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Phytochemistry 68 (2007) 41–51
PHYTOCHEMISTRY
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Review

Nitrogen transport in the ectomycorrhiza association: The *Hebeloma cylindrosporum–Pinus pinaster* model

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Received 28 April 2006; received in revised form 26 June 2006
Available online 2 November 2006

Abstract

The function of the ectomycorrhizal mutualism depends on the ability of the fungal symbionts to take up nutrients (particularly nitrogen) available in inorganic and/or organic form in the soil and to translocate them (or their metabolites) to the symbiotic roots. A better understanding of the molecular mechanisms underlying nutrient exchanges between fungus and plant at the symbiotic interface is necessary to fully understand the function of the mycorrhizal symbioses. The present review reports the characterization of several genes putatively involved in nitrogen uptake and transfer in the *Hebeloma cylindrosporum–Pinus pinaster* ectomycorrhizal association. Study of this model system will further clarify the symbiotic nutrient exchange which plays a major role in plant nutrition as well as in resistance of plants against pathogens, heavy metals, drought stress, etc. Ultimately, ecological balance is maintained and/or improved with the help of symbiotic associations, and therefore, warrant further understanding.
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Keywords: *Hebeloma cylindrosporum*; *Pinus pinaster*; Amino acids; Ammonium; Ectomycorrhiza; Nitrate; Nitrogen; Peptides; Transport; Transporters

Contents

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doi:10.1016/j.phytochem.2006.09.021

1. Introduction

Ectomycorrhizal fungi have evolved in N limiting ecosystems (Read and Perez-Moreno, 2003), and can utilize a range of both inorganic and organic N sources including ammonium, nitrate, amino acids, di-tripeptides, proteins and secondary metabolites (Smith and Read, 1997). The ability to use different N forms varies for each species (Smith and Read, 1997), and the ability to use organic N can vary within a species between strains (Finlay et al., 1992; Keller, 1996; Anderson et al., 1999; Rangel-Castro et al., 2002; Guidot et al., 2005). It is important to study N transporters since N translocation from the soil through the fungus and to the plant is a defining characteristic of this mutualism. In order to have a full picture of functioning of this mutualism we need to understand what forms of N are being taken up by the fungus and transferred to the plant, how they are being transported, and lastly what regulates this uptake and transport system.

There are three membrane barriers that N must pass through before it can be assimilated into the plant: the soil/fungus membrane, the fungus/apoplast membrane, and the apoplast/plant root membrane (Chalot et al., 2002). The current working model is that inorganic N is taken up into the fungal hyphae and converted to an amino acid, either as glutamine or alanine (Smith and Read, 1997) and transferred to the plant. However, recent studies have suggested that ammonium may also be transferred from the fungus to the plant in ectomycorrhizae (Selle et al., 2005) as well as in arbuscular mycorrhizae (Jin et al., 2005). This translocation of ammonia has been recently discussed (Chalot et al., 2006). Only when all N transporters are identified, will we be able to determine how and in what forms N is preferentially taken up from the soil and transferred to the plant symbiont.

Hebeloma cylindrosporum is a well-studied ectomycorrhizal fungus (Marmeisse et al., 2004), and it has been one of a few model ectomycorrhizal species chosen for in-depth genetic analysis (Wipf et al., 2003; Lambilliotte et al., 2004). *H. cylindrosporum* is a pioneer species found throughout Europe that prefers sandy soils of coastal sand dune ecosystems (Gryta et al., 1997; Marmeisse et al., 2004). The life cycle of *H. cylindrosporum* includes multiallelic mating types where clamp connections between haploid mycelia form dikaryotic mycelia (Debaud et al., 1986), and it is only the dikaryotic mycelia that form mycorrhizas and sporocarps (Fig. 1). *H. cylindrosporum* can utilize both inorganic and organic N sources (Plassard et al., 2000; Wipf et al., 2002a; Guidot et al., 2005; Benjdia et al., 2006) and can form fruiting bodies *in vitro*, allowing controlled experiments to understand life cycle regulation (Debaud and Gay, 1987), which are desirable characteristics for a model species. Also, its mutualistic partner, *Pinus pinaster*, and closely related *Pinus taeda* have over 11,500 sequenced ESTs between the two of them (Marmeisse et al., 2004), creating a situation where genes from both partners in the mutualism can be identified and the interactions of their products studied.

One method to discover new transporter genes is yeast complementation. Fischer et al. (1998) used this method to identify 13 *Arabidopsis* amino acid transporters, belonging to two super-genefamilies. Wipf et al. (2003) and Lambilliotte et al. (2004) created a cDNA library for *H. cylindrosporum* and includes sequencing of ESTs and use of yeast complementation to identify new genes. Radio labelling of substrates can further be used to study uptake capacities of transporters found and cloned into yeast (Wipf et al., 2002a; Benjdia, 2004). Once N transporters are cloned, it is possible to create GFP fusion proteins to study the localization of their expression. (Mueller et al., 2006) recently demonstrated that GPF fusion proteins can be transformed into *H. cylindrosporum* and their expression visualized.

2. Inorganic nitrogen uptake by *H. cylindrosporum*

2.1. Isolation of ammonium transporters from H. cylindrosporum: HcAMT1, 2 and 3

Ammonium mobilization by hyphae from soil sources is directly linked to hyphae uptake capacities. Using [^{14}C]-methylamine as an analog of ammonium, kinetics of ammonium/methylammonium transport in ectomycorrhizal fungi have been characterized (Jongbloed et al., 1991; Javelle et al., 1999). A saturable uptake was obtained, which conformed to simple Michaelis–Menten kinetics, and was consistent with carrier-mediated transport. Three ammonium transporters, *HcAmt*1, *HcAmt*2 and *HcAmt*3 (*Am*monium *t*ransporter) were further cloned in *H. cylindrosporum* (Javelle et al., 2001, 2003a) and their functional expression in the yeast strain 31019b, *mep1*Δ*mep2*Δ*mep3*Δ

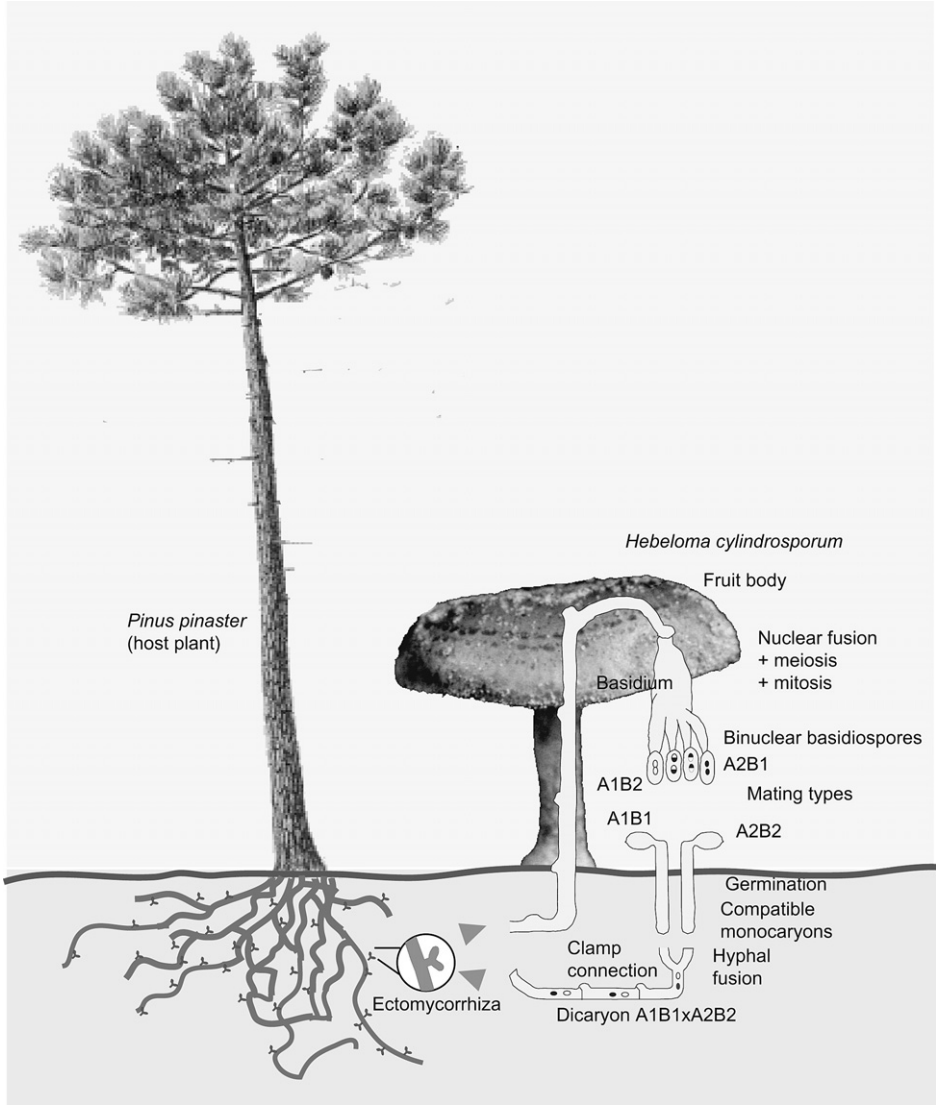

Fig. 1. Life cycle of *Hebeloma cylindrosporum* (modified from Debaud et al. (1997) with permission).

resulted in complementation of growth defect in the presence of less than 1 mM ammonium as sole nitrogen source. Thus, HcAMTs cDNA encode functional NH_4^+ transporters. HcAmt3, Mep1 and Mep3 belong to the low affinity ammonium transporter family whilst HcAmt1, HcAmt2, Mep2, UmMep1 and CaAmt2 belong to the high affinity ammonium transporter and sensor family (TC 2A.49.3.2).

We hypothesize that ammonium transporters belonging to the latter family could complement the pseudohyphal growth defect of a yeast strain defective in the *MEP2* gene and tested this using the three HcAmts from the ectomycorrhizal fungus *Hebeloma cylindrosporum*. In experiments with a short-term incubation (4 days), we found that HcAmt1, and somewhat less effectively HcAmt2, were able to complement the pseudohyphal deficiency.

The high affinity ammonium transporter gene *AMT1* of *H. cylindrosporum* is expressed only under N-deficient conditions. Its transcription is strongly repressed by glutamine and therefore this gene is subject to nitrogen repression in *H. cylindrosporum*. By contrast, the low affinity ammonium transporter gene *AMT3* is highly expressed but not highly regulated. We therefore propose that the high affinity ammonium transporters from mycorrhizal fungi might sense the environment and induce, via yet unidentified signal transduction cascades, a switch of the fungal growth mode observed during mycorrhiza formation. Further progress in identifying the components of this pathway will be advanced by genetic approaches.

2.2. Isolation of nitrate assimilation genes from *H. cylindrosporum*

It has been shown that ectomycorrhizal fungi are able to utilize NO_3^- and, for a few species, it is capable of promoting better growth than ammonium (Scheromm et al., 1990). Further, it has been shown that *H. cylindrosporum*

affects nitrate nutrition of its natural host plant, *P. pinaster* (Plassard et al., 2000, 2002). In *H. cylindrosporum*, three structural genes coding for the nitrate assimilation pathway have been cloned. They code for a nitrate transporter (*HcNrt2*) and the nitrate (*HcNR*) and nitrite reductase (*HcNIR*) (Jargeat et al., 2000, 2003). This indicates that clustering of genes participating to a common metabolic pathway, which is well known for ascomycetes (Keller and Hohn, 1997) is also probably widespread among the basidiomycetes. The nitrate transporter polypeptide (NRT2) is characterised by 12 transmembrane domains and presents both a long putative intracellular loop and a short C-terminal tail, two structural features which distinguish fungal high-affinity transporters from their plant homologues (Jargeat et al., 2003). Interestingly, transcriptional regulation of the three nitrate assimilation genes in *H. cylindrosporum* is under ammonium repression but does not need nitrate for induction.

3. Organic nitrogen uptake by *H. cylindrosporum*

Amino acid transporters have been characterized in detail in animals, plants, and yeasts (Fischer et al., 1998; Van Belle and Andre, 2001; Williams and Miller, 2001; Wipf et al., 2002b). On the basis of physiological studies, the existence of a large number of transporters has been postulated differing in their substrates, tissue specificity, and transport mechanism (i.e., the ions used in co-transport). In *Saccharomyces cerevisiae* amino acids are taken up by a set of 24 secondary active influx systems (Van Belle and Andre, 2001; Wipf et al., 2002b). All 24 members contain 12 putative membrane-spanning domains and have been characterised functionally; for example CAN1 functions as a proton-arginine symporter (Opekarová et al., 1993). APC family members are not highly specific but transport several related, in some cases even a wide spectrum of structurally different (including D-isomers) amino acids (Wipf et al., 2002b). The ability of ectomycorrhizal fungi to take up amino acids was previously described (Abuzinadah and Read, 1988; Chalot and Brun, 1998; Näsholm et al., 1998) and Nehls et al. (1999) isolated the first amino acid transporter from *Amanita muscaria* with an EST project.

3.1. Isolation of an amino acid transporter from *H. cylindrosporum*: *HcGAP1*

Wipf et al. (2002a) investigated the ability of *H. cylindrosporum* to use amino acids as single nitrogen source, and found that on glutamine and asparagine *Hebeloma* mycelia grew better than on an ammonium control. Further, growth, measured as dry weight of hyphae, was comparable to that on ammonium for glutamine, aspartate, alanine and valine. These findings show dominant soil amino acids, such as glutamine, glutamate and alanine (Abuzinadah and Read, 1988), are readily assimilated by *H. cylindrosporum*.

HcGAP1, a gene coding for an amino acid transporter was isolated by functional complementation of a yeast amino acid uptake mutant (strain JT16; Tanaka and Fink, 1985). The *HcGAP1* cDNA (AF521906) is 1785 bp long and codes for a 594-amino acid protein, and has the amino acid permease conserved domain (RPS-BLAST 2.2.1 (August-1-2001)). The calculated mass is 65.7 kDa and TMHMM transmembrane domain predictions (http://www.cbs.dtu.dk/services/TMHMM/) suggest that the protein has 12 transmembrane domains with both ends protruding in the cytoplasm. Phylogenetic analyses by maximum of parsimony (Fig. 2) illustrated homologies to other fungal amino acid transporters like UfAAT1p (*Uromyces fabae*, amino acid transporter AAT1p) (Hahn and Mendgen, 1997) and AmAAP1 (*Amanita muscaria*, general amino acid permease 1) (Nehls et al., 1999). The cDNA also showed homology to the APC family in yeast mediating H^+-coupled amino acid uptake, which could be correlated with the [^{14}C]-aspartate uptake results (see below) and indicate a similar mechanism for the HcGAP1 mediated transport.

[^{14}C]-labeled aspartate uptake studies resulted in a 150 μM K_m-value for the transport for aspartate which is within the range of amino acid concentrations found in the soil (Scheller, 1996), making it very likely that HcGAP1 is involved in soil amino acid uptake. HcGAP1 activity was strictly pH-dependent with an optimum at approximately pH 4 (Table 1), which is consistent with the pH optimum described for the uptake of glutamate and glutamine by mycelia of the ectomycorrhizal fungus *Paxillus involutus* (Chalot et al., 1995). A strong dependence on the presence of glucose and a proton gradient indicates that HcGAP1 transport is mediated by a secondary active transport mechanism similar to its yeast homologs (Opekarová et al., 1993). All 20 proteogenic amino acids bind to HcGAP1 as revealed by competition studies (Wipf et al., 2002a) indicating a putative broad substrate spectrum, which is the same as its yeast homolog ScGAP1.

3.2. Expression of *HcGAP1*

HcGAP1 is expressed in mycelia grown on a standard medium. However, no transcripts could be detected in mycorrhiza, as the expression of HcGAP1 to take up amino acids from plant cells would be counterproductive. The results suggest that HcGAP1 plays a role in the uptake of amino acids from the soil for fungal nutrition. In free living mycelia *HcGAP1* expression pattern is similar to the one described for the di- and tripeptide transporter *HcPTR2A* (see 3.3), indicating that *HcGAP1* regulation involves mechanisms able to sense both extra- and intracellular nitrogen source availability in order to rapidly adapt to the environmental conditions (Benjdia et al., 2006).

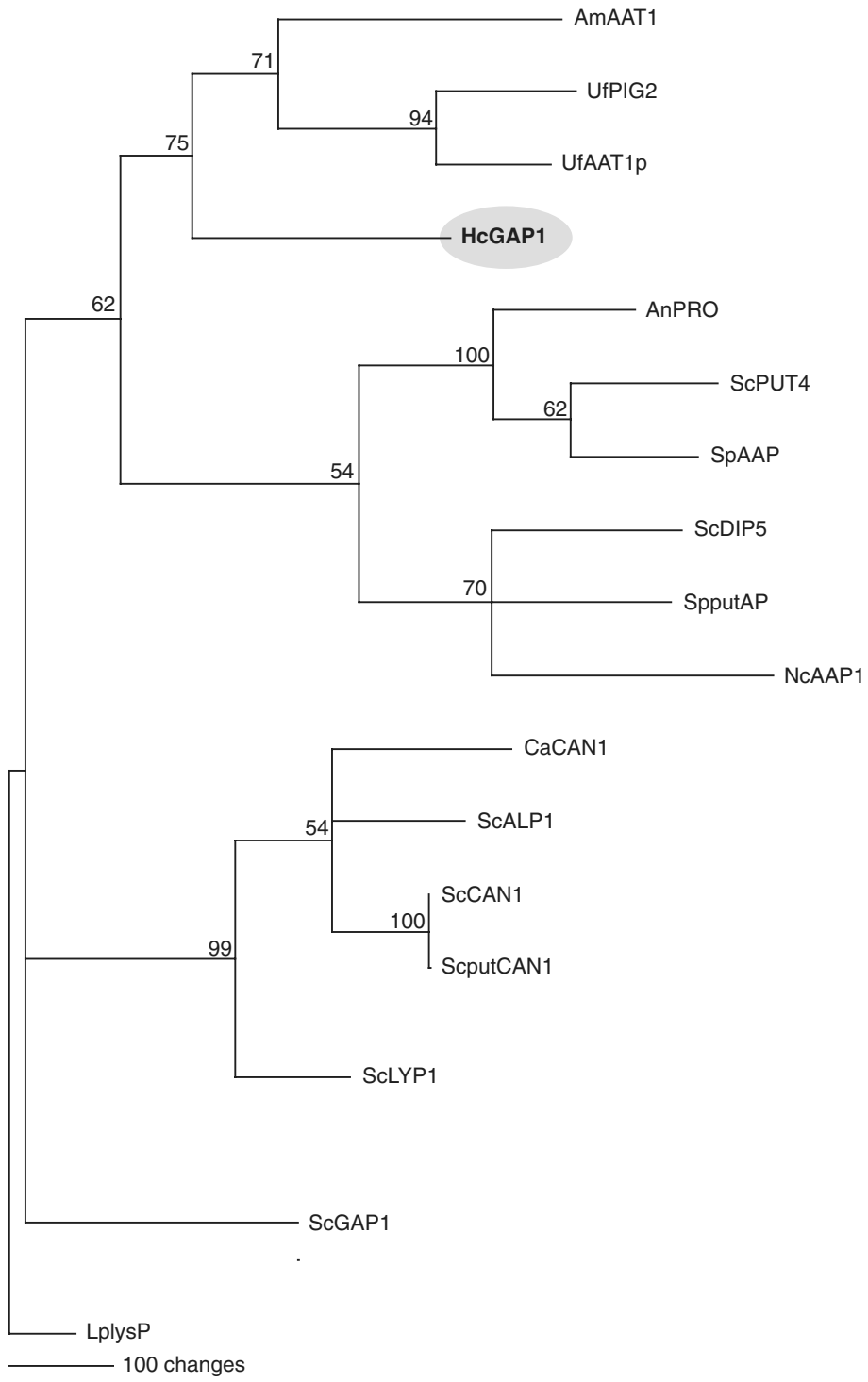

Fig. 2. Phylogenetic analyses of a multiple alignment of the deduced protein sequence of HcGAP1 and other fungal amino acid permeases (Am = *Amanita muscaria*; An = *Aspergillus nidulans*; Ca = *Candida albicans*; Hc = *Hebeloma cylindrosporum*; Lp = *Lactobacillus plantarum*; Nc = *Neurospora crassa*; Sc = *Saccharomyces cerevisiae*; Sp = *Schizosaccharomyces pombe*; Uf = *Uromyces fabae*). Maximum parsimony analysis were performed using PAUP 4.0b10 (Swofford, 1998). Numbers indicate bootstrap values (in %) out of 1000. The complete alignment was based on 665 sites; 546 were phylogenetically informative. The distantly related *Lactobacillus plantarum* lysine transport protein was defined as outgroup.

3.3. Isolation of di- and tripeptide transporters from H. cylindrosporum: HcPTR2A and 2B

Known peptide transporters fall into three families (Stacey et al., 2002): the ATP binding cassette family ABC transporters; (Higgins, 1992), the oligopeptide transporter family, OPT; (Lubkowitz et al., 1997; Hauser et al., 2001), and the peptide transporter (PTR) or *P*roton-coupled *O*ligopeptide *T*ransporter family (POT) transporting di- and tripeptides, and also includes nitrate transporters

Table 1
Properties of the different nitrogen compound transporters isolated from *Hebeloma cylindrosporum*

Gene	Acc. no	K_m (μM)	V_{max}	TMD	Source	pH opt.	References
GAP1	AF521906	150 μM Asp at pH 4.5	0.59 nmol Asp/mg protein/min	12	Yeast functional complementation	~4	Wipf et al. (2002)
PTR2A	DQ078993	1.5 μM LeuLeu at pH 5	0.24 nmol LeuLeu/mg protein/min	10–12	Yeast functional complementation	5–5.5	Benjdia et al. (2006)
PTR2B	DQ078994	~100–250 μM LeuLeu at pH 5	n.d.	10–12	Yeast functional complementation	n.d.	Benjdia et al. (2006)
OPT1	n.d.	n.d.	n.d.	14–15	EST Montpellier	n.d.	–
AMT1	AY094982	91 μM methylamine	101 nmol methylamine/mg protein/min	11	Yeast functional complementation & PCR	n.d.	Javelle et al. (2003)
AMT2	AAk82416	54 μM methylamine	71 nmol methylamine/mg protein/min	11	Yeast functional complementation & PCR	n.d.	Javelle et al. (2001)
AMT3	AAK82417	290 μM methylamine	71 nmol methylamine/mg protein/min	11	Yeast functional complementation and PCR	n.d.	Javelle et al. (2001)
NRT2	CAB60009	n.d.	n.d.	12	Gene-walking on genomic DNA library	n.d.	Jargeat et al. (2003)

Acc. no = GenBank accession number, TMD = number of predicted transmembrane domains, pH opt. = pH optimum, n.d. = not determined.

(Steiner et al., 1995; Paulsen and Saier, 1997). In yeast, the transport of di- and tripeptides involves three genes *PTR1*, *PTR2* and *PTR3* (Island et al., 1991; Perry et al., 1994; Barnes et al., 1998). *ScPTR2* is an integral membrane protein which can transport peptides, while *ScPTR1* and *ScPTR3* are cytosolic regulators of peptide transport (Alagramam et al., 1995; Barnes et al., 1998).

Uptake experiments with [^{3}H]-LeuLeu demonstrated the ability of *H. cylindrosporum* to take up small peptides, which led us to investigate the molecular basis of peptide transport. A yeast mutant deficient in peptide uptake (strain LR2; Rentsch et al., 1995) was transformed with a *H. cylindrosporum* cDNA library (Lambilliotte et al., 2004).

Two cDNAs with strong homology to other fungal peptide transporter genes (Fig. 3) were identified and were named *H. cylindrosporum* peptide transporter 2A and 2B (*HcPTR2A* and *HcPTR2B*). The *HcPTR2A* and *HcPTR2B* cDNAs are 1770 bp and 1806 bp long, and encode 590 and 602 amino acid proteins with a calculated molecular mass of 65.2 and 65.9 kDa, respectively. The HcPTR2A sequence includes the PTR2-signature 1 conserved domain (YmyFYLlINIGAL) and HcPTR2B includes the PTR2-signature 2 conserved domain (GGILADtMWGrykTImifSiVcliG). Phylogenetic analysis of protein sequences of the PTR-family from animals, plants, yeast and bacteria underlined the fungal origin of the isolated genes, as they were located in a cluster comprising only fungal PTRs (Fig. 3).

Transport properties of HcPTR2A were determined by radiotracer uptake studies with [^{3}H]-labeled LeuLeu (Benjdia et al., 2006). The K_m-value for LeuLeu transport was about 1.5 μM at pH 5, which is much lower than those previously found for VfPTR1, a peptide transporter from *Vicia faba* (20 mM) (Delrot et al., 2001). The HcPTR2A activity was pH-dependent with an optimum at around pH 5 (Table 1). [^{3}H]-LeuLeu uptake was dependent on the presence of glucose and was sensitive to the protonophores 2,4 DNP and CCCP, indicating a proton co-transport mechanism, similar to its yeast homologs (Perry et al., 1994). Competition studies showed that HcPTR2A binds di- and tripeptides. The uptake rates observed when expressing HcPTR2B in yeast were too low to determine kinetic parameters. Preliminary results indicate that the kinetic parameters could be determined by heterologous expression in *Xenopus laevis* oocytes (Müller et al., unpublished results).

3.4. Expression of HcPTR2A and B

Fungal colonies were grown for 10 days on rich media (YMG), then transferred for 12 h to minimal media without N, and finally grown on minimal media containing different N sources. Transcript levels for *HcPTR2A* and *HcPTR2B* and the intracellular concentration of amino acids and ammonium content in the medium were investigated (Benjdia et al., 2006). *HcPTR2B* is constitutively expressed and is independent of the N-source and the

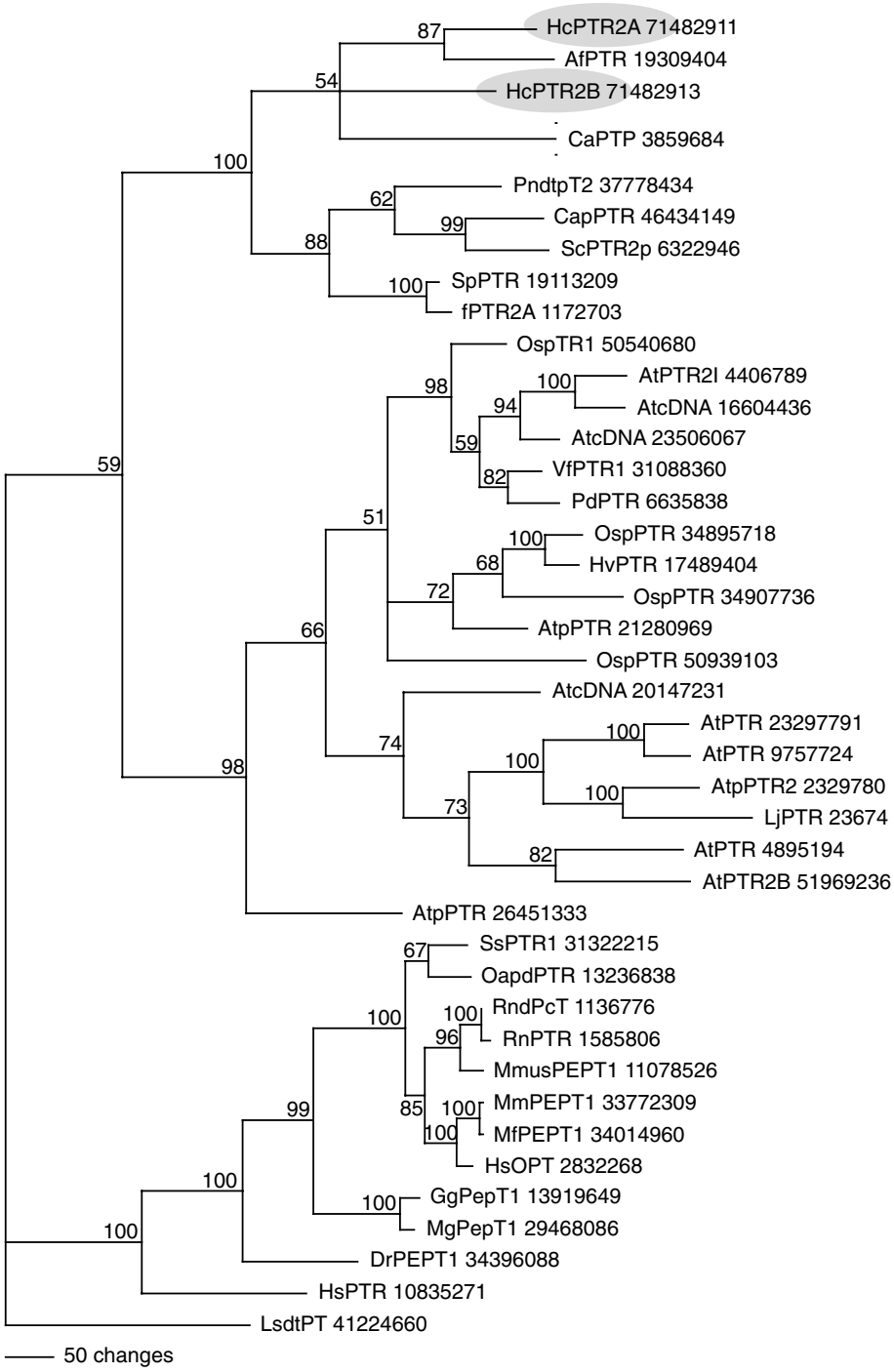

Fig. 3. Phylogenetic tree of peptide transporters from the PTR family. Maximum parsimony analysis were performed using PAUP 4.0b10 (Swofford, 1998). Numbers indicate bootstrap values (in %) out of 1000. The complete alignment was based on 798 sites; 658 were phylogenetically informative. Numbers behind organisms are according to the GI accession at NCBI. (Af = *Aspergillus fumigatus*; At = *Arabidopsis thaliana*; Ca = *Candida albicans*; Dr = *Danio rerio*; f = *fungal*; Gg = *Gallus gallus*; Hc = *Hebeloma cylindrosporum*; Hs = *Homo sapiens*; Hv = *Hordeum vulgare*; Lj = *Lotus japonicus*; Ls = *Lactobacillus sakei*; Mf = *Macaca fascicularis*; Mg = *Meleagris gallopavo*; Mm = *Macaca mulatta*; Mmus = *Mus musculus*; Oa = *Ovis aries*; Os = *Oryza sativa*; Pd = *Prunus dulcis*; Pn = *Phaeosphaeria nodorum*; Rn = *Rattus norvegicus*; Sc = *Saccharomyces cerevisiae*; Sp = *Schizosaccharomyces pombe*; Ss = *Sus scrofa*; Vf = *Vicia faba*). The transporters are shortened like following: OPT = oligopeptide transporter; PTR, PTP or PEPT = peptide transporter; pPTR = putative peptide transporter; dtPT = di/tripeptide transporter; dPcT = proton-coupled dipeptide cotransporter; pTR = putative transport protein; pdPTR = putative proton-dependent peptide transporter. All names and following numbers are the originally at NCBI published transporter names. The distantly related *Lactobacillus sakei* di/tripeptide transporter was defined as outgroup.

incubation time. In contrast, *HcPTR2A* is strongly expressed during N deficient conditions or in the presence of a secondary N source. The observed *HcPTR2A* expression pattern suggests that peptide uptake in *H. cylindrosporum* is regulated by mechanisms sensing both extra- and intracellular nitrogen source (Benjdia et al., 2006).

Taken together this suggests that HcPTR2A and 2B must have different roles in *H. cylindrosporum* peptide acquisition from the soil. We propose that HcPTR2B is involved in the constitutive uptake of peptides whereas HcPTR2A is responsible for peptide uptake under stress conditions, e.g., nitrogen deficiency.

3.5. Isolation of an oligopeptide transporter from H. cylindrosporum: HcOPT1

An oligopeptide nitrogen transporter, HcOPT1, has also been isolated from *H. cylindrosporum*. OPT members transport peptides with a length of at least four to five amino acid residues. HcOPT1 was isolated by RACE–PCR based on an EST from the *H. cylindrosporum* library constructed by Lambilliotte et al. (2004). HcOPT1 shows similarities to previously isolated plant and fungal OPTs but is not yet characterized.

3.6. Protease excretion by H. cylindrosporum

The ability of several ectomycorrhizal fungi to grow on media containing proteins as sole nitrogen source (Bajwa et al., 1985; Abuzinadah and Read, 1986) in correlation with an production of extracellular protease (Leake and Read, 1990; Botton and Chalot, 1991) has been reported. Intermediate products of protein breakdown such as small peptides could also be directly taken up by fungal cells. In addition to the uptake of amino acids and peptides, *H. cylindrosporum* was also able to excrete proteases in the nutrient medium in the presence of BSA or ammonium (Fig. 4). Under stress conditions (i.e., nitrogen starvation) *H. cylindrosporum* was also excreting proteases in the medium (Fig. 4). By analysing an EST library, two sequences were found that could correspond to excreted aspartic proteases. On BSA and ammonium two protease activity bands were observed, which could be related to the two bands previously described for the ectomycorrhizal fungus *Amanita muscaria* (Nehls et al., 2001). Expression studies using one of these fragments led to the same expression pattern as those observed for HcGAP1 and HcPTR2A indicating a putative involvement in stress (nitrogen deficiency) response (Müller, unpublished results).

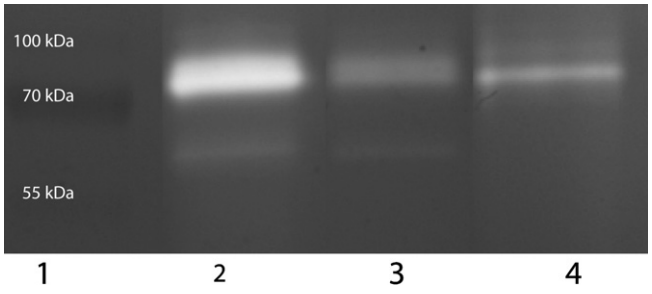

Fig. 4. Protease excretion by *H. cylindrosporum*. Excreted protease activity was analysed by zymogram SDS–PAGE according to Kleiner and Stetler-Stevendon (1994). Lane 1 – PageRuler™ Prestained Protein Ladder (Fermentas), Lane 2 – *H. cylindrosporum* grown for 5 days on BSA, Lane 3 – *H. cylindrosporum* grown for 5 days on ammonium as single N source, Lane 4 – *H. cylindrosporum* grown for 24 h under N starvation.

4. Nitrogen transfer in the ectomycorrhizal association

4.1. Nitrogen export from H. cylindrosporum

The most difficult mechanism to perceive will be the release of nitrogen into the apoplast by the fungus.

The processes involved in the further transfer of N within the symbiotic tissues are still poorly understood (Fig. 5). The exchange of nutrients between the fungus and the plant requires passage across the fungal plasmalemma, the interfacial matrix and the plant plasmalemma. Indirect evidence from ^{15}N labelling experiments supports the view of amino acid transfer from the fungus to the host (Smith and Read, 1997; Chalot and Brun, 1998; Selle et al., 2005). However a direct transfer of NH_4^+, as hypothesized by Selle et al. (2005) cannot be excluded. This hypothesis has been recently discussed in a review paper (Chalot et al., 2006). A successful ammonia transfer from the fungus to the plant will greatly depend on the lack of NH_4^+ retrieval systems in the plasma membrane of intraradical fungal cells. However, the ammonium retrieval capacity of *H. cylindrosporum* AMTs was clearly demonstrated (Javelle et al., 2003b) and thus suggests that other transport systems are needed at the fungal plasma membrane to sustain large ammonia efflux.

The traditional view hypothesizes that amino acids will be released from fungal cells to the apoplast (Chalot and Brun, 1998). Whether the mechanisms involved are specifically located at the symbiotic interface remains an intriguing question. The lack of yeast mutants deficient in amino acid excretion greatly hampered the study of amino acid excretion in other organisms. Recently Aqr1, an internal membrane transporter involved in excretion of amino acids has been characterized in *Saccharomyces cerevisisiae* (Velasco et al., 2004). ScAqr1 is assumed to be present in the membrane of intracellular vesicles and would act as an amino acid/H^+ antiporter to load the vesicles with amino acids from the cytosol. The amino acids would then be released into the external medium by exocytosis. A similar model has been recently proposed for auxin transport in plants (Baluska et al., 2003). Research in these directions could give hints to the characterization of amino acid exporters in mycorrhizal fungi in the near future (Fig. 5).

However, both hypothesis (inorganic versus organic N transfer) may co-exist, and one major factor to consider is the availability of carbon substrates. Under C depletion, the synthesis of organic N might be strongly down-regulated thus inducing the accumulation of large amounts of free ammonia. Under C sufficiency, one might expect larger incorporation of free ammonia into C skeletons, and

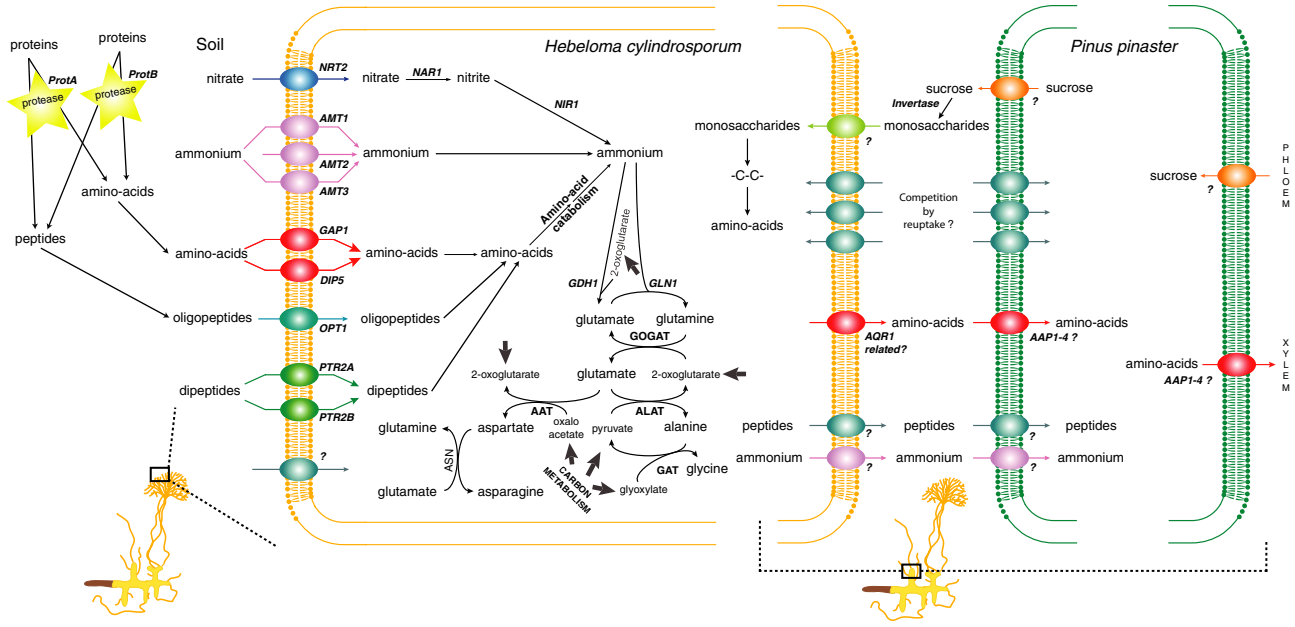

Fig. 5. Current knowledge of N uptake and assimilation pathways in *Hebeloma cylindrosporum* and the *Hebeloma cylindrosporum–Pinus pinaster* association. Enzymes and transport systems for which the corresponding coding genes have been at least partially cloned are indicated in blue. Genes indicated in green have not yet been cloned. AAT, aspartate aminotransferase; ALAT, alanine aminotransferase; ASN, asparagine synthetase; GAT, alanine glyoxylate aminotransferase; GOGAT, glutamate synthase. Enzymes participating to amino acid catabolism include an NAD-specific glutamate dehydrogenase, an arginase and an ornithine carbamoyl transferase. NRT2, NAR1 and NIR1 are, respectively, a nitrate transporter, a nitrate and nitrite reductase (Jargeat et al., 2003); AMT1, 2 and 3, GDH1 and GLN1 are, respectively, three ammonium transporters, a NADP-specific glutamate dehydrogenase and a glutamine synthetase (Javelle et al., 2001, 2003b); GAP1 is a general amino acid permease (Wipf et al., 2002a) and PTR2A and B are two peptide transporters (Benjdia et al., 2006).

hence, larger fluxes of organic N from the fungus to the plant (Chalot et al., 2006).

4.2. Isolation of amino acid transporters from the plant partner, *Pinus pinaster*

By using degenerated primers based on plant amino acid transporters from the AAP (Amino Acid Permeases) family (for review see Wipf et al., 2002b) four amino acid transporters from the plant partner were isolated (PpAAP1 to 4; Olivi et al., unpublished results). The expression and role of these transporters in the mycorrhizal association is currently under investigation.

Little is known about the regulation of nitrogen fungal and plant transporters in the mycorrhizal association. We know that HcGAP1 is undetected in the mycorrhiza (Wipf et al., 2002a), and we hypothesise that this is to minimize the re-uptake of excreted amino acids, assuming that a competition for nitrogen based nutrients exists in mycorrhizal root tips. Moreover, we are interested in expression profile differences between the *P. pinaster* amino acid transporters. Some may only be expressed to load the phloem and xylem, as in *Arabidopsis*, while others may only be expressed in mycorrhizal root tips. All this will be answered with future research.

Recently, we have blasted our known nitrogen transporters against the genome of *Laccaria bicolor*, another ectomycorrhizal fungus, and found evidence for many more nitrogen transporter genes. This suggests that *H. cylindrosporum* has more transporters than currently known to aid in the uptake of organic nitrogen.

5. Concluding remarks

In recent years progresses on nutrient uptake in ectomycorrhizal fungi at a molecular level have been made. Nitrogen uptake and metabolism have been extensively studied and several key genes have been isolated from *H. cylindrosporum*: three ammonium transporters (Javelle et al., 2001, 2003b), a nitrate transporter (Jargeat et al., 2003), an amino acid transporter (Wipf et al., 2002a) and two di-tripeptide transporters (Benjdia et al., 2006), and most recently an oligopeptide transporter (Müller et al., unpublished). In addition to these transporters several N processing genes have also been characterized: a glutamine synthetase and NADP-dependant dehydrogenase (Javelle et al., 2003b) and nitrate and nitrite reductases (Jargeat et al., 2003). Four amino acid transporters have been isolated from the plant partner (*i.e.*, *P. pinaster*). By piecing these proteins together we can begin to create a picture of the localization of transporters and what substrates are transferred at which membrane barriers (Fig. 5). However, the precise function and localization of these putative proteins in mycorrhizal tissues remain unknown, and proteins involved at the apoplastic interface remain to be uncovered. The overall question to be considered is whether the symbiotic nutrient transfer processes are

analogous to processes of partners in the non-mycorrhizal state or whether new transport events are switched on and new genes recruited as a result of interactions between the organisms.

Acknowledgement

This work was supported by grants from Deutsche Forschungsgemeinschaft (Gottfried–Wilhelm–Leibniz; DFG WI1994/2-1 and 2-2).

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