT GACGCTGAAT GATGTAAAAT TAGGAAGTAT

      TCCTACGATA TTTCCACTCA TCTACACCAT GACAACCCCT GGACAACTCC
      AGGATGCTAT AAAGGTGAGT AGATGTGGTA CTGTTGGGGA CCTGTTGAGG

 1101 TAGTAAGTCG CCATTAGTAG TAGGCTGGAC ACTTCCATCT GATGATTTCA
      ATCATTCAGC GGTAATCATC ATCCGACCTG TGAAGGTAGA CTACTAAAGT

      CGGCAGTCGG AAACAGCGAT GCCAGCCTGC ATGCGGGGGC CCGTCGTTGG
      GCCGTCAGCC TTTGTCGCTA CGGTCGGACG TACGCCCCCG GGCAGCAACC

 1201 TGGTAAATTG GTAATCGGTG ATCATGACAG GGAAAGTGTT GTCGGCGCAG
      ACCATTTAAC CATTAGCCAC TAGTACTGTC CCTTTCACAA CAGCCGCGTC
      ATACCCTAAA GCGGTGGTTG GCGTGATATT AGCGTGACTC GCCGACGTTA
      TATGGGATTT CGCCACCAAC CGCACTATAA TCGCACTGAG CGGCTGCAAT

 1301 GTCACTGTCA CCTGCCACCT GAAATCAACC TCGTGAACCC CCTCTTAAAG
      CAGTGACAGT GGACGGTGGA CTTTAGTTGG AGCACTTGGG GGAGAATTTC

      TTTAAATAAA GCGAGTACTG GCCACGGCAG GTTCGCACCA TTAACCCAGG
      AAATTTATTT CGCTCATGAC CGGTGCCGTC CAAGCGTGGT AATTGGGTCC

 1401 CTTGCTATGG AACACGAATA TAAATCCGAT AATACGCCGC TTTCACGTCA
      GAACGATACC TTGTGCTTAT ATTTAGGCTA TTATGCGGCG AAAGTGCAGT

      CCTGCGACAG GCTGCAATCG AGCTTCATCG CGCAAAACAG CACCTACGAC
      GGACGCTGTC CGACGTTAGC TCGAAGTAGC GCGTTTTGTC GTGGATGCTG

 1501 GATTACACGT TTAGCCTGGA CCAAGAATAA AACCACGGCA ACTTAGCTCA
      CTAATGTGCA AATCGGACCT GGTTCTTATT TTGGTGCCGT TGAATCGAGT

      CCTACCCAGA CCTACCCACG ACCTTGGTTT TATTTTGCTA CTTGACATGG
      GGATGGGTCT GGATGGGTGC TGGAACCAAA ATAAAACGAT GAACTGTACC

 1601 CCCCCCCGAT GCTGCGCTGC AATGGAATCA GTGGGCGCGA GCTTTATCTG
      GGGGGGGCTA CGACGCGACG TTACCTTAGT CACCCGCGCT CGAAATAGAC

      CGAATCTTAT TCCATATGTG GTGTACTTAG TTGGTCCTGC CACTGCCTCA
      GCTTAGAATA AGGTATACAC CACATGAATC AACCAGGACG GTGACGGAGT

 1701 TGAGGCCTTC ATTATTTTGC TCTTGACATA ACCGACGATC TGTGATGAAC
      ACTCCGGAAG TAATAAAACG AGAACTGTAT TGGCTGCTAG ACACTACTTG

      GACCAGTCTG GCTTGGAATT CCTTCTTGAG ATATATTTAT TCTCAAAATT
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        CTGGTCAGAC CGAACCTTAA GGAAGAACTC TATATAAATA AGAGTTTTAA
1801    ATTCGACAGA GACCAAAGGG TACCAATATA CTTACCACCA GCTGCACGTC
        TAAGCTGTCT CTGGTTTCCC ATGGTTATAT GAATGGTGGT CGACGTGCAG
        CAGGGACATG GGGATACCTG GCGTCCGCAG GGCTGCCGAT GACGCGATGC
        GTCCCTGTAC CCCTATGGAC CGCAGGCGTC CCGACGGCTA CTGCGCTACG
1901    GAATGTATCA GGCTGATCAC TGATAGCCAA AGGCACTGCA TTCGAATCCA
        CTTACATAGT CCGACTAGTG ACTATCGGTT TCCGTGACGT AAGCTTAGGT
        GGTGCGCCCC TCCCATTATC TGACAGCACC ATGTGAAGAG GATTTTCTAT
        CCACGCGGGG AGGGTAATAG ACTGTCGTGG TACACTTCTC CTAAAAGATA
2001    TTTTAACGCC TGTCAGCCAT AGCCTCACAC GCCGGTGGTG CAAAATACAC
        AAAATTGCGG ACAGTCGGTA TCGGAGTGTG CGGCCACCAC GTTTTATGTG
        TTCCCCGAAG CCACATTAAC TCCTGTGCAG GTGACTATAT AGGTCGGGCA
        AAGGGGCTTC GGTGTAATTG AGGACACGTC CACTGATATA TCCAGCCCGT
2101    AACCCGCGGC CTTCTTTTCG TTCCTTCCTC CCAACCCCAG CAGTCACACG
        TTGGGCGCCG GAAGAAAAGC AAGGAAGGAG GGTTGGGGTC GTCAGTGTGC
        ATGGAAAACG AGAAAACCGA GCATGAGAA
        TACCTTTTGC TCTTTTGGCT CGTACTCTT
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Figure 3.12: *HcGAP1* promoter sequence 5´→ 3´. In red, putative GATA transcription binding site. In blue, ATG start codon. Bold, putative TATA box.

The promoter sequence was analyzed *in silico*. A TATA box could be predicted with the program PROSCAN (http://bimas.dcrt.nih.gov/molbio/proscan).

The oligonucleotide ATG start codon context (in blue in the figure 3.12) was also analyzed and compared to the consensus sequence described in eukaryotes by (Kozak M., 1999) : "$A^{A}/_{C}{}^{A}/_{C}$**ATG**G". *HcGAP1* shows a G in position +4 and an A in position –3 which is conform to the consensus sequence.

The sequence was further analyzed with the program TRANSFAC (http://transfac.gbf.de/TRANSFAC) to identify putative transcription factor binding sites. Eight putative GATA or TATCT sites were found which could be binding sites for GATA transcription factors like AreA from *Aspergillus Nidulans* (Caddick *et al.*, 1986) or Nit-2 from *Neurospora crassa* . AreA and Nit-2 belong to the GATA family of DNA-binding proteins. They contain a DNA-binding domain, consisting of a single zinc finger and basic region, which binds to specific motifs that are located in the promoter region of nitrogen-regulated genes (Nitrogen Catabolic Repression). This analysis led us to conclude that *HcGAP1* might be regulated via the Nitrogen

Catabolic Repression like other genes involved in nitrogen uptake or metabolism (Arst & Cove, 1973; Arst *et al.*, 1997; Caddick, 1994; Caddick *et al.*, 1994; Marzluf, 1997; Peters & Caddick, 1994; Wiame *et al.*, 1985).

3.3.1.3 Analysis of the HcGAP1 genomic sequence

From the screening of the *Hebeloma* genomic library, *HcGAP1* genomic sequence could be determined from clones which hybridize with the ^{32}P-labelled *HcGAP1* probe. A PCR was carried out using the DNA prepared from the clones A, E, L, I and the primers HcBAP1gfor/HcBAP1grev. A 2.8 kb fragment was obtained, cloned in a pGEM-T vector and sequenced using gene-walking sequencing strategy with the following primers T7/SP6 in a first step, then cpl hcgap1 for/cpl hcgap1.

HcGAP1 genomic sequence was analyzed. It contained 19 introns and 20 exons (Fig. 3.13.A). The high number of introns has already been described for other *Hebeloma* genes (Corratge, 2003; Jargeat *et al.*, 2000; Tatry, 2003) and in basidiomycetes (Schuren, 1992).

The introns are found as regularly distributed along the coding regions, occupying 40% of them. Their size ranges over 38 - 118 bp but most of the introns are from 60 to 50 bp (average size : 58 bp) (Fig 3.13.B). This small size of the introns and their uniformity were again observed in *H. cylindrosporum* : nitrate reductase gene *HcNAR1* (12 introns), nitrate transporter gene *HcNRT2* (7 introns), nitrite reductase gene *HcNIR1* (19 introns) (Jargeat *et al.*, 2000; Jargeat *et al.*, 2003), the phosphate transporter genes *HcPT1* (10 introns) and *HcPT2* (16 introns) (Tatry, 2003) and the potassium transporter gene *HcTRK* (20 introns) (Corratgé, 2003).

All introns start with GT and end with AG. In general, the 5´ and 3´ border sequences of the introns follow the pattern 5´- $GT^{A}/_{G}{}^{A}/_{C}G^{T}/_{C}$…..$^{C}/_{T}$AG - 3´, which is very similar to the conserved sequence identified in basidiomycetes fungi (Schuren, 1992) and in other *Hebeloma* genes (Jargeat *et al*., 2000; Jargeat *et al*., 2003; Corratgé, 2003; Tatry, 2003). The sequence $^{C}/_{T}$AG in 3´ was observed in 95% of the introns and in 5´, the sequence $GT^{A}/_{G}{}^{A}/_{C}G^{T}/_{C}$ was found in more than 60% of the introns.

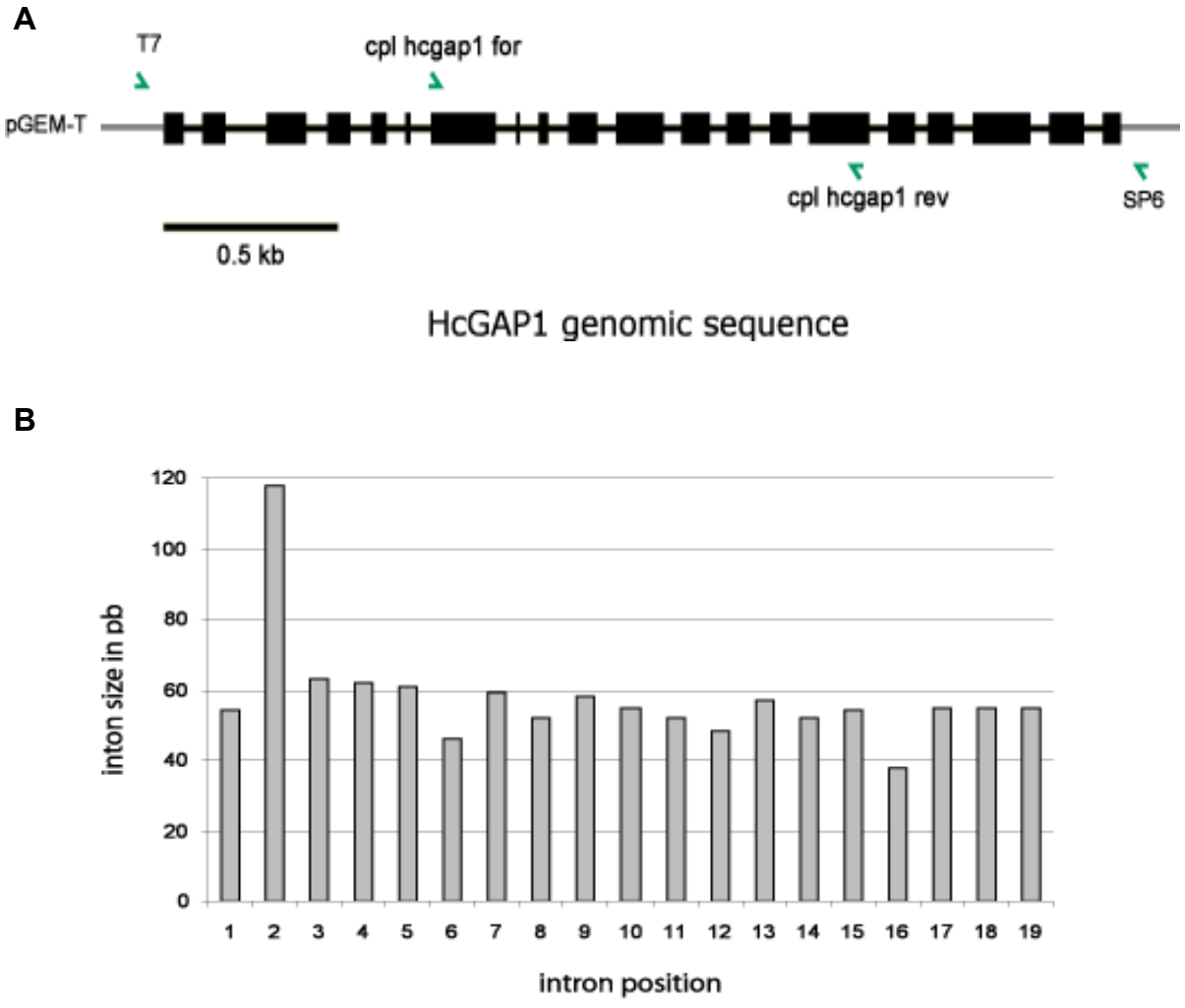

Figure 3.13: Size and position of intron in the *HcGAP1* genomic sequence
A. Genomic structure of *HcGAP1*. Exons are represented by black rectangle. **B**. Size of the introns

The exon size is variable, the biggest one is about 185 bp and the smallest is 14 bp (Fig 3.13.B). Presence in the gene of really small introns (20 bp, 14 bp) was also observed by Jargeat for *HcNTR2* and *HcNIR1* (Jargeat *et al*., 2000; Jargeat *et al*., 2003) and by Corratgé for *HcTRK* (Corratgé, 2003).

3.3.2 Characterization and regulation of two peptide transporters, HcPTR2A and HcPTR2B, from *Hebeloma cylindrosporum*

A first step towards analysis of organic nitrogen transport in the *Hebeloma cylindrosporum/Pinus pinaster* ectomyccorhizal association, was the isolation and the characterization of an amino acid transporter from the fungal partner.

Because of the ability of *Hebeloma* to use dipeptides as single nitrogen source (Rikirsch, 2002), transport systems involved in peptide uptake were investigated. Two peptide transporters from *Hebeloma*, *HcPTR2A* and *HcPTR2B*, were cloned by suppression cloning in a yeast mutant deficient in peptide uptake, using a yeast expression cDNA library generated from mycelia of *Hebeloma* D2 strain (Rikirsch, 2002).

3.3.2.1 Functional characterization of HcPTR2A and HcPTR2B

The transport activity of HcPTR2A and HcPTR2B were tested directly by using ^{3}H-labeled Leucine-Leucine in a yeast mutant deficient in peptide uptake (LR2).

Dipeptide uptake by yeast cells expressing *HcPTR2A* and cells expressing *HcPTR2B* was linear for at least the first four minutes of the assay (Fig 3.14.A). HcPTR2A-mediated Leu-Leu uptake was 15-fold higher compared to the cells expressing the empty vector pFL61, whereas the transport rate for cells expressing *HcPTR2B* was only 2-fold increased (Fig 3.14.A). Due to the very low uptake rate of yeast cells expressing *HcPTR2B*, only HcPTR2A transport properties could be further investigated.

Kinetic studies of Leu-Leu uptake by HcPTR2A showed that the transport rate was concentration dependent and displayed saturation kinetics (Fig 3.14.B). A K_M value for Leu-Leu transport of 1.46 μM could be determined for *HcPTR2A*. HcPTR2A activity was pH dependent with an optimum around pH 5,5 (Fig 3.14.C). In addition HcPTR2A-mediated ^{3}H-LeuLeu uptake was sensitive to the protonophores 2,4 DNP and CCCP and the plasma membrane H^{+} - ATPase inhibitors DES and was dependent on the presence of glucose, indicating that energization is required for transport (Fig 3.14.D). To determine the substrate specificity of HcPTR2A, ^{3}H-LeuLeu uptake was competed with other peptide (di-, tri-, tetra-peptide), amino acids, nitrate and ammonium. Among the variety of substrates used, dipeptides with leucine in N-terminal position, the dipeptide GlnGln, tri- peptides containing only leucines and to a lesser extent the dipeptide HisLeu were able to significantly compete with ^{3}H-LeuLeu for uptake (Fig 3.14.E). It seems that HcPTR2A-mediated transport is substrate-specific and that the N-terminal amino acid might be essential in the substrate recognition by the transporter.

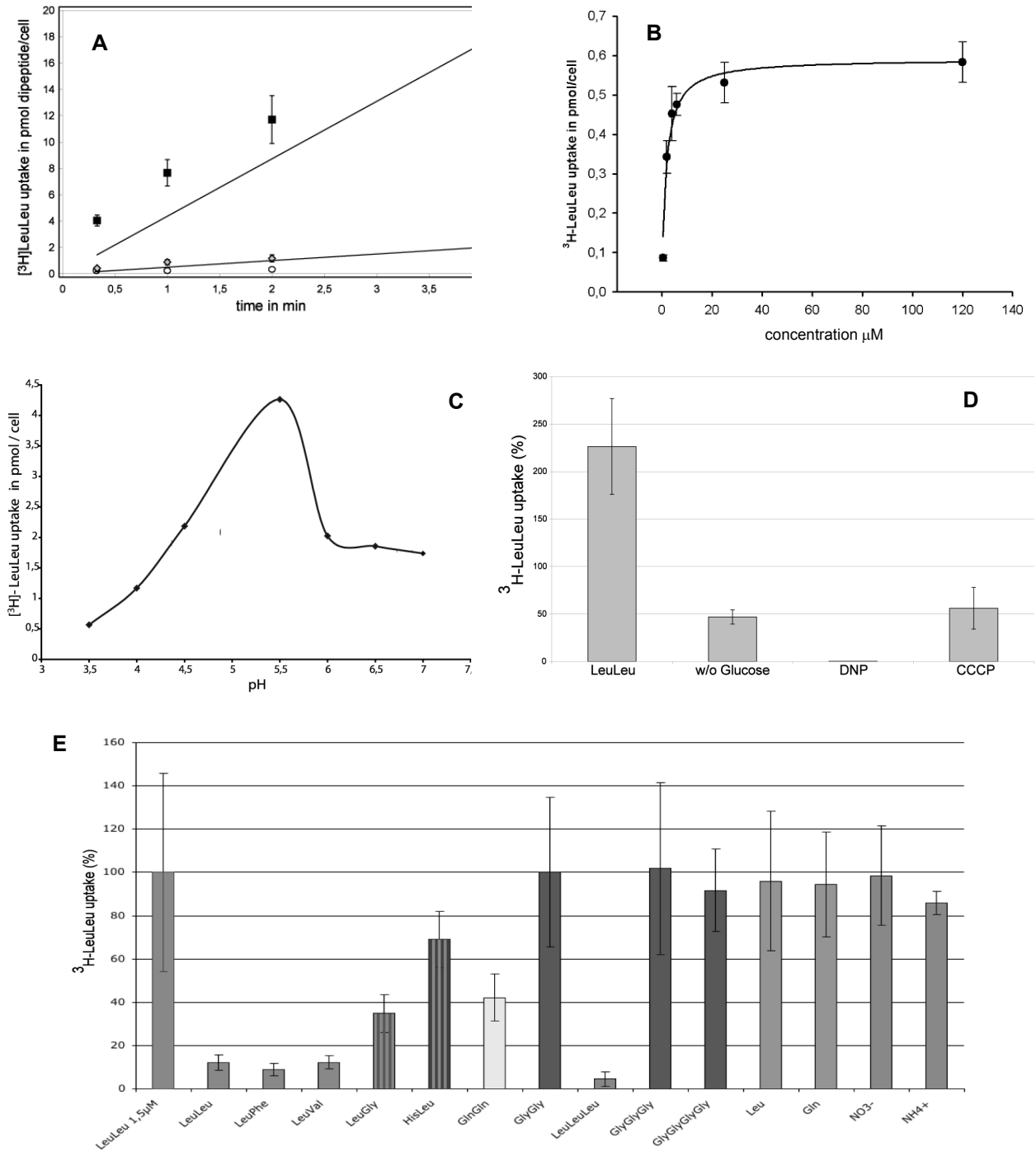

Figure 3.14: Kinetic of ^{3}H-LeuLeu uptake by the yeast mutant LR2 expressing *HcPTR2A* and *HcPTR2B*. **A**. Time-course of ^{3}H-LeuLeu uptake. Mutants were transformed with the empty vector pFL61 (white circles) or with pFL61 expressing *HcPTR2A* (black square) or *HcPTR2B* (grey square). Yeast cells were assayed for ^{3}H-LeuLeu uptake at 1,5 μM and pH 5. **B**. HcPTR2A-mediated ^{3}H-LeuLeu uptake at different substrate concentrations. Experiments were performed at pH 5. **C** pH dependence. Yeast expressing *HcPTR2A* in pFL61 measured at different pH values and 1,5 μM substrate concentration. Values represent the mean of three independent experiments ± S.D. **D**. Influence of plasma membrane energization on the uptake rate of ^{3}H-LeuLeu uptake by yeast cells expressing *HcPTR2A*. 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. Substrate specificity of HcPTR2A.**E** Inhibition of 1.50 μM ^{3}H-LeuLeu uptake by a five-fold molar excess of competing nitrogen source . Data are expressed as percentage of the uptake rate in presence of 1.5 μM ^{3}H-LeuLeu. Values represent the mean of three independent experiments ± S.D.

3.3.2.2 Expression pattern of *H. cylindrosporum* peptide transporter

To understand how the two peptide transporters HcPTR2A and HcPTR2B are regulated in *H. cylindrosporum*, their expression was analyzed by RNA gel-blot hybridization. As for *HcGAP1*, the transcript level on different nitrogen sources was investigated (Fig 3.15). The experiment was carried out in the same way as already described for *HcGAP1*.

HcPTR2B is constitutively expressed independently of the N-source and the time. In contrast, *HcPTR2A* is strongly expressed in mycelia grown on NO_3^-, under nitrogen starvation and surprisingly also on complete media. A weak expression of *HcPTR2A* could be observed in mycelia grown on Gln, GlnGln or NH_4^+ (after 6h and 12h). Like *HcGAP1*, *HcPTR2A* seems to be up-regulated under organic nitrogen deficiency and its transcription is repressed on primary nitrogen sources.

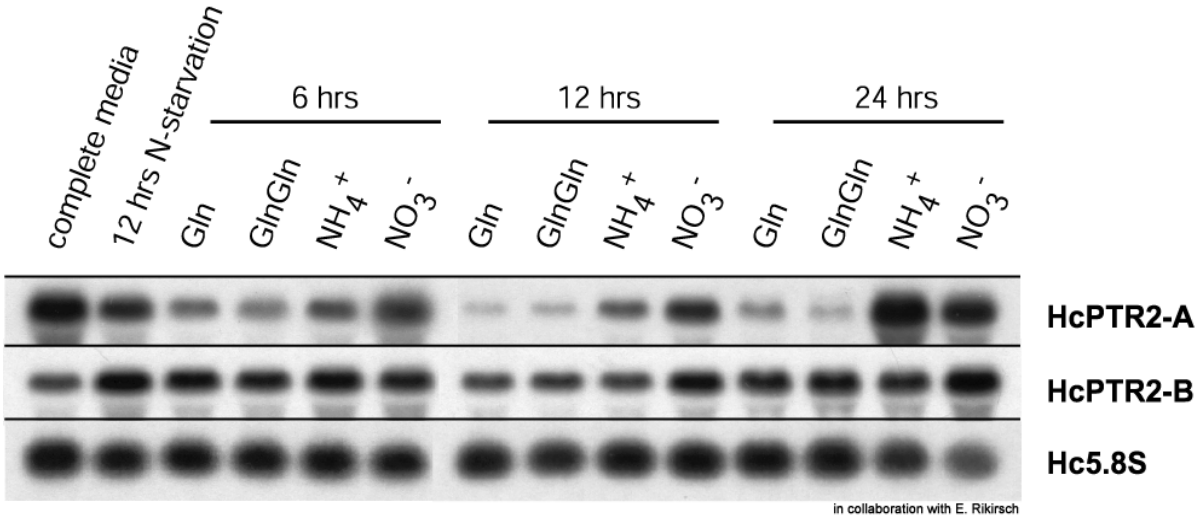

Figure 3.15: Regulation of *HcPTR2A* and *HcPTR2B* expression by nitrogen. Expression level of *HcPTR2A* and *HcPTR2B* according to the nitrogen source in the medium. *H.cylindrosporum* 5.8S rRNA probe was used as a loading control. The nylon membrane used in § 3.3.1.2 was stripped and successively hybridized with ^{32}P-*HcPTR2A* cDNA and ^{32}P-*HcPTR2B* cDNA.

3.3.3 Proteinase activity in *Hebeloma cylindrosporum*

In the boreal and temperate forests soil, nitrogen availability is mainly the result of litter decomposition. Moreover, as the competition between soil micro-organisms for nitrogen is high, the organisms having the ability to use more complex organic compounds (proteins) will be favored. Ericoid fungi (for review, Smith & Read 1997), but also some ectomycorrhizal fungi (e.g. *Amanita muscaria*, Abuzinadah & Read, 1986a; Nehls *et al.*, 2001a) can use protein as nitrogen source.
As *Hebeloma cylindrosporum* can take up peptides and amino acids from the soil, it was interesting to know if it can degrade proteins. Therefore we investigated the proteinase activity in *Hebeloma*.

3.3.3.1 Proteinase activity measurement

H. cylindrosporum mycelia were grown in 20 ml MMN medium. The experience was carried out at pH 4 as it was shown that the pH optimal of extracellular proteinase from ectomycorrhizal fungi is 4–4.5. Nitrogen was supplied as $(NH_4)_2SO_4$ (16 $\mu g.ml^{-1}$), which is comparable with the concentration of NH_4 in the soil for much of the year (Abuarghub & Read, 1988), or as BSA (0.1 $mg.ml^{-1}$). There were three replicate flasks for each treatment, and harvests were taken 1, 2, 3, 4, 5, 7, 12 and 16 days after inoculation and proteinase activity in cultures filtrates was measured (Figure 3.16).
From the first to the fifth harvest the proteinase activity showed no real difference between the mycelium grown on $(NH_4)_2SO_4$ and the one grown on BSA. After five days culture, the proteinase activity of the filtrate where the mycelium was grown on BSA increased considerably and attained peak values. In these cultures, maximum activity is three times higher than that obtained with filtrates harvested from cultures grown on ammonium. Here where proteinase activity already reached peak values at 5 days. In both case, activity declined rapidly after maximum had been attained.

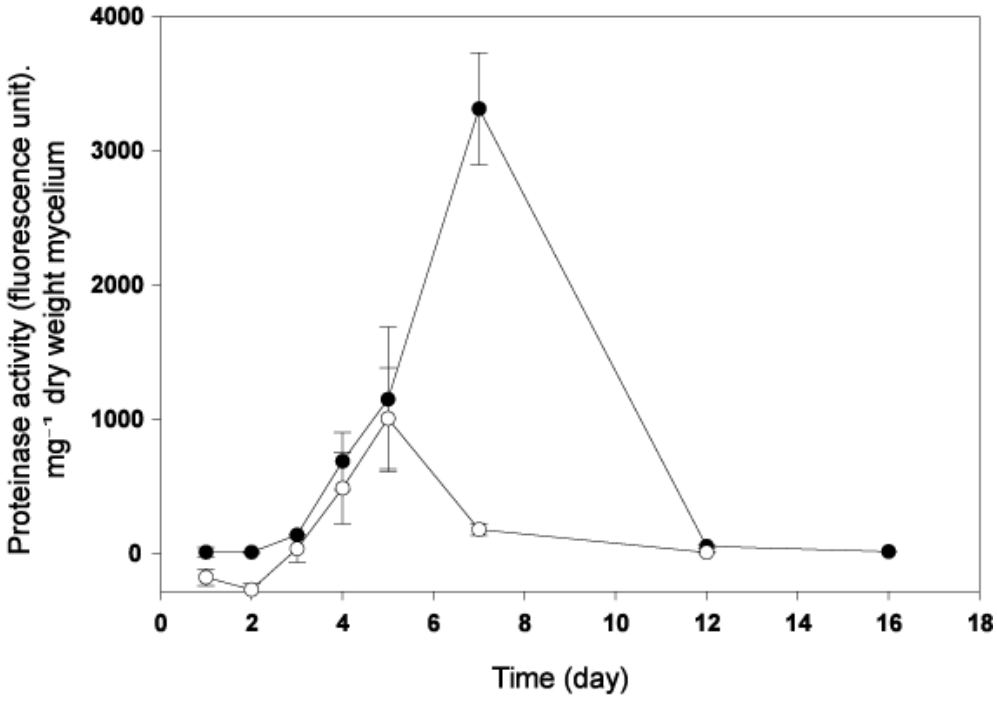

Figure 3.16: Specific enzyme activity (fluorescence units released in 3h per ml culture filtrate per mg mycelium dry weight) in culture of *Hebeloma cylindrosporum* grown on $(NH_4)_2SO_4$ (white circle, ○) or on BSA (black circle,●). Values represent the mean of three independent experiments ± S.D..

This result led us to think that *Hebeloma* can produce extracellular proteinase, this production might be induced by proteins. Moreover, the identification from the cDNA library from Lambilliote et al. (submitted) and the cloning of one putative extracellular proteinase gene *HcProtB* seems to confirm these results. BLAST analysis of the *HcProtB* sequence showed similarity to various proteinase gene from yeast and animals (see alignment in appendix 6). However, expression of HcProtB protein in a yeast mutant (BJ2168) deficient in the three vesicular proteinases (*pep4, prb1* and *prc1*), and used for expression of extracellular proteinase, could not show functional complementation and proteinase activity was not detected in the growth medium.

3.3.3.2 Expression of *HcProtB*

The expression of *HcProtB* was also investigated under different nitrogen concentration (Fig 3.17). The northern blot analysis was performed as described in § 3.3.1.2.

HcProtB gene was only poorly transcribed under nitrogen starvation and when amino acid, dipeptide or nitrate were used as nitrogen source. In medium containing ammonium, a weak transcript was detectable after 6h and 12h growth, but after 24h the expression of *HcProtB* gene was increased. In complete medium a strong expression could also be detected.

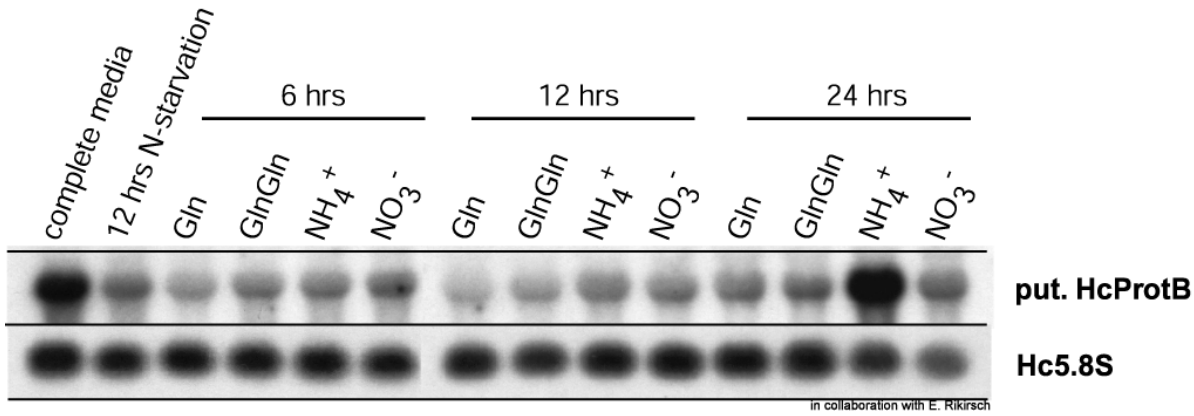

Figure 3.17: Regulation of *HcProtB* expression. Expression level of *HcProtB* were investigated under different nitrogen conditions. The nylon membrane used in § 3.3.1.2 and § 3.3.2.2 were stripped and hybridised with *32*P- *HcProtB* cDNA o the nitrogen source in the medium. *H.cylindrosporum* 5.8S rRNA probe was used as a loading control.

3.4 *Hebeloma* transformation

Hebeloma cylindrosporum is the only symbiotic fungus that can be genetically transformed using the conventional protoplast method (Marmeisse *et al.*, 1992). However, the transformation generally results in several plasmid integrations, which represent a strong limitation for gene tagging and subsequent molecular characterization of inactivated genes. The main advantage of *A. tumefacien*s-mediated over plasmid-mediated transformation of fungi is that it alleviates protoplast isolation as spores, hyphae, sporocarp or gill fragments can be transformed (Chen *et al.*, 2000; de Groot *et al.*, 1998). Furthermore, *A. tumefacien*s-mediated transformation (ATMT) generates a high percentage of transformants with a single T-DNA insert in the fungal genome (Combier *et al.*, 2003; de Groot *et al.*, 1998) which facilitates the genetic analysis of transformants and the identification of disrupted sequences.

*A. tumefacien*s-mediated transformation was carried out as described before (see Materials and Methods). *Hebeloma* transformation was carried out with the *A. tumefaciens* AGL-1 strain containing pBGghg plasmid (see appendices). The transforming plasmid has a disarmed T-DNA into which the hygromycin B phosphotransferase gene (*hph*) fused to the *Agaricus bisporus gpd* promoter sequence was inserted. This modified T-DNA confers hygromycin resistance. Moreover, the T-DNA contains also a *EGFP* gene under the control of *Agaricus bisporus gpd* promoter. One hundred transformation were realised. One transformation consisting in a co-cultivation of 50 µL of *Hebeloma* mycelium suspension and 100 µL of a bacterial culture on a disc which was placed on a co-cultivation solid medium (Fig.3.18). Hygromycin-resistant colonies appeared at the margins of the discs after 15 to 21 days on selective medium.

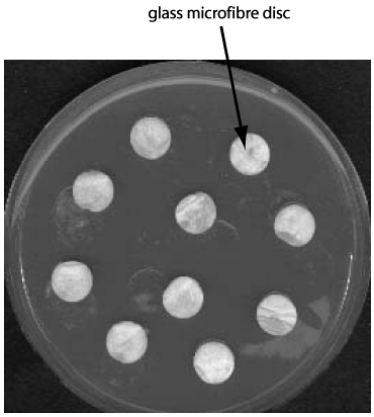

Figure 3.18: *Hebeloma* transformation plate

3.4.1 Molecular analysis of the transformants

Thirteen transformants were obtained showing resistance on 200 µg.mL^{-1} hygromycin after several propagations. Genomic DNA was extracted from the 13 transformants and the presence of *hph* gene was tested by PCR using specific primers (hygro5´/hygro3´). The expected 850 bp PCR product was detected in the 13 transformants (Fig. 3.19), indicating that T-DNA integrated into the fungal genome.

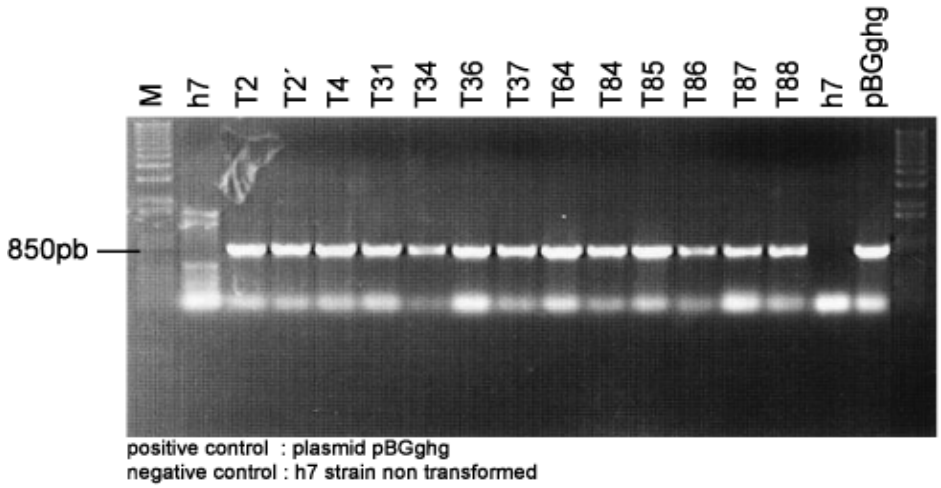

Figure 3.19: PCR analysis of DNA isolated from putative hygromycin-resistant Hebeloma transformants . PCR amplify- cation was carried out on genomic DNA using primers (hygro 5´and hygro 3´) defining an 850 bp sequence in the hph gene. M :DNA molecular size markers (1kb plus DNA ladder).

Southern blot analyses using the ^{32}P-*hph* as a probe confirmed that the hygromycin resistance gene was integrated into the genome of *H. cylindrosporum* (Fig. 3.20). We detected no false positives by Southern blot analysis or PCR amplification, though for T2 the hybridization signal was very weak. This could be due to the small quantity of genomic DNA loaded on the gel. The transformed cultures appeared to have a single integrated copy of the gene, although some showed evidence of up to three integration events. However, a precise determination of copy number would require further analysis, especially as several transformants showed the same hybridization pattern.

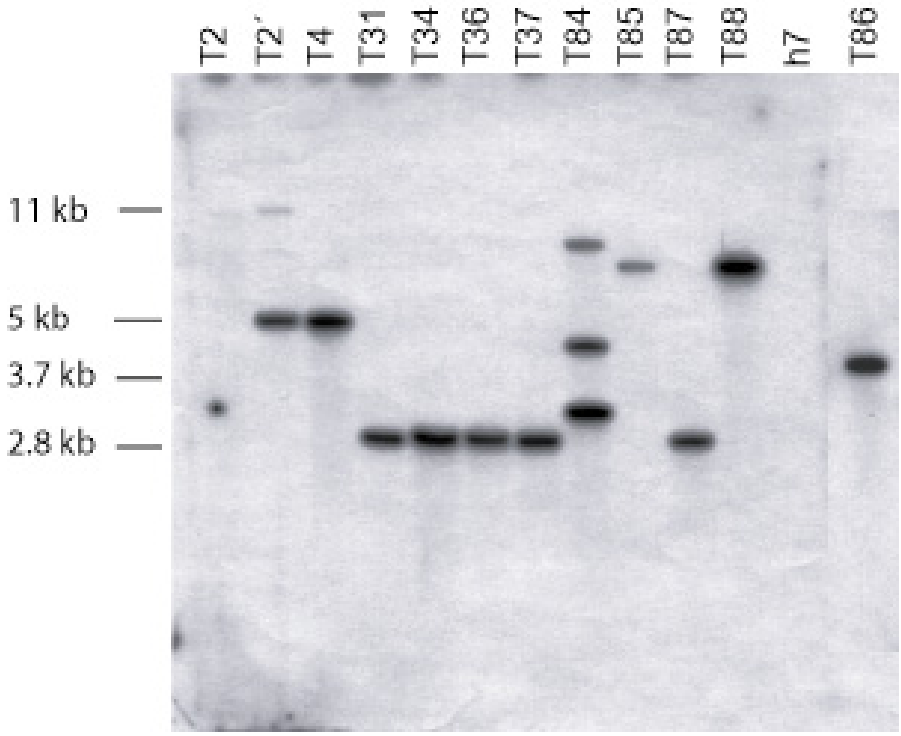

Figure 3.20: Southern blot analysis of transformants of *Hebeloma cylin -drosporum*.
Genomic DNA of 12 transformants was digested with *Eco*R*V*, gel-size fragmented, transferred onto a nylon membrane and probed with the radiolabeled *hph* gene. Molecular weights are indicated as bp, on the left. h7:untransformed wild type strain

3.4.2 GFP expression in the transformant

As pBGghg contains *EGFP* gene under the control of the constitutive *gpd* promoter, GFP expression was investigated in fast growing mycelia of four transformants T2, T4, T36 and T86 by fluorescence confocal microscopy. Only T86 could show a GFP expression. Fluorescence appeared uniformly distributed throughout the cytoplasm of the hyphae. In control experiments with nontransformed mycelia, we were unable to detect any hyphae showing fluorescence (Fig. 3.2.1).

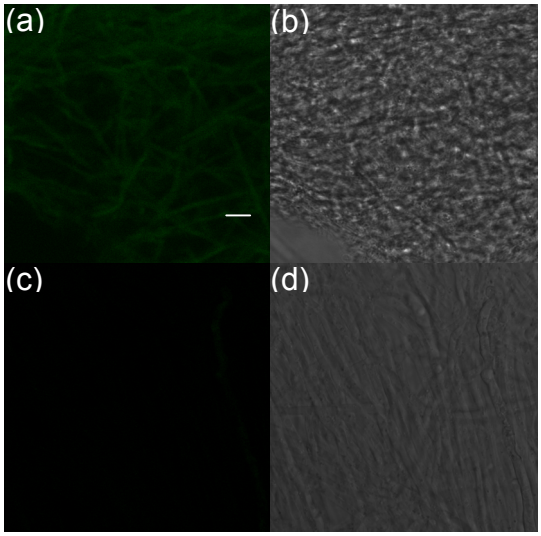

Figure 3.2.1: Fluorescence microscopy of *Hebeloma cylindrosporum*. (a) Hyphae from transformed *Hebeloma cylindrosporum* with the pBGghg vector. (c) Hyphae from wild-type strain h7. (b) and (d) transmission images. The bar represent 8μm.

Discussion & Conclusion

4 Discussion and conclusion

4.1 Analysis of the EST library from *Hebeloma cylindrosporum*

We have constructed a *Hebeloma cylindrosporum* cDNA library, from mycelia grown under different N-nutrition conditions. Analysis of ESTs proved the high quality of this library with a large proportion of full-length cDNAs (www.uni-tuebingen.de /plantphys/hebeloma/index.html). In parallel, we have shown that the library is also suitable for functional genomics using suppression cloning strategies. It was possible to clone the *HIS4* homologue from *H. cylindrosporum* and a general amino acid transporter HcGAP1. Thus, the cDNA library is efficient for cloning even large fungal cDNAs by complementation of yeast mutants.

An analysis of 500 ESTs suggests that *Hebeloma* contains a significant proportion of genes that have no counterpart in yeast, indicating differences in the gene pools of different fungi. A larger EST project will thus not only be useful for characterization of the ectomycorrhizal symbiosis, but also as a reference for other fungi. In addition, another *Hebeloma* cDNA library has been constructed, in a yeast expression vector, from a dicaryotic mycelia under different culture conditions (Wipf *et al.*, 2003). Thus, with these two libraries, efficient molecular tools are now available to identify genes likely to play crucial roles in ectomycorrhizal symbiosis, e.g. genes encoding proteins involved in N transport and metabolism, or P and K transport (uptake from the soil and secretion towards the host tree).

4.2 Characterization and regulation of *HcGAP1*

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 different nitrogen sources. The uptake characteristics allowed description of HcGAP1 as a high-affinity, secondary active, proton coupled, general amino acid permease. The K_M value for transport for aspartate of 150 μM is in the range of amino acid concentrations found in the soil (Scheller, 1996). Thus, the main function of *HcGAP1*, as also indicated by gene expression, may be the uptake of amino acids from the soil solution.

HcGAP1 is regulated by nitrogen sources; the presence of amino acids, dipeptides or ammonia, which are N sources imported by the fungus, induced a weak and constitutive expression of *HcGAP1*. Thus, in the presence of primary nitrogen sources, strong expression of high affinity transporter is not needed. So either the transporter is not working at its "maximum" or other transport systems might be used in this context. In contrast, when mycelia were grown without N source or on nitrate, which can be used by *Hebeloma* but with a slow uptake rate (Jargeat, 1999), *HcGAP1* expression was increased. Indeed, in condition of nitrogen deficiency or in the presence of a secondary nitrogen source like nitrate, high affinity transport systems (like HcGAP1), allowing uptake of amino acid, will be regulated positively. This led us to that *HcGAP1* gene might be regulated at a transcriptional level by the Nitrogen Catabolism Repression (NCR) mechanism, which has already been described for yeast and other fungi (Coffman *et al.*, 1997; Fu & Marzluf, 1990; Kudla *et al.*, 1990; Minehart & Magasanik, 1991). The strong expression of *HcGAP1* during N starvation could also indicate that *HcGAP1* may be involved, as it was suggested for the amino acid transporter *AmAAP1* (Nehls *et al.*, 1999), in the control of loss of amino acid by hyphae leakage. As was shown for a yeast mutant deficient in arginine uptake, the incapacity of arginine import resulted in a loss of arginine from the cells (Nehls *et al.*, 1999).

It is worthwhile to notice that when mycelia were grown on ammonium as a nitrogen source, strong expression of *HcGAP1* could be seen after 24h. This was not observed for *AmAAP1*. One explanation for this observation could be that, after 24h, NH_4^+ uptake and assimilation by the fungus, ammonium concentration is reduced in the medium, (similar to N deficiency) thus leading to a condition of nitrogen deficiency. Therefore *HcGAP1* expression is positively regulated.

The presence of several GATA (8) sequences in *HcGAP1* promoter also correlates with a possible regulation of *HcGAP*1 by NCR. However, to prove that these GATA sequences are really involved in NCR mechanism, further experiments, like promoter-reporter gene studies and electrophoretic mobility shift assays (EMSA) have to be performed.

HcGAP1 seems to be regulated by the NCR mechanism, like *AmAAP1* from *Amanita* (Nehls *et al.*, 1999) and *Gap1* from yeast (Jauniaux & Grenson, 1990). Additionally, the yeast *Gap1* is the target of post translational regulation, designated as nitrogen-regulated ubiquitination. When the yeast is grown on proline or urea (secondary nitrogen sources) as a sole nitrogen source, Gap1 activity is maximal, when ammonium is added *Gap1* is inactivated (Grenson, 1983a; Grenson, 1983b) internalized and subsequently degraded in the vacuole (Springael & Andre, 1998). *HcGAP1* may also be regulated, at the translational level, in the same way as *Gap1* (Bruno André, personal communication.

Analysis of the *HcGAP1* genomic sequence revealed an important number of introns (19) with an average size of 58 bp. The high number of introns, their small size and their uniformity were observed in *H. cylindrosporum* : nitrate reductase gene *HcNAR1* (12 introns), nitrate transporter *HcNRT2* (7 introns), nitrite reductase gene *HcNIR1* (19 introns) (Jargeat *et al.*, 2000; Jargeat *et al.*, 2003), the phosphate transporter genes *HcPT1* (10 introns) and *HcPT2* (16 introns) (Tatry, 2003) and the potassium transporter gene *HcTRK* (20 introns) (Corratgé, 2003). In other basidiomycete genes (Schuren, 1992), a high number of introns was also reported, for instance *PIG2* gene, an amino acid transporter from *Uromyces fabae*, presents 17 introns (Hahn *et al*., 1997).

4.3 Characterization of two peptide transporters from *Hebeloma*

Two peptide transporters from *Hebeloma*, *HcPTR2A* and *HcPTR2B*, the first peptide transporters described in mycorrhizal fungi, were isolated by functional complementation of the yeast strain LR2, deficient in peptide uptake (Rikirsch, 2002). In this work, biochemical properties of *HcPTR2A* and *HcPTR2B* were characterized. Tritium labeled LeuLeu uptake experiments showed that yeast expressing *HcPTR2A* and *HcPTR2B* could transport ^{3}H-LeuLeu. HcPTR2A-mediated uptake was higher than the one observed in yeast cells expressing *HcPTR2B*. Since HcPTR2B uptake rate is really low, further kinetics studies could not be performed and only *HcPTR2A* was characterized. *HcPTR2A* codes for a high affinity H^{+}/peptide transporter, which mediates uptake of di- and tripeptides, preferentially peptides containing leucine at the N terminal position but also other dipeptides like GlnGln and, to a less extent, the dipeptide HisLeu. It seems that HcPTR2A-mediated transport is substrate-specific

and that the N-terminal amino acid might be essential in the substrate recognition by the transporter.

Although PTR transporters have been shown to transport a wide range of nitrogen-containing substrates, including nitrate, amino acids and peptides (Williams & Miller, 2001), $NO3^-$ and amino acids, such as leucine and glutamine could not compete against LeuLeu uptake mediated by HcPTR2A. However, further peptide transporters, such as the *Arabidopsis PTR2*, were showed to transport peptides but not $NO3^-$ (Song *et al.*, 1996). In leaves of faba bean, peptide uptake systems do not import $NO3^-$ (Jamai *et al.*, 1996) and it was shown that PTR members from *A. thaliana* and *B. napus* have a low affinity for basic amino acids (Frommer *et al.*, 1994; Zhou *et al.*, 1998).

The K_M value for LeuLeu transport of 1.46 μM could be determined. This value, in the range of peptide concentrations found in the soil (1-2 μM), allows us to conclude that HcPTR2A may be involved in peptide uptake for fungal nutrient acquisition from the soil. It is worthwhile to notice that HcPTR2A is the first peptide transporter described with a so high affinity when compared to all described PTR members. For instance the K_M value of AtPTR2 for ^{3}H-LeuLeu was found to be 14 μM (Song *et al.*, 1996) and the K_M of VfPTR1, peptide transporter from *Vicia faba*, showed a value of 20 μM (Delrot *et al.*, 2001).

Expression of *HcPTR2A* and *HcPTR2B* were also analyzed. *HcPTR2A* showed a different expression pattern according to the N source present in the medium. In the absence of a nitrogen source or in the presence of $NO3^-$, *HcPTR2A* is highly expressed. This confirms the competition experiments where it was suggested that nitrate is not a substrate for *Hebeloma PTR2A.* According to Perry et al. (1994), yeast *PTR2* gene, which *HcPTR2A* is close to, falls under the regulatory control of the NCR system. Thus, like the yeast *PTR2* (Barnes *et al.*, 1998) and *HcGAP1*, *HcPTR2A* is strongly expressed in condition of N deficiency or in the presence of secondary N source. In mycelia grown on amino acid, dipeptide or ammonia (after 6h and 12h), considered as “preferred” sources, *HcPTR2A* mRNA level is weak, which may confirm its regulation by the NCR system. In contrast to *HcGAP1*, *HcPTR2A* is surprisingly strongly expressed on complete medium. This expression pattern was also observed for *VfPTR1* (peptide transporter from *Vicia faba*); Miranda et al., (2003)

investigated the *VfPTR1* mRNA level in seedlings grown on medium supplemented with 1mM or 50 mM sorbitol (control, equivalent to N starvation), Gln, LeuLeu or nitrate. At high amino acid or peptide concentrations the transcript level was higher than the control. At low concentrations of Gln or LeuLeu, transcription of *VfPTR1* decreased and was lower than the control. *HcPTR2A* might be regulated as the *Vicia faba* peptide transporter; in complete medium, where the amino acid and the peptide concentration is higher, *HcPTR2A* transcript level is increased. However, Perry et al. (1994) mentioned that yeast *PTR2* was always expressed at a basis level when yeasts were grown on full medium.

Interestingly, *HcPTR2A* was positively regulated after 24h on medium containing NH_4^+ as sole nitrogen source. As in the case of *HcGAP1* it is hypothesized that, after 24h, NH_4^+ uptake and assimilation by the fungus reduce the ammonium concentration in the medium and produce a condition of nitrogen deficiency, therefore *HcPTR2A* expression is positively regulated. This hypothesis was confirmed by Javelle A., who could observed a decrease of ammonium concentration in a fungal culture after 24h (personnal communication).

HcPTR2B seems to be a low affinity transporter, since its uptake capacity is low. The northern analysis could show that its regulation is independent from the nitrogen source present in the medium. *HcPTR2B* seems to behave like the yeast *PTR3* gene, which is not nitrogen dependent regulated (Barnes *et al.*, 1998). *PTR3* is one of the three identified components of the Ssy1p-Ptr3p-Ssy5 (SPS) sensor of extracellular amino acids (Klasson *et al.*, 1999);. The SPS amino acid sensor affects the transcription of several encoding proteins involved in amino acid uptake (*GAP1*, *BAP2, BAP3, AGP1, DIP5, GNP1, TAT1* and *TAT2*); (Forsberg & Ljungdahl, 2001; Iraqui *et al.*, 1999) and other genes, i.e. *PTR2* (Barnes *et al.,* 1998) or a gene encoding for an arginase (*CAR1)* (Forsberg & Ljungdahl, 2001). Thus, *HcPTR2B* may be involved in peptide and amino acid uptake regulation.

4.4 Proteinase activity of *Hebeloma cylindrosporum*

The ability of ectomycorrhizal fungi to explore the soil and to use organic debris as a nutrient source is important for the plants in nutrient poor soil (Smith & Read, 1997). A number of ericoid (Smith & Read, 1997) and ectomycorrhizal fungi can use proteins as a nitrogen and carbon source. However, proteins are not taken up directly but must be degraded to peptides and amino acid by proteinase.

In this work, a proteinase activity was measured for the first time in the ectomycorrhizal fungus *H. cylindrosporum*. This activity was induced strongly in presence of protein (BSA) and attained a maximum after 7 days. Ammonium, to a less extent, could also induce proteinase activity which attained maximum at 5 days and was three times lower than that induced by the presence of protein. After 7 days, the proteinase activity in the presence of BSA declined rapidly, apparently due to the diminution of substrate availability.

In the ericoid fungi *Hymenoscyphus ericae* Leake and Read (1990) a strong induction by BSA was observed and a weak induction by ammonia, amino acids and peptides. El Badaoui and Botton (1989) measured, for *Cenococcum geophilum,* a proteinase activity in presence of BSA, which was twice as high as than the one observed in the presence of ammonium. Zhu et al (1990), showed that proteins and a few amino acids, like glycine can induce proteinase production. Ammonium leads to weak proteinase activity which is, nevertheless, approximally twice as high as than that induced by glutamine and casein hydrolysate.

Taken together *Hebeloma cylindrosporum*, like other mycorrhizal fungi, can produce extracellular proteinases which are regulated by induction.

Moreover, a putative extracellular proteinase gene, *HcProtB,* was cloned, which confirms the detected extracellular proteinase activity. However, *HcProtB* could not complement the yeast strain BJ2168 which is deficient in vesicular proteinases and no specific proteinase activity was detected in the growth medium. One hypothesis to explain this result, is that *HcProtB*, which shows a similarity to the *PEP4* gene (precursor of the *S. cerevisiae* vesicular proteinase A, PrA), was isolated from the Hebeloma cDNA library prepared by Lambilliote et al. (submitted). In this library, inserts were cloned in the pFL61 yeast vector, thus, *HcProtB* is under the control of a

weak promoter. Furthermore, Wolff et al. (1996) showed that overexpression of *PEP4* leads to the secretion of mature PrA. Therefore it would be interesting to clone *HcProtB* under the control of a strong promoter to transform yeast mutants with this construct and see whether or not extracellular proteinase activity can be detected in the growth medium.

In parallel, expression of *HcProtB* was investigated, transcripts were weakly detectable when the mycelia were grown on Gln, GlnGln, or NH_4^+ (after 6h and 12h), but the mRNA level seemed to be slightly higher when ammonium was the sole nitrogen source. These results confirm the biochemical observations made in other mycorrhizal fungi (EL-Badaoui & Botton, 1989; Leake & Read, 1990; Zhu *et al.*, 1990); on amino acid, peptides and ammonium, the fungus showed a low secretion of extracellular proteinase.

In N-starvation conditions, *HcProtB* is weakly expressed, as it is on nitrate. Thus, as mentioned in (Smith & Read, 1997), extracellular proteinase are not subject to NCR. A high level of transcripts was detected only when the mycelia were grown on complete medium and on NH_4^+, after 24h. Complete media contains a mixture of amino acids, peptides of different size, vitamins…, it is likely that one or several compounds, i.e. glycine, present in the medium, are able to induce proteinase transcription.

It is worthwhile to notice, that these results differ partially from the expression analysis of *AmProt1*, an extracellular proteinase isolated from *A. muscaria* (Nehls *et al*., 2001). In medium containing glucose and nitrate, phenylalanine or no nitrogen source, a high level of *AmProt1* transcript was detected; in contrast on ammonium or casein hydrolysate, *AmProt1* was weakly expressed.

4.5 *Hebeloma* transformation

Hebeloma transformation was carried out with the *A. tumefaciens* AGL-1 strain transformed with the binary plasmid, pBGghg. In this vector, the T-DNA contains the hygromycine resistance gene (*hph*) and the *EGFP* gene, both under the control of the *Agaricus bisporus gpd* promoter.

Agrobacterium tumefaciens was used successfully to transform *Hebeloma cylindrosporum*. The efficiency of transformation was about 13% (percentage of discs regenerating colonies on hygromycin medium).

PCR amplification and Southern blot analysis could show that the *hph* gene was present and integrated into the genome of the transformants. For most of the transformants (except T84 transformant) only one band was detected on the Southern blot, indicating that a single copy of the T-DNA might be integrated in the genome. However, determination of the copy number would require further analysis. Interestingly, several transformants showed the same hybridization pattern. It is unlikely that the T-DNA was integrated at preferential sites, since Combier et al. (2003) showed that the integration occurred at random sites. One possibility to explain this hybridization pattern is that the transformants were the same. As described in the results, the transformation was performed on one disc and the transformed mycelium appeared at the margins of the discs, growing in every direction (Fig 4.1), some faster than the other. Fungal agar plug, surrounding one disc, was cut from the plate and transferred onto selective medium. However, on some plates, some mycelia were grew from one disc to the next, making it difficult to distinguish if the mycelium was growing from this disc or from the next disc. Therefore, it could be that mycelium appearing at the margin of several discs were from the same transformation which overgrow and reached other discs. Therefore, subsequent transformations, were carried out on compartmented Petri dishes.

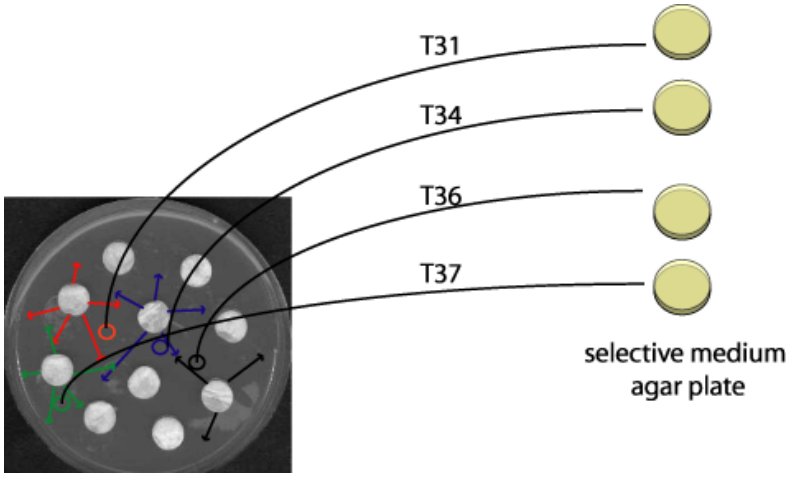

Figure 4.1: *Hebeloma* transformation

As the transforming plasmid pBGghg contain an *EGFP* gene inserted in the T-DNA, transformants were screened for GFP expression. From 4 transformants screened, one (T86) showed GFP expression, showing for the first time that GFP can be detected in *Hebeloma*. However , *EGFP* gene integration in the genome has to be confirmed.

4.6 Conclusion and perspectives

As a first step towards analysis of organic nitrogen nutrition, especially uptake and transport of organic nitrogen, by the mycorrhizal model fungus *Hebeloma cylindrosporum*, three genes involved in organic nitrogen uptake (*HcGAP1, HcPTR2A and HcPTR2B*) were isolated by functional complementation of yeast mutants.

HcGAP1 encodes a general amino acid permease mediating high affinity 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. Furthermore, Northern blot analysis showed that *HcGAP1* expression is dependent on available nitrogen sources. *HcPTR2-A* and *HcPTR2-B* encode peptide transporters and display strong identity to peptide transporters in yeast, plant and mammalian. HcPTR2A mediates a strong dipeptide uptake and, like HcGAP1, its expression is highly regulated under organic nitrogen deficiency, whereas HcPTR2B mediates a weak uptake and is constitutively expressed.

To characterize in more detail these transporters for their substrate specificity, it will be necessary to express them in *Xenopus laevis* oocytes and to carry out electrophysiology experiment, as several substrates can be tested in the same experiment.

Evidence led us to think that *HcGAP1, HcPTR2A* and *HcPTR2B* expression is regulated by the Nitrogen Catabolic Repression regulatory system. However, this data has to be confirmed by promoter-reporter gene studies and electrophoretic mobility shift assay (EMSA).

Production of extracellular proteinase from *Hebeloma cylindrosporum* could be demonstrated and led us think that *Hebeloma*, by producing extracellular proteinase, can degrade proteins, present in the soil, into amino acids and peptides which can be taken up.

Furthermore, the development of the *Hebeloma* transformation provides an important tool for the molecular genetic analysis of biological processes in this fungus.

The next step towards mycorrhizal organic nitrogen transport is the identification of new transporters at the soil/fungus interface (importers) and at the fungus/plant interface (exporters). It will be also interesting to characterize mutants for N-transport.

4.6.1 A new amino acid transporter

Recently we isolated a clone, which has a high similarity to the yeast □- amino butyric acid transporter gene, UGA4. However, the function of this transporter has to be confirmed.

4.6.2 Amino acid exporter

Read et al., (1989) showed that amino acids could be translocated to the colonized roots through mycorrhizal mycelia. Thus, transport systems localized at the fungus/plant interface and involved in the transfer of amino acids from the fungus to the plant (exporters to the apoplast) need to be characterized.
Preliminary experiments have already been carried out.
In order to isolate a putative amino acid exporter, a suppression of toxicity approach was used. In fact, if yeast cells are grown on a medium containing an amino acid toxic analog (AATA) and import it, there are three solutions for the yeast to suppress the toxicity: import in the vacuole (export from the cytosol), export out of the cell or enzymatic degradation. Thus, yeast transformed with an *H. cylindrosporum* cDNA library and showing growth on AATA lethal concentration, may express an amino acid exporter (Fig. 4.2).
As a first step, toxicity level, on yeast growth, of several AATA was tested in order to determine AATA toxic concentrations which could then be used for screening. The preliminary test were realized with the yeast “wild type” strain 23344c. The toxicity of different AATA was tested (Fig. 4.3).

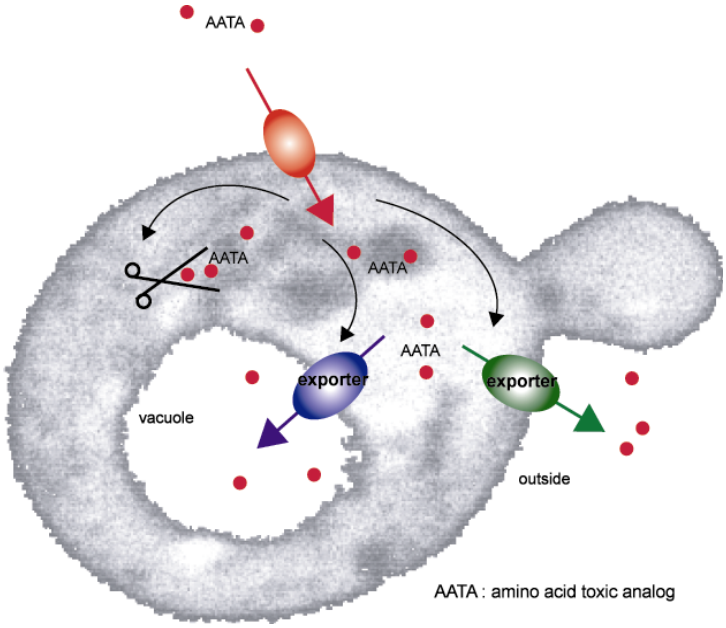

Figure 4.2 : Scheme representing the suppression of toxicity apprroach in a yeast cell

The yeast strain 23344c was transformed with the *Hebeloma* cDNA library and plated on L methionine sulfoxime at the concentration of 1mM for screening. However, all the transformants obtained were sequenced, but no insert showed similarity with any known amino acid exporters. As this cDNA library was realized from the saprophyte mycelia, genes encoding amino acid exporters might not be expressed. So it is necessary to screen a mycorrhizal cDNA library to get higher possiblity to isolate this gene. In collaboration with the INRA Montpellier (France), mycorrhized plants were prepared under different conditions of potassium, phosphate and nitrogen. Mycorrhizas were harvested and a cDNA library will be constructed.

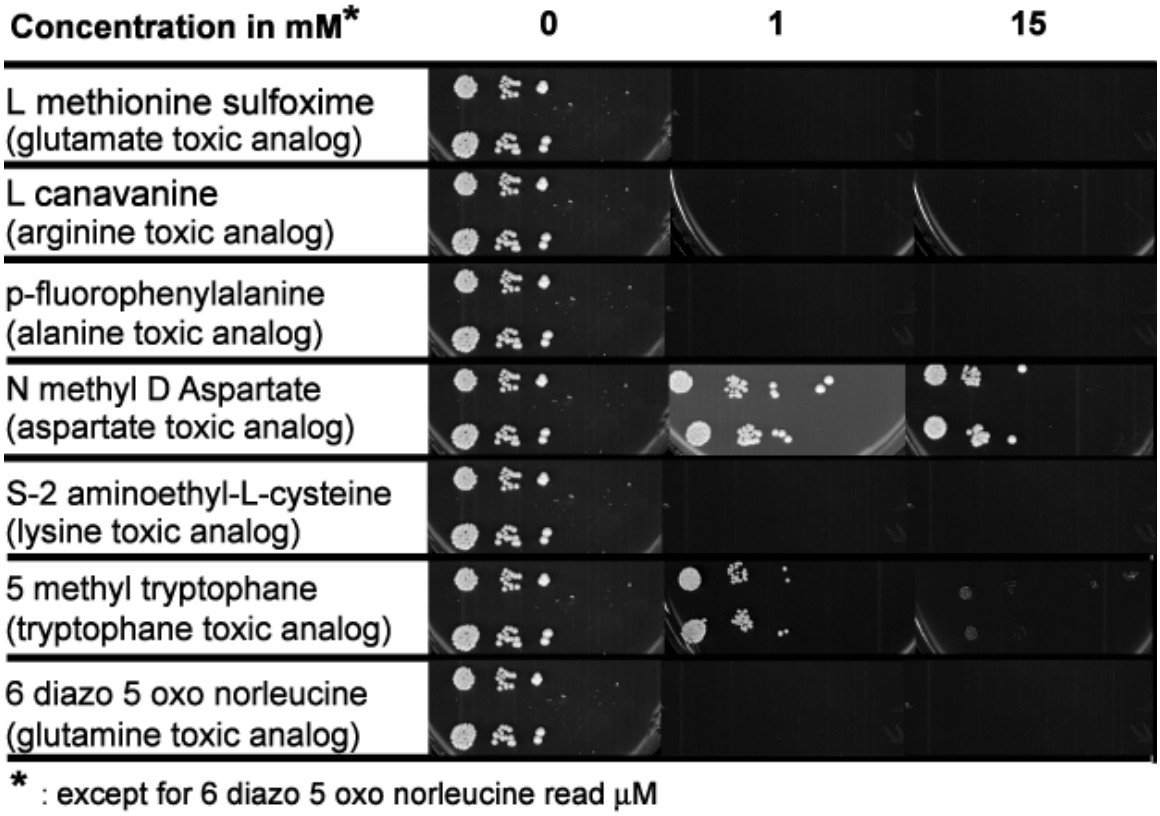

Figure 4.3: Growth test of the yeast wild type strain 23344c on different amino acid toxic analogs. Several dilutions from a yeast culture grown until OD_{595} of 4 were dropped on the plate; the dilution factor is decreasing from the left to the right.

4.6.3 Characterization of *Hebeloma* mutants

Mutants of *Hebeloma* can be produced by UV mutagenesis, and since the development of *Agrobacterium* mediated transformation it is possible to obtain mutants by gene replacement or to silence a gene by RNAi technique. These *Hebeloma* mutants can then be tested for their N transport capacity and characterized.

Thus, in order to characterize *in vivo* the role of HcGAP1, HcPTR2A and HcPTR2B, it would be interesting to silence *HcGAP1, HcPTR2A and HcPTR2B* gene and to observe the consequences on fungal growth and on the establishment of mycorrhiza.

However, as a collection of insertional *Hebeloma* mutants (2000 mutants) is now available (Combier *et al*., 2003) a screening method was set up in order to characterize mutants deficient in amino acid transport. This technique is also based on the utilization of amino acid toxic analogs. Different amino acid toxic analogs were

tested on fungal growth, the results of the test are shown figure 4.4 These results will then be used to screen *Hebeloma* mutants for resistance to ATAA.

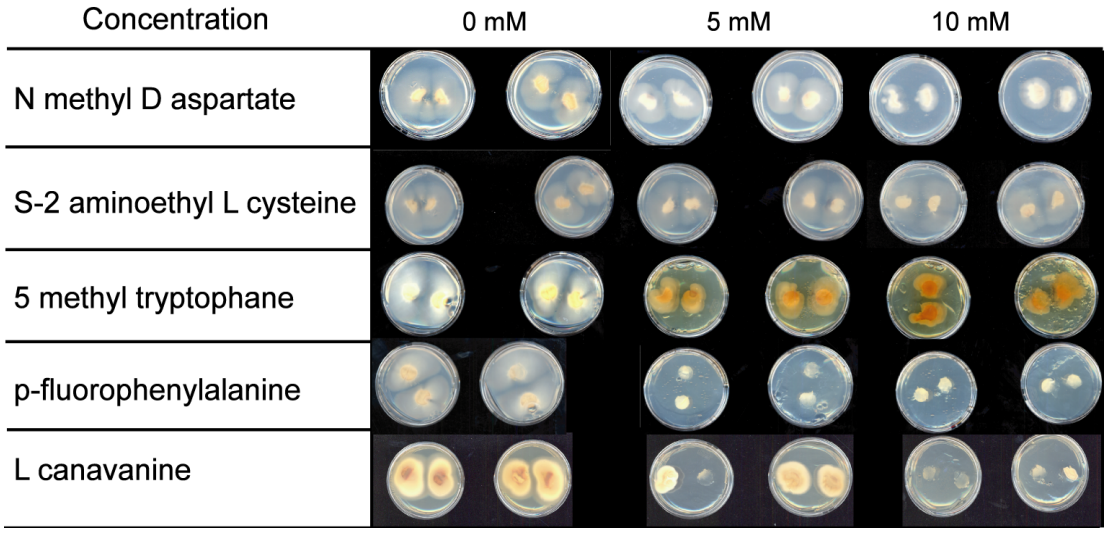

Figure 4.4: Growth test of *Hebeloma* mycelium on different amino acid toxic analog. Each concentration was tested twice.

⁂

⁂ ⁂

To conclude, data are now available to analyze how the isolated transporters are involved in nitrogen nutrition of the ectomycorrhizal fungus and will allow us to understand to which extent they participate to plant nutrition.

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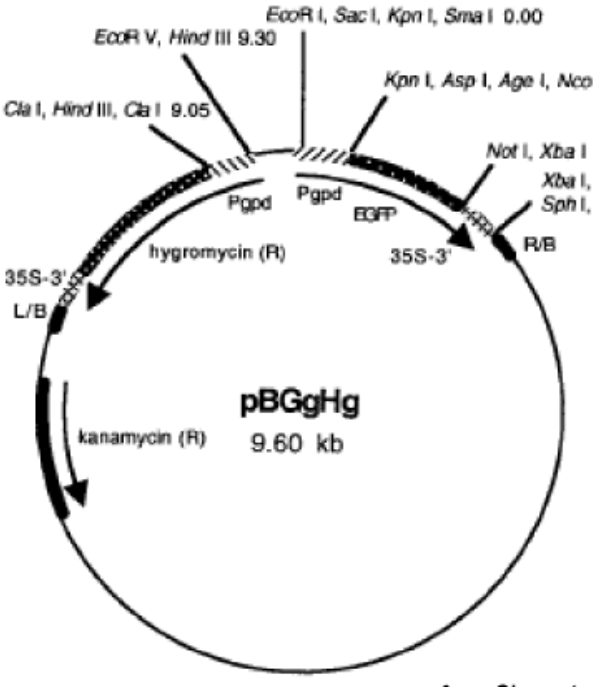
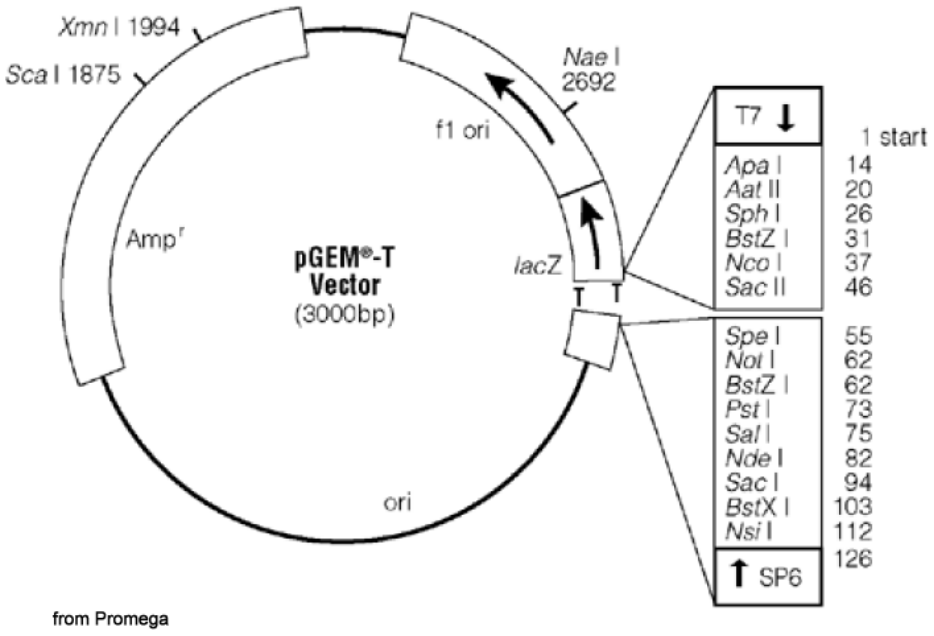
Appendices

6 Appendices

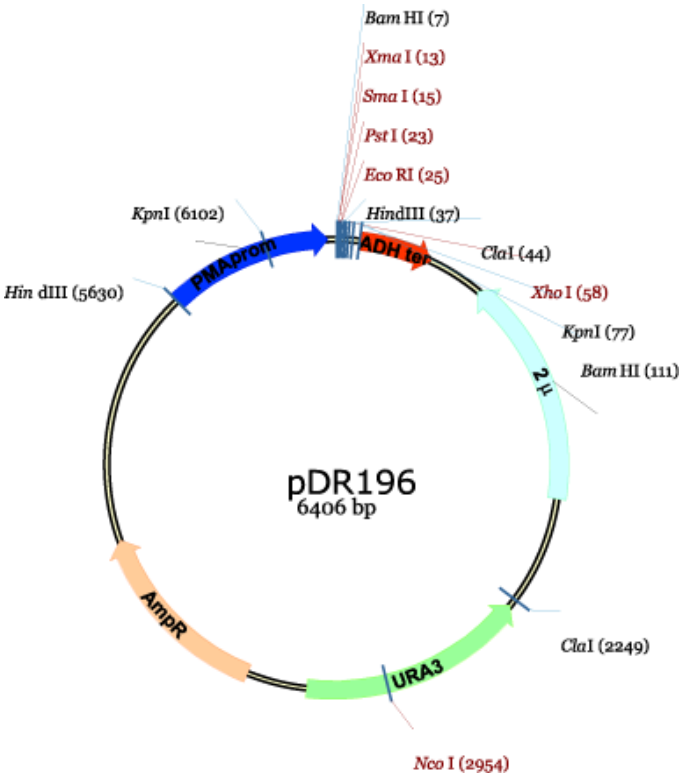
6.1 Appendix 1: Map of pBGghg vector



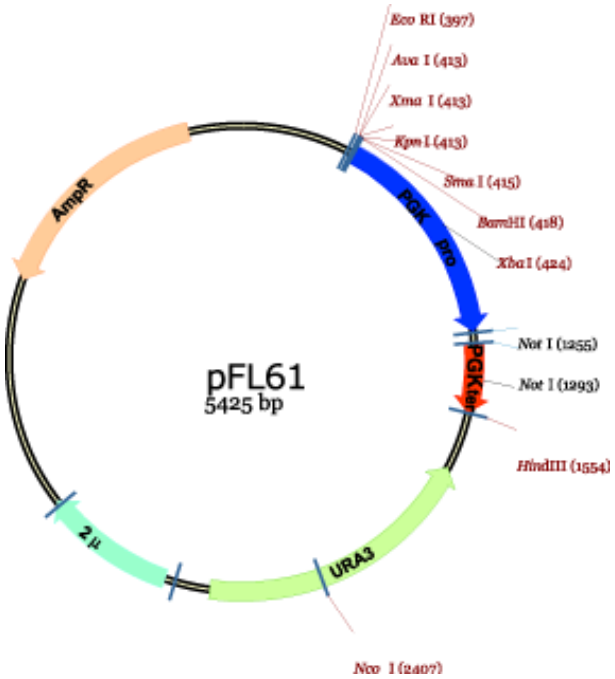
6.2 Appendix 2: Map of pGEM-T vector



6.3 Appendix 3: Map of the yeast expression vector pDR196



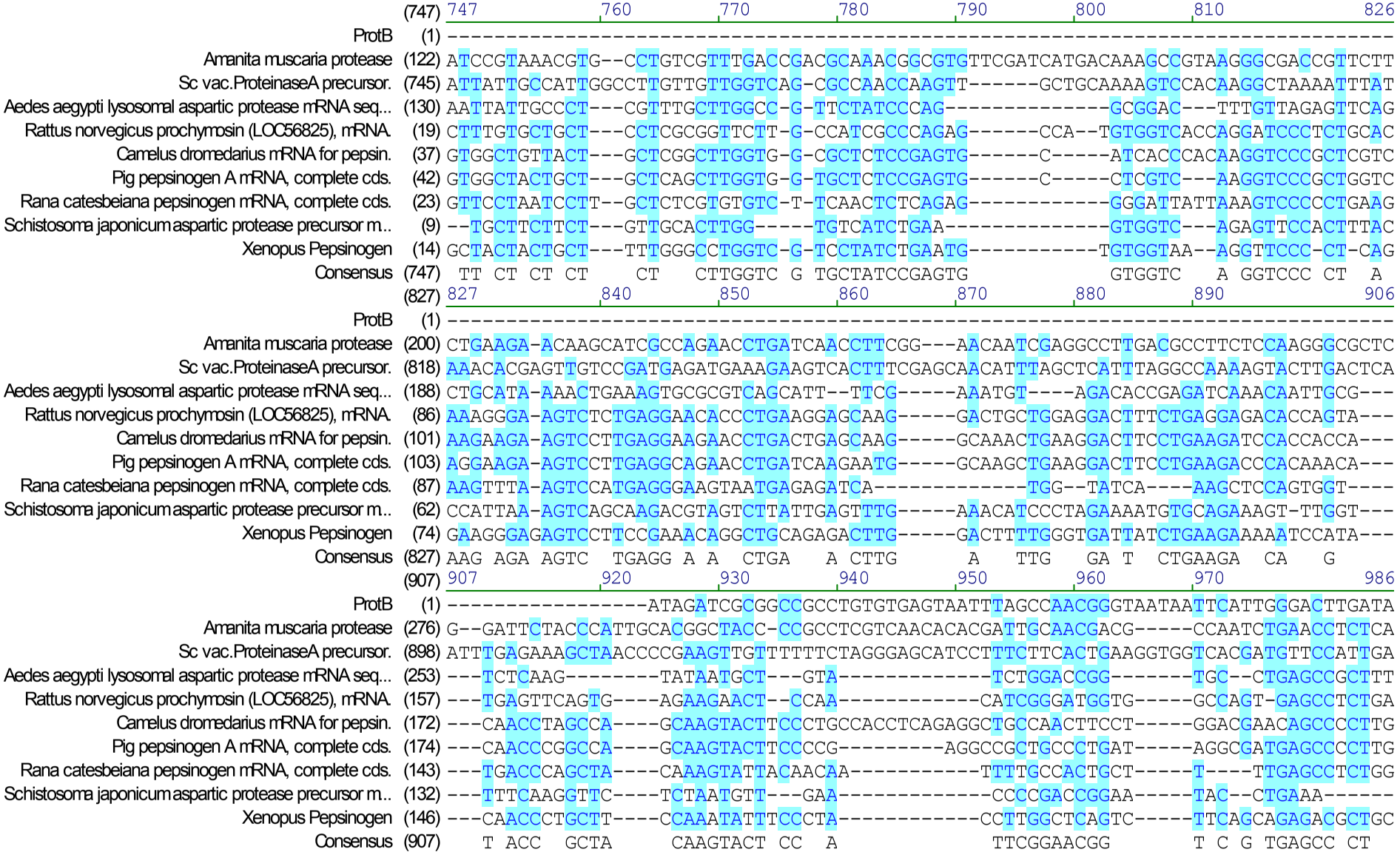
6.4 Appendix 4: Map of the yeast expression vector pFL61



6.5 Appendix 5: List of primers used in this work

Name	Sequence 5´→3´
promHcrev-50	AGA TTA GAG GAA CTT TCA CG
5´- HcGAP1	ATT AAT CAT GCA GAC CAA CAC A
SP6	ATT TAG GTG ACA CTA TAG
T7	CGC GTA ATA CGA CTC ACT ATA GGG
promHcGAP1+724	GCA GGT GAC GTG AAA GCG GC
promHcGAP1+1281	CTG AAA TCT AGA TAC GAG GTG T
HcBAP1gfor	ATG GAA AAC GAG AAA ACC GAG CAT GAG AAA CG
HcBAP1grev	TTA GAA GAT GAT ATT GGC AAT ACG TTC TCC G
Cpl hcgap1 for	CGA CAC CTG TTG AAA TCA CGG
Cpl hcgap1 rev	CGT GAG TAC CTT GGG AGC TTG ACC
hygro 5´	GGAGGGCGTGGATATGTCCTGCGGG
hygro 3´	CGCTTCTGCGGGCGATTTGTGTACG

6.6 Appendix 6: Comparison of the nucleotides sequence of several proteinases.

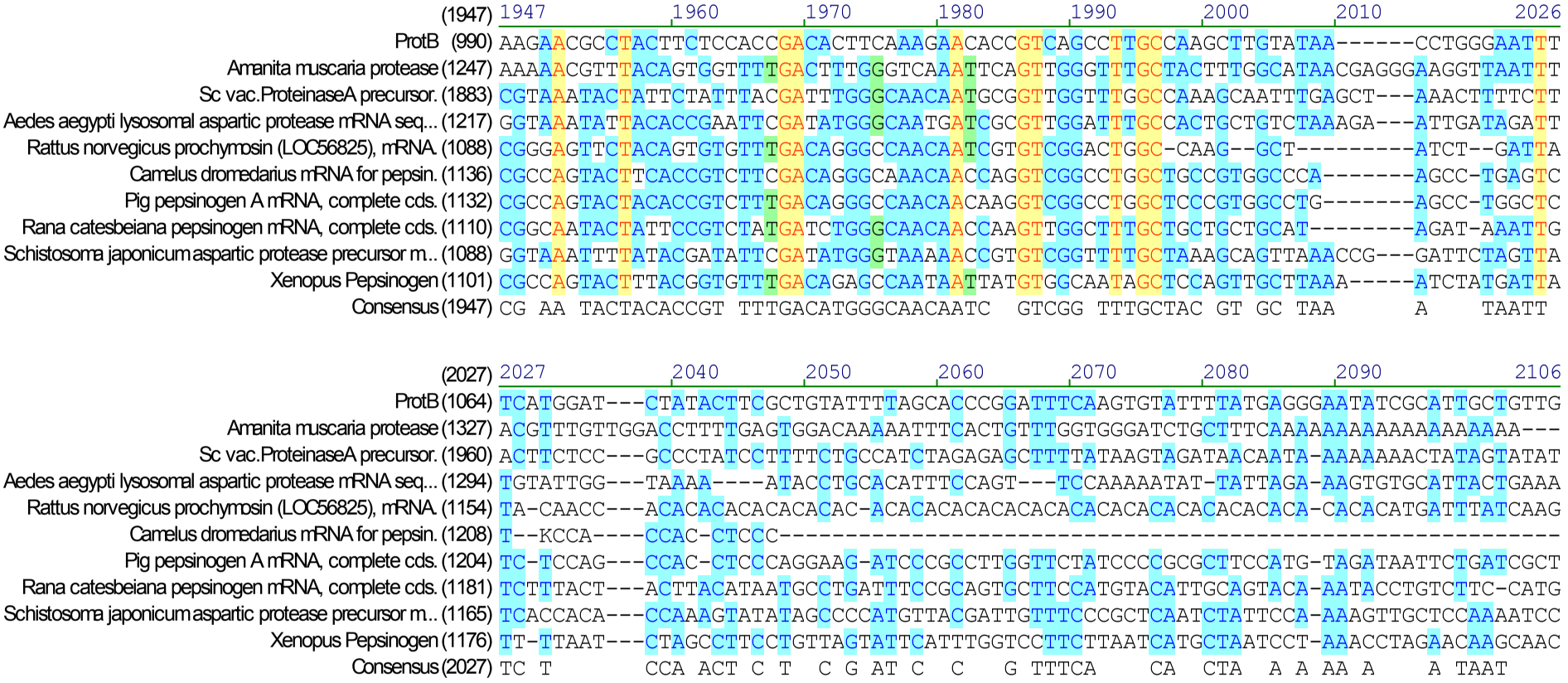



(987) 987 1000 1010 1020 1030 1040 1050 1066
ProtB (64) TTGAAGC---GCAGGATGTTGGGTATCTCGCAACCGTGCAGATCGGAACCCCTCCTCGTGACTTTTCGATTCTTATGGAC
Amanita muscaria protease (348) CCGACCAAAACGGTGATACTGAATGGACAGGAACGATCTCAATAGGCACACCTGGTACAGAGTTCTTGATCGACTTTGAT
Sc vac.ProteinaseA precursor. (978) CAAATTA---CTTGAACGCACAATATTACACTGACATTACTTTGGGTACTCCACCTCAAAACTTCAAGGTTATTTTGGAT
Aedes aegypti lysosomal aspartic protease mRNA seq... (297) CGAACTA---TTTGGATGCCCAATACTATGGAGCTATCACCATCGGAACACCACCGCAGAGCTTCAAAGTTGTGTTCGAT
Rattus norvegicus prochymosin (LOC56825), mRNA. (210) CCAACTA---TCTAGATAGTGAGTACTTCGGACTGATCTACGTTGGAACACCACCCCAGGAGTTCAAGGTGGTGTTTGAC
Camelus dromedarius mRNA for pepsin. (240) AGAACTA---CCTGGATACGGAGTACTTTGGCACCATCAGCATCGGAACCCCGGCTCAGAACTTCACCGTCATCTTTGAC
Pig pepsinogen A mRNA, complete cds. (233) AGAACTA---CCTGGATACTGAGTACTTTGGCACCATCGGCATTGGAACCCCCGCTCAGGACTTCACCGTCATCTTTGAC
Rana catesbeiana pepsinogen mRNA, complete cds. (196) CAAACTA---CATGGATATGTCTTACTATGGTGAGATCAGTATTGGAACGCCTCCTCAGAACTTCCTCGTTCTGTTTGAC
Schistosoma japonicum aspartic protease precursor m... (176) --AACTA---CTTAGATGCTCAGTATTATGGTGATATAACGATTGGAACACCACCTCAGACATTCAGCGTTGTATTTGAT
Xenopus Pepsinogen (202) AGAACTA---TATGGATATTGAATATTATGGGACCATTTCCATTGGGACCCCTCCCCAGGAATTCACTGTTATCTTTGAC
Consensus (987) C AACTA C TGGATACTGAGTACTATGG ACCATCACCATTGGAAC CC CCTCAGGACTTCA GTT T TTTGAC
(1067) 1067 1080 1090 1100 1110 1120 1130 1146
ProtB (141) TCTGGTTCGGCCGATTTCTGGGTCGGAGCAGAGAACTGTCAGTCTGACGCTGGTGGTGGATGTGGGAATCACAAGTTCCT
Amanita muscaria protease (428) ACCGGCTCTTCCGATCTCTGGGTACCATCCGCAGCTTGCTCC------AGCTCAATTTGCGAGCCCAAGCACAAGTACGA
Sc vac.ProteinaseA precursor. (1055) ACTGGTTCTTCAAACCTTTGGGTTCCAAGTAACGAATGTGGT------TCCTTGGCTTGTTTCCTACATTCTAAATACGA
Aedes aegypti lysosomal aspartic protease mRNA seq... (374) ACGGGATCATCTAACCTTTGGGTGCCCTCGAAGGAGTGCTCATTCACCAACATCGCTTGCTTGATGCACAACAAATACAA
Rattus norvegicus prochymosin (LOC56825), mRNA. (287) ACAGGCTCCTCAGAACTCTGGGTGCCCTCTGTGTACTGCAGC------AGCAAAGTTTGCAGAAACCACAACCGCTTTGA
Camelus dromedarius mRNA for pepsin. (317) ACTGGCTCTTCCAACCTGTGGGTGCCCTCCATCTACTGCTCC------AGCTCTGCCTGCACCAACCACAACCGCTTCAA
Pig pepsinogen A mRNA, complete cds. (310) ACCGGCTCCTCCAACCTGTGGGTGCCCTCTGTCTACTGCTCT------AGTCTCGCCTGCAGCGACCACAACCAGTTCAA
Rana catesbeiana pepsinogen mRNA, complete cds. (273) ACTGGGTCCTCAAACCTGTGGGTACCCTCAACCTACTGTCAA------AGCCAGGCCTGCACCAATCATCCTCAGTTTAA
Schistosoma japonicum aspartic protease precursor m... (251) ACTGGTTCATCCAATCTTTGGGTGCCGTCGAAACACTGCAGCTATTTTGATATTGCTTGCCTGCTACATCGTAAATATGA
Xenopus Pepsinogen (279) ACAGGATCTGCTAATCTGTGGGTTCCATCTGTGTACTGCTCC------AGCCAAGCCTGCTCAAATCACAACAGGTTTAA
Consensus (1067) ACTGG TC TCCAACCT TGGGTGCCCTC G TACTGCTCC AGC GCTTGC AA CACAACAAGTTCAA
(1147) 1147 1160 1170 1180 1190 1200 1210 1226
ProtB (221) CGGAGCGCAGTCCAGTAGTTCCTTCCAGGACACCGGCGCACCTTTCTCCGTTACATACGGA-ACAGGG--CAGGTTTCTG
Amanita muscaria protease (502) TCCTACTGCCTCCAGCA---CCTCTCAACTGCAGAATGGAACATTCACTATCCAATATGGCGACAAGTCAACCGTTTCGG
Sc vac.ProteinaseA precursor. (1129) TCATGAAGCTTCATCAA---GCTACAAAGCTAATGGTACTGAATTTGCCATTCAATATGGT-ACTGGT--TCTTTGGAAG
Aedes aegypti lysosomal aspartic protease mRNA seq... (454) TGCCAAGAAGTCATCGA---CGTTCGAAAAGAACGGAACAGCTTTCCATATTCAATATGGA-TCTGGT--AGCTTATCTG
Rattus norvegicus prochymosin (LOC56825), mRNA. (361) CCCATCCAAGTCCTTCA---CCTTCCAGAACCTGAGCAAGCCCCTGTTTGTCCAGTATGGT-ACTGGC--AGTGTGGAGG
Camelus dromedarius mRNA for pepsin. (391) CCCTGAGGAATCCTCCA---CCTACCAGGGCACCGACGAGACGCTCTCCATCACCTATGGC-ACCGGC--AGCATGACAG
Pig pepsinogen A mRNA, complete cds. (384) CCCTGATGACTCCTCCA---CCTTCGAGGCCACCAGCCAGGAGCTGTCCATCACCTATGGC-ACCGGT--AGCATGACAG
Rana catesbeiana pepsinogen mRNA, complete cds. (347) CCCCAGCCAGTCTTCCA---GCTATTCCTCAAACCAGCAGCAGTTCTCTCTGCAGTACGGC-ACTGGT--AGTCTGACTG
Schistosoma japonicum aspartic protease precursor m... (331) CAGCTCAAAGTCCACCA---CTTATGTTCCGAATGGCACTGATTTCAGCATTCGTTACGGT-ACTGGC--AGTCTTAGTG
Xenopus Pepsinogen (353) CCCACAGCAGTCATCCA---CTTTCCAAGCAACAAACACTCCAGTGTCTATTCAATATGGA-ACCGGC--AGCATGAGTG
Consensus (1147) CCC A AGTCCTCCA CCTTCCA GC AACGGCAC C TTCTCTATTCAATATGG ACTGGT AGC TGACTG


(1227) 1227 1240 1250 1260 1270 1280 1290 1306
ProtB (298) GTAATATTATCAAGGATAATATCGCTATTGCTGGACTCGCACTCGATGCTCAAACTTTCGGAGTCGCTACTTCGGAAAGC
Amanita muscaria protease (579) GCCCAGTGTACACTGATGCAGTCAATGTTGCTGGTGTCCAGGTGACAGGTCAATATCTCTCTC-CGG--TCACAAA---T
Sc vac.ProteinaseA precursor. (1203) GTTACATTTCTCAAGACACTTTGTCCATCGGGGATTTGACCATTCCAAAACAAGACTTCGCTGAGGCTACCAGCGA--GC
Aedes aegypti lysosomal aspartic protease mRNA seq... (528) GTTACTTGTCAACTGACACCGTTGGTTTGGGAGGGGTTTCCGTTACGAAACAAACCTTCGCTGAAGCCATCAATGA--AC
Rattus norvegicus prochymosin (LOC56825), mRNA. (435) GCTTCCTGGCCTATGACACTGTCACGGTCTCTGACATTGTAGTTCCCCATCAGACTGTGGGCCTGAGTACTGAGGA--AC
Camelus dromedarius mRNA for pepsin. (465) GCATCCTCGGATATGACACTGTCCAGGTTGGAGGCATCAGCGATGTCAACCAGATCTTTGGCCTGAGTGAGACAGA--GC
Pig pepsinogen A mRNA, complete cds. (458) GCATCCTTGGATACGACACCGTCCAGGTCGGAGGCATCAGCGACACCAACCAGATCTTTGGCCTGAGCGAGACAGA--GC
Rana catesbeiana pepsinogen mRNA, complete cds. (421) GAATCCTTGGATATGACACAGTTCAGATCCAGAATATAGCTATTTCCCAGCAAGAATTTGGCTTGAGTGTGACTGA--GC
Schistosoma japonicum aspartic protease precursor m... (405) GATTTCTAAGTACTGACTCCCTTCAGCTAGGTTCACTCGGAGTAAAAGGACAAACATTTGGAGAAGCGACAAAACA--GC
Xenopus Pepsinogen (427) GGTTCCTGGGATATGATACTTTGCAAGTGGGAAATATCCAGATTTCTAACCAAATGTTCGGTCTTAGTGAGTCAGA--GC
Consensus (1227) G TTCCT GGATATGACACTGTCCAGGT GG GG ATC GTT C AA CAAA TTCGG CTGGGTA ACAGA GC
(1307) 1307 1320 1330 1340 1350 1360 1370 1386
ProtB (378) GTGGATTTCTCATCTAATACTACTCCCTTTGATGGGCTGATGGGACTTGCGCAGGCGACCATTTCGCAACAACGAACTCC
Amanita muscaria protease (653) CTTTCATCGACCTTTGGTACCGATCCTATCGACGGGCTTCTCGGCCTGGCCTTCCCTGCGATCTCAAACATGAAACAAAG
Sc vac.ProteinaseA precursor. (1281) CGGGCTTAACATTTGCATT-TGGCAAGTTCGATGGTATTTTGGGTTTGGGTTACGATACCATTTCTGTTGATAAGGTGGT
Aedes aegypti lysosomal aspartic protease mRNA seq... (606) CAGGATTGGTATTCGTTGC-GGCCAAGTTTGACGGAATTCTCGGATTAGGCTACAGCTCGATTTCAGTAGATGGCGTCGT
Rattus norvegicus prochymosin (LOC56825), mRNA. (513) CAGGTGACATCTTCACCTA-CTCTCCATTTGATGGCATCCTGGGCCTGGCCTACCCTACTTTGCCTCCAAGTACTCAGT
Camelus dromedarius mRNA for pepsin. (543) CCGGCTCCTTCCTGTATTA-TGCCCCCTTCGATGGCATCCTGGGTCTGGCTTACCCCAGCATCTCCTCCTCCGGGGGCAC
Pig pepsinogen A mRNA, complete cds. (536) CCGGCTCCTTCCTGTACTA-CGCCCCCTTCGACGGCATCCTGGGTCTGGCCTACCCCAGCATCTCCGCCTCCGGGGCCAC
Rana catesbeiana pepsinogen mRNA, complete cds. (499) CTGGAACCAACTTTGTCTA-TGCGCAGTTTGATGGTATTTTGGGTCTAGCCTACCCATCTATTGCTGAGGGTGGGGCTAC
Schistosoma japonicum aspartic protease precursor m... (483) CAGGGTTAGTTTTCGTCAT-GGCAAAATTTGATGGGATTCTGGGTATGGCTTATCCTTCACTTGCTGTTGGTGGTGTTAC
Xenopus Pepsinogen (505) CTGGTTCATTCCTCTACTA-CTCACCATTCGATGGAATCCTTGGTCTAGCTTTTCCCAGCATTGCATCATCTCAAGCCAC
Consensus (1307) C GG TTC TCTTCT CTA GC CC TTTGATGG ATTCTGGGTCTGGCCTACCC ACCATTTC G TGG GC AC
(1387) 1387 1400 1410 1420 1430 1440 1450 1466
ProtB (458) AACGCCGGTTGACTCGCTCGCTACTGCGGGATTGATCCCAGACTCCATCGTTTCATTCAAAATCTCTCGCCTTGCTGATA
Amanita muscaria protease (733) CCCATTCTTCAACACCGCCATCGCACAAGGCAGCGTGTCAAAACCCAGCTTTGGTTTTAAACTCGCCAAAAACGAT----
Sc vac.ProteinaseA precursor. (1360) CCCTCCATTTTACAACGCCATTCAACAAGATTTGTTGGACGAAAAGAGATTTGCCTTTTATTTGGGAGACACTTCAAAGG
Aedes aegypti lysosomal aspartic protease mRNA seq... (685) ACCAGTATTCTACAATATGTTCAACCAGGGTCTCATCGATGCTCCCGTTTTCTCTTTCTATTTGAATCGTGATCCAAGTG
Rattus norvegicus prochymosin (LOC56825), mRNA. (592) ACCCATATTTGACAACATGATGAACAGGCACCTGGTGGCCCAAGACCTGTTCTCCGTTTACATGAGCAGGAATGAC----
Camelus dromedarius mRNA for pepsin. (622) CCCTGTCTTTGACAACATCTGGGACGAGGGTCTGATTTCCGAAGACCTCTTCTCTGTCTACCTGAGCTCCAATGAC----
Pig pepsinogen A mRNA, complete cds. (615) CCCTGTCTTTGACAACCTGTGGGACCAGGGCCTGGTTTCCCAAGACCTCTTCTCCGTCTACCTGAGCTCCAATGAC----
Rana catesbeiana pepsinogen mRNA, complete cds. (578) CACTGTGATGCAAGGCATGATTCAACAGAATCTCATCAACCAACCTCTCTTTGCATTCTACCTTAGTGGGCAACAGAACT
Schistosoma japonicum aspartic protease precursor m... (562) TCCTGTATTTGTAAACATGATCAAACAAGGTGTAGTTGATTCCCCAGTGTTTTCCTTTTATTTAAGCAGGAATATAACGA
Xenopus Pepsinogen (584) ACCAGTGTTTGACAACATGTGGAGCCAAGGACTCATACCACAGAACCTCTTTTCTGTGTATCTCAGCTCTGATGGT----
Consensus (1387) CCCTGT TTTGACAACATGAT AACCAGGGTCTGAT CC AA CCCTCTTTTC TTCTA CTGAGC G AATGA


(1467) 1467 1480 1490 1500 1510 1520 1530 1546
ProtB (538) ---ACCTGAATGACGGA-GAGATTACTTTGGAGGACTGGATGACACCAAGTTCGATCCAAAGACCCTGATTACCGTGCC
Amanita muscaria protease (809) ----------TCAGAACTTTACCTGGGCGGTGCCAAATGCAAGCTTTACACCGGGGC--CATTGAATCGCACAGC
Sc vac.ProteinaseA precursor. (1440) ATACTGAAAATGGCGGT-GAAGCCACCTTTGGTGGTATTGACGAGTCTAAGTTCAAGGGCGA---TATCACTTGGTTACC
Aedes aegypti lysosomal aspartic protease mRNA seq... (765) ---CTGCTGAGGGTGGC-GAAATTATTTTCGGTGGATCAGACTCGAATAAGTATACTGGGGA---CTTTACTTATCTGTC
Rattus norvegicus prochymosin (LOC56825), mRNA. (668) -----CAA---GGGAGC-ATGCTCACGCTGGGGGCCATCGATCAGTCCTACTTCATAGGCTC---ACTGCACTGGGTGCC
Camelus dromedarius mRNA for pepsin. (698) -----GAGAGTGGAAGC-GTGGTGATATTTGGTGGCATCGATTCTTCTTACTATACAGGAAG---CCTCAACTGGGTGCC
Pig pepsinogen A mRNA, complete cds. (691) -----GATAGCGGCAGT-GTGGTGTTGTTGGGCGGCATCGATTCTTCTTACTACACAGGAAG---CCTGAACTGGGTGCC
Rana catesbeiana pepsinogen mRNA, complete cds. (658) ---CACAGAATGGCGGTGAAGTTGCTTTTGGTGGCG-TTGACCAAAACTACTACTCTGGACA---AATTTATTGGACTCC
Schistosoma japonicum aspartic protease precursor m... (642) ---ATGTATTGGGTGGT-GAGCTGATGATTGGTGGTATAGATGACAAATATTATACTGGTGA---AATTAACTATGTGAA
Xenopus Pepsinogen (660) -----CAAACCGGCAGCTATGTTCTCTTTGGAGGAG-TTGACAATTCTTACTACAGTGGCAG---CCTAAACTGGGTCCC
Consensus (1467) GA A GGCGGC GAG T AT TTGGG GG AT GAT A TCTTACTACACTGG A CCT AATTGGGTGCC
(1547) 1547 1560 1570 1580 1590 1600 1610 1626
ProtB (614) AAATGTCAACCAACAAG-GATTCTGGGAGGCTGCCCTCGACGCTGCCACCGTTGACGGCACGGACA--CG----GGTTTG
Amanita muscaria protease (872) --CTTTCTTCCACCACTGGGTACTGGCAAATTGGCAACGCCTCCATTTCCGTAGGCGGACAAACCC-------CAGTATT
Sc vac.ProteinaseA precursor. (1516) ---TGTT-CGTCGTAAGGCTTACTGGGAAGTCAAGTTTGAAGGTATCGGTTTAGGCGACGAGTACGCCGAATTGGAGAGC
Aedes aegypti lysosomal aspartic protease mRNA seq... (838) ---GGTGGACCGTAAAG-CCTACTGGCAATTCAAAATGGACTCCGTTAAGGTTGGCGATACTGAGT-----TC-TGCAAC
Rattus norvegicus prochymosin (LOC56825), mRNA. (736) ---TGTG-ACTGTACAAGGATATTGGCAGTTCACAGTGGACAGGATCACAATCAATGATGAGGTGG-TGGCT--TGTCAA
Camelus dromedarius mRNA for pepsin. (769) ---TGTT-TCTGTTGAGGGTTACTGGCAGATCACCGTGGACAGCATCACCATGGAAGGAGAGTCCA-TCGCT--TGCAGC
Pig pepsinogen A mRNA, complete cds. (762) ---TGTT-TCCGTCGAGGGTTACTGGCAGATTACCTTGGACAGCATCACCATGGACGGAGAGACCA-TCGCT--TGCAGC
Rana catesbeiana pepsinogen mRNA, complete cds. (731) ---CGTG-ACTTCTGAAACATACTGGCAAATTGGAATACAAGGATTCTCAGTTAACGGACAAGCTACTGGATGGTGTAGC
Schistosoma japonicum aspartic protease precursor m... (715) ---CCTC-ACAGAAAAGTCCTATTGGTTATTCAAAATGGACAATTTGACTATCTCAGATTTATCCA-----TT-TGTACG
Xenopus Pepsinogen (731) ---CTTA-ACTGCTGAAACCTACTGGCAAATTACTCTTGACAGCGTATCAATAAATGGGCAGGTTA-TTGCT--TGCAGT
Consensus (1547) TGT ACTG AAGGG TACTGGCAAATTAC TGGACAGCATCAC AT GACGG AGGCCA T G T TGTAGC
(1627) 1627 1640 1650 1660 1670 1680 1690 1706
ProtB (687) ACTGGCCGCACCGCCATTCTTGACACTGGAACAACCCTCCTCGTCGTCCCCGCAGCCGATGCCGTAACGTTGCACAAACA
Amanita muscaria protease (943) TCAGTTTGAAA--CCATTATCGATTTTGGCACAACTGTTATTTACGGGCCCGCTGACGGTGTCCAGCAACTTTACGGGTC
Sc vac.ProteinaseA precursor. (1592) CATGG-TGCC--GCCAT---CGATACTGGTACTTCTTTGATTACCTTGCCATCAGGATTAGCTGAAATGATTAATGCTGA
Aedes aegypti lysosomal aspartic protease mRNA seq... (908) AATGGATGCGAAGCAATTGCCGATACCGGCACCAGCTTGATTGCCGGCCCAGTGTCGGAGGTCACCGCTATCAACAAGGC
Rattus norvegicus prochymosin (LOC56825), mRNA. (809) GGTGGCTGCCCTGCTGTTCTGGACACAGGCACTGCCCTGCTGACAGGGCCTGGTAGAGACATCCTCAATATTCAGCACGC
Camelus dromedarius mRNA for pepsin. (842) AGTGGCTGCCAGGCCATTGTTGACACCGGCACCTCTCTGCTGGCCGGCCCAACCGACGCCATTTCTAACATCCAGAGCTA
Pig pepsinogen A mRNA, complete cds. (835) GGTGGCTGCCAGGCCATTGTGGATACGGGCACCTCTCTGCTGACCGGCCCCACCAGTGCCATTGCCAACATCCAGAGCGA
Rana catesbeiana pepsinogen mRNA, complete cds. (807) CAAGGATGTCAGGGTATTGTGGATACTGGTACTTCTCTGTTGACCGCTCCTCAATCAGTCTTCTCCTCCCTCATGCAGTC
Schistosoma japonicum aspartic protease precursor m... (785) GATGGATGCCAGGCAATAGCTGATACTGGTACTTCAATGATCGCCGGTCCAACTGACGAAGTAAAGCAGATTAATCAGAA
Xenopus Pepsinogen (804) CAATCCTGCCAGGCCATTGTAGATACTGGAACCTCTCTGATGACTGGCCCCTCTACTCCAATTGCTAACATCCAGAACTA
Consensus (1627) ATGGCTGCCAGGCCATTGT GATACTGGCAC TCTCTGATGACCGGCCC C G G GTC C AA ATCCAGAA A


(1707) 1707 1720 1730 1740 1750 1760 1770 1786
ProtB (767) GATCCAAGGCGCCCAG---TCTGACGGACAGGGTGGGTTTACCATACCCTGCACGACTAA--TGCC-AGCGTCGCGTTGA
Amanita muscaria protease (1021) AGTACCTGGAGCTACTTTGTTTGATTCATCGAACGGGTTTTATTCGTTCCCGTGTGACTCTGTTCCCTCTGTTTCGTTCA
Sc vac.ProteinaseA precursor. (1666) AATTGGGGCCAAGAAGGGTTGGACCGGTCAATATACTCTAGACTGTAACACCAGAGACAATCTACCTGATCTAATTTTCA
Aedes aegypti lysosomal aspartic protease mRNA seq... (988) TATCGGTGGCACTCCTATTATGAACGGAGAATACATGGTTGACTGCTCGTTGATTCCCAAACTGCCAAAGATCTCATTCG
Rattus norvegicus prochymosin (LOC56825), mRNA. (889) CATTGGAGCTGTGCAGGGCCAACATGACCAGTTTGACATTGACTGCTGGAGGCTGAACTTCATGCCCACGGTTGTCTTTG
Camelus dromedarius mRNA for pepsin. (922) CATCGGAGCCAGTGAGGACTCATACGGTGATATGGTGGTCAGCTGCTCCTCCATCAGCAGCCTGCCCAACATCGTCTTCA
Pig pepsinogen A mRNA, complete cds. (915) CATCGGAGCCAGCGAGAACTCATATGGCGAGATGGTGATCAGCTGCTCCTCCATCGACAGCCTGCCTGACATCGTCTTCA
Rana catesbeiana pepsinogen mRNA, complete cds. (887) CATTGGTGCTCAACAAGATCAGAATGGACAGTATGCTGTCAGCTGTAGCAACATCCAGAGTCTTCCCACCATCAGTTTTA
Schistosoma japonicum aspartic protease precursor m... (865) GCTCGGTGCTACCCATCTACCTGGTGGAATTTATACCGTTTCTTGTGACGTTATAAATAATCTTCCATCAATTGATTTTG
Xenopus Pepsinogen (884) CATTGGAGCAAGTCAGGACTCTAATGGACAGTATGTGATCAACTGCAATAACATCAGCAACATGCCAACTATTGTCTTTA
Consensus (1707) CATCGGAGCCA CAGG TC ATGGACAGTATG G TTAACTGCT C CAT AACAA CTGCC AC ATCGT TTCA
(1787) 1787 1800 1810 1820 1830 1840 1850 1866
ProtB (841) CATTCGGCGGACAAGAGTTCACTATCGATCCGAGGGATATGGCGTTCCAGCCTGTGGATCCCAATAACCCCCAAGGCGAC
Amanita muscaria protease (1101) ACTGGGGGTGGTCAGGATTGGCCTATTACATCG-----GACAACTTCAATATGGGAGAAAC--------GACAAAGGGATT
Sc vac.ProteinaseA precursor. (1746) ACTTCAATGGCTACAACTTCACTATTGGGCCA-----T-ACGATTACACGCTTGAA----------GTTTCAGGCTCC
Aedes aegypti lysosomal aspartic protease mRNA seq... (1068) TTTTGGGAGGAAAATCATTCGATCTCGAAGG------TGCTGATTACGTACTGCGTGTGGCTCAAATGGGTAAAACCATC
Rattus norvegicus prochymosin (LOC56825), mRNA. (969) AGATCAATGGTAGGGAGTTCCCACTGCCACCC-----T-CTGCCTATACCAACCAG----------TTCCAGGGCTCC
Camelus dromedarius mRNA for pepsin. (1002) CCATCAACGGCGTCCAGTACCCTCTGTCCCCC-----A-GTGCCTACATCCTGGAG----------AGCGACGACAGC
Pig pepsinogen A mRNA, complete cds. (995) CCATCAACGGTGTCCAGTACCCTCTGAGCCCC-----A-GTGCCTACATCCTGCAG----------GACGATGACAGC
Rana catesbeiana pepsinogen mRNA, complete cds. (967) CCATAAGTGGAGTTTCTTTCCCACTTCCACCC-----T-CTGCCTATGTACTTCAGCAA---------AACAGTGGCTAC
Schistosoma japonicum aspartic protease precursor m... (945) TAATAAATGGGAAA-CATATGACCTTAGAGCC-----TACAGATTACATCATGAAGGTATCTAAATTGGGTTCTGAAATT
Xenopus Pepsinogen (964) CTATCAATGGTGTCCAATATCCATTGTCACCC-----T-CTGCATATGTGCGCCAG----------AATCAACAAGGC
Consensus (1787) CCATCAATGG A A TTCCCTCT CACCC T CTGC TACAT CTGCAG G C AAGGCA C
(1867) 1867 1880 1890 1900 1910 1920 1930 1946
ProtB (921) TGTATCTCTGGAATC--ACTTCTGGATCGGTTGGCGGGGCAA--AGG-------AGTGGCTTGTTGGAGATGTTTCCTC
Amanita muscaria protease (1168) TGG-TCAATGGGTTGGCGCTATCGCTGCTCAAAATTTTGGTTTAGGCAGTAAAGTTTGGGTTTTTGGCGACAGCTTTATT
Sc vac.ProteinaseA precursor. (1808) TGTATCTCTGCAATTACACCAATGGATTTCCCAGAACCTGTT--GG--CCCA-CTGGCCATCGTTGGTGATGCCTTCTTG
Aedes aegypti lysosomal aspartic protease mRNA seq... (1142) TGCCTGTCTGGGTTCATGGGAATCGATATTCCACCGCCTAAT--GG--ACCG-TTGTGGATTTTGGGAGACGTTTTCATT
Rattus norvegicus prochymosin (LOC56825), mRNA. (1031) TGCTCCAGTGGCTTC-AGGCATGG---CTCCC----------AGA---TGTGGATCCTTGGAGATGTGTTCATT
Camelus dromedarius mRNA for pepsin. (1064) TGCACCAGTGGCTTCGAGGGCATGGACCTCTCCAGCTCC-----GAAGAGC---TCTGGATCCTGGGTGACGTCTTCATC
Pig pepsinogen A mRNA, complete cds. (1057) TGCACCAGCGGCTTCGAGGGCATGGATGTCCCCACCTCCTCC--GGAGAGC---TCTGGATCCTGGGTGACGTCTTCATC
Rana catesbeiana pepsinogen mRNA, complete cds. (1032) TGCACCATTGGCATCATGCCTACTTACCTGCCTTCCCAGAAT--GGACAGCCTCTCTGGATCCTGGGTGATGTCTTCCTC
Schistosoma japonicum aspartic protease precursor m... (1019) TGTCTAACCGGTTTTATTGGTATGGATTTGCCGAGGAAGAAA----------TTATGGATATTAGGGGATGTATTCATT
Xenopus Pepsinogen (1026) TGTTCAAGCGGATTCCAGGCAATGAACCTCCCCACCAATTCA--GGGGATT---TGTGGATCCTGGGAGATGTATTTATC
Consensus (1867) TGCATCA TGG TTC GG AATGGATCTCCC AC GG A C TGTGGATCCTGGG GATGTCTTCATC



Alignement of the putative *Hebeloma cylindrosporum* proteinase *Hc*ProtB.gene with different protease gene: *Amanita muscaria* prot1 gene for protease (AJ295271). *S.cerevisiae* vacuolar ProteinaseA precursor (PEP4) gene (M13358), *Aedes aegypti* lysosomal aspartic protease, mRNA (M95187), *Camelus dromedarius* pepsin, mRNA (AJ131678), Pig pepsinogen A mRNA, complete cds (J04601), *Rana catesbeiana* pepsinogen mRNA, complete cds (M73750), *Rattus norvegicus* prochymosin, mRNA (NM_020091), *Schistosoma japonicum* aspartic protease precursor mRNA partial (U90750), *Xenopus laevis* mRNA for pepsinogen A, complete cds (AB045380). The alignment was performed with the program ClustalW (Thompson *et al*., 1994).

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