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ORIGINAL PAPER

Regulation of genes involved in nitrogen utilization on different C/N ratios and nitrogen sources in the model ectomycorrhizal fungus *Hebeloma cylindrosporum*

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Abstract Nitrogen (N) utilization by ectomycorrhizal fungi is an essential aspect of their ecosystem function. N deposition changes both the N pools and the carbon/nitrogen (C/N) ratio of the substrates where ectomycorrhizal fungi are found, and it is important to understand how these changes affect the N forms used by ectomycorrhizal fungi. To overcome the difficulties of studying ectomycorrhizal fungi *in situ*, we investigated all known N genes in the model fungus, *Hebeloma cylindrosporum* in a culture study. In addition to studying the regulation of all known N utilization genes, we aimed to understand whether there are gene clusters that undergo similar regulation. Lastly we studied how C/N ratio, N transporter type, and N source affected relative gene expression levels. We grew the D2 strain of *H. cylindrosporum* on a range of inorganic and organic N sources under low, medium, and high C/N ratios. We found three gene clusters that were regulated in a similar pattern. Lastly, we found C/N ratio, N source and N transporter type all affected gene expression levels. Relative expression levels were highest on the high C/N ratio, BSA and diLeucine N sources, and inorganic N transporters were always expressed at higher levels than organic N transporters. These results suggest that inorganic N sources may always the default preference for *H. cylindrosporum*, regardless of both the N sources and the C/N ratio of the substrate.

Keywords Nitrogen transporters · Inorganic nitrogen · Organic nitrogen · Gene expression · Carbon/nitrogen ratio

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Introduction

Ectomycorrhizal fungi are present in boreal and northern temperate forests where nitrogen (N) is the limiting nutrient (Read and Perez-Moreno 2003). A principle function of the ectomycorrhizal mutualism is the transfer of N from the soil to the host plant through the fungus (Smith and Read 2008). Thus, understanding N utilization—including uptake and metabolism—in ectomycorrhizal fungi is important to reveal how this mutualism operates (Buscot et al. 2000; Chalot et al. 2002; Müller et al. 2007). Ectomycorrhizal fungi can utilize a range of inorganic and organic N sources including ammonium, nitrate, amino acids, di- tri-peptides, proteins, and secondary metabolites (Smith and Read 2008), however, ammonium is generally the preferred source of N for fungi because of its low assimilation cost (Salsac et al. 1987). Despite preference for ammonium, ectomycorrhizal fungi have been demonstrated to take up a range of organic N sources (Talbot and Treseder 2010). The recently sequenced genome of *Laccaria bicolor* supports the idea that ectomycorrhizal fungi are able to utilize a variety of N sources as it encodes many inorganic and organic N transporter proteins (Lucic et al. 2008; Martin et al. 2008).

Although much is known about which forms of N ectomycorrhizal fungi prefer out of the range of forms of N they are able to utilize, it remains unclear whether this preference is affected by external environmental factors.

Studying N utilization remains difficult *in situ*, but molecular advancements allow for the direct study of membrane bound N transporter proteins—that determine which sources of N are taken up by the fungi—and N metabolism proteins—that convert forms of N taken up from the environment to a more usable form within the fungus (Müller et al. 2007; Lucic et al. 2008). As the expression of these genes contribute greatly to fungal N utilization (Müller et al. 2007), studying the genetic regulation of N processing genes could provide insight to strategies and mechanisms ectomycorrhizal fungi use when responding to changing environmental conditions *in situ*.

Previous research on model fungal organisms has demonstrated how N source affects regulation of N utilization genes. Work on yeast and non-mycorrhizal filamentous fungi have demonstrated that nitrogen regulation by the fungus is highly dependent on the source of N in the substrate. Nitrogen catabolite control (NCR; yeast) and nitrogen metabolite regulation (NMR; filamentous fungi) are important in N gene regulation (Wiame et al. 1985; Caddick et al. 1994; Marzluf 1997; Hofman-Bang 1999; Wong et al. 2008) where transcripts for proteins involved in N uptake are down-regulated when "good" N sources, such as glutamine and ammonium, are present in the substrate. Pathway specific regulation has also been demonstrated (Marzluf 1997; Wong et al. 2008), which occurs in the absence of a preferred N source and when another secondary N source is present in the substrate; only transcripts for proteins involved in the utilization of the secondary N source are up-regulated. For ectomycorrhizal fungi, Talbot and Treseder (2010) found the use of more complex forms of organic N to be dependent on their abundance in the substrate. Less is known, however, about how N source interacts with other environmental factors to affect N gene regulation in ectomycorrhizal fungi.

The carbon (C) to nitrogen ratio (C/N) of the substrate has been hypothesized to affect preferred N source by soil microorganisms (Geisseler et al. 2010). At high C/N ratios (above 40) where N is limiting to C, microorganisms should directly take up whatever N sources are available, initially depleting inorganic N sources, at which point microorganisms will take up organic N sources. At low C/N ratios (below 20) where C is limited relative to N, microorganisms also prefer direct uptake of the substrate after external ammonium levels have been depleted. Organic N, such as amino acids, could serve as a C source for the fungi (Chalot et al. 1994; Chalot and Brun 1998) and eventually N will be shed as ammonium, with the organic N being just used for the C sources. Lindahl et al. (2007) demonstrated that ectomycorrhizal fungi are spatially found in areas with higher C/N ratios, above 30, but the C/N ratio of forest soils where ectomycorrhizal fungi develop can range 14 to 52 (Hogberg et al. 2003; Hogberg et al. 2007). Atmospheric N deposition reduces the soil C/N ratio of a forest floor (Aber et al. 2003), and has increased in many N-limited temperate ecosystems since the 1940's (Vitousek et al. 1997). Rates of N depositions can be as high as 3 $gN\ m^{-2}\ yr^{-1}$ is some places (Reay et al. 2008). This environmental change may affect preferred N source by ectomycorrhizal fungi. Geisseler et al. (2010) hypothesize that N uptake from forest soils by microorganisms is dependant on the form (inorganic or organic) and source (ammonium, nitrate, glutamine, ect.) of N and the C/N ratio of the soil. How these factors interact to affect N utilization in ectomycorrhizal fungi remains uninvestigated.

Here, we study the regulation of transcripts for N uptake and metabolism proteins in *H. cylindrosporum,* a model ectomycorrhizal fungus that utilizes both inorganic and organic N sources (Chalot and Brun 1998; Marmeisse et al. 2004; Guidot et al. 2005; Müller et al. 2007). Many nitrogen-processing genes have been characterized in *H. cylindrosporum* (Table 1; Fig. 1; see (Müller et al. 2007; Chalot and Plassard 2011). *H. cylindrosporum* is typically found in sandy soils and is known to be efficient at scavenging inorganic N, including nitrate (Marmeisse et al. 2004). For example, nitrate transporter proteins are expressed in the absence of nitrate (Jargeat et al. 2003), which is not true for other mycorrhizal fungal species, such as *Tuber borchii* (Guescini et al. 2003). We study the dikaryotic strain (D2) grown on a range of N sources and C/N ratios to investigate how environmental factors could affect the regulation of N utilization genes. There were three objectives to this study, 1) to examine the effect of C/N ratio and form of N source on the regulation of individual genes; 2) to determine if there are clusters of genes that undergo similar regulation and whether these clusters respond to C/N ratio and N source; and 3) to investigate which factors had the greatest effect on gene expression patterns, the C/N ratio, N transporter (inorganic versus organic), and N source. With our last objective, we hoped to gain better insight into the regulation N uptake genes under complex environmental conditions. NCR control of N genes will be detected by the down-regulation of N transporters in the presence of a preferred N source (Wong et al. 2008). When a preferred N source is not in the media, pathway specific regulation will be evidenced by an up-regulation of N transporters that take up the specific N source in substrate (Wong et al. 2008). We hypothesize, however, that preferred N source could depend on the C/N ratio of the substrate, where fungi will prefer organic N sources on a low C/N ratio, and inorganic N sources on a high C/N ratio. Thus we expect to observe a complex pattern of N transporter gene regulation. Understanding how the C/N ratio affects the uptake and

Table 1 Nitrogen genes for metabolism and uptake proteins studied. Listed is the genes' known or hypothesized (denoted by a question mark) regulation, the primers used to study them, and the size of the PCR product from RNA templates. Subscript numbers next to gene name refer to the paper that originally isolated the gene, listed below the table

Name	Gene	Regulation	Primers (5'-3')	PCR Product (bp)
HcAMT1[5]	Ammonium transporter 1	NCR	AMT1F - ATTCATCCTCGGCGGTCTCGATT AMT1R - CACCAGGGATTTCGGTGATACCA	590
HcAMT2[2]	Ammonium transporter 2	NCR	AMT2F - GGTTTCTCGCTTGCTTTTAGCGAC AMT2R - TGTAAGCACTTCAGTGCCGTATCC	408
HcAMT3[2]	Ammonium transporter 3	Constitutively expressed	AMT3F - CGCAGGAACGAACGAGAATTGCTT AMT3R - CACCACGAACTCCGATGGTAACA	496
HcGDHA[5]	NADP-dependent glutamate dehydrogenase	Regulated like NCR	GDHAF - AAACATTCAAGAACGCCCTCACCG GDHAR - GGCTTCCTCCCTTCAACTTGAGGT	549
HcGLNA[5]	Glutamine synthetase	Constitutively expressed	GLNAF - CGGAGATAACATCCTCGTTCTCG GLNAR - GTTTGGGGTGGAAGGAAACCTTGA	453
HcNAR1[1]	Nitrate reductase 1	Nitrate/NCR?	NAR1_2F - AACGCAACGTACACGGTGAAAG NAR1_2F - TTTGATGGATCGAGCCCTTGGT	575
HcNRT2[4]	Nitrate transporter 2	Nitrate/NCR?	NRT2F - AAGACCTCGCGCTCGTCAAATCCA NRT2R - CCGAGGTTCGGTTGTTTTGCATGT	479
HcPTR2A[6]	Peptide transporter 2A	NCR and external N	PTR2AF - GGGAGCGCGTGATTGTAGACCCAT PTR2AR - TCGGAACGCCGTTAAGGGTCATTG	528
HcPTR2B[6]	Peptide transporter 2B	NCR and external N	PTR2BF - TGCGTGTCATTGGCTTTGCTCTTG PTR2BR - GCTCGTTATCGTGGCAGGGAGACT	501
HcGAP1[3]	General amino acid permease 1	NCR and external N	GAP1_2F - GTCCTTTACGGACTGGCTCTTC GAP2_2R - TTCCAGCGGCTTCCAGAACTTG	471
HcOPT	Oligopeptide transporter 1	NCR and external N	OPTF - ATTTCATCCCGTGGCTCGTTCA OPTR - GAGACCGACTTGTTGATTCGTG	459
HcTUB	Tubulin	N.A.	TUBF - ATCTCTGCAGAGAAAGCTCACCACG TUBR - CATACCCTCACCGACGTACCAGTGC	400

1 Jargeat et al. 2000; 2 Javelle et al. 2001; 3 Wipf et al. 2002; 4 Jargeat et al. 2003; 5 Javelle et al. 2003; 6 Benjdia et al. 2006

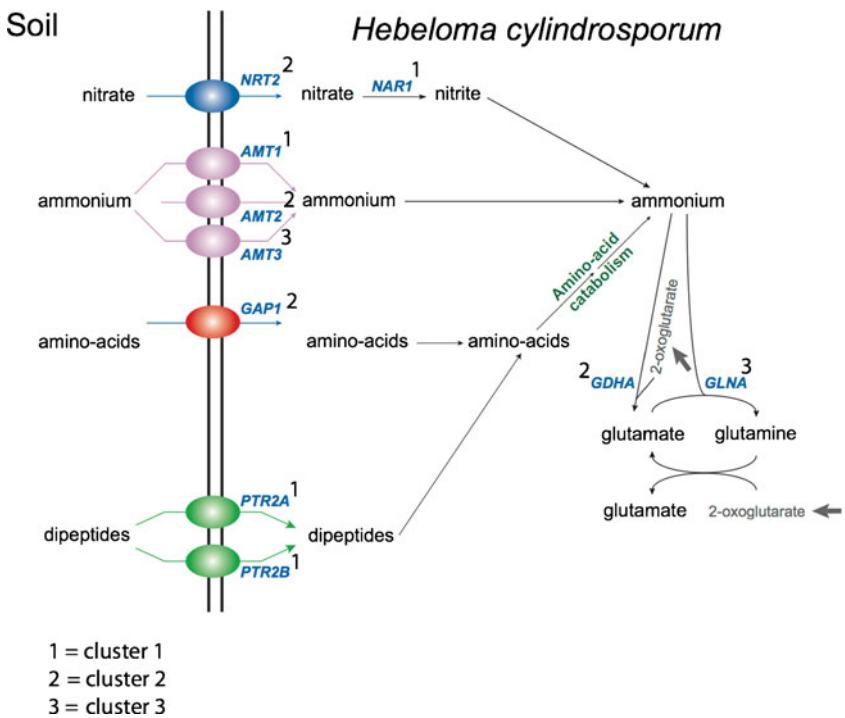

Fig. 1 Schematic of the soil/fungal interface detailing the utilization of nitrogen (N) within ectomycorrhizal fungus. We studied a range of genes involved in the uptake (ovals) and internal metabolism (shape) of both inorganic and organic N forms. Numbers refer to groups of genes whose patterns of expression clustered together (see Results)

assimilation of N sources, both inorganic and organic, can provide insight into how fungi respond to their changing environment.

Material and methods

Culture growth and strains

The *H. cylindrosporum* Romagnesi dikaryotic strain D2 (consisting of the h1 and h7 strains; (Debaud and Gay 1987) was used. All fungal cultures were grown in a dark room at 23°C. 10 days before the start of the experiment fungal cultures were transferred onto YMG (Rao and Niederpruem 1969) plates covered with sterilized cellophane membranes. Plugs were taken from the growing edge of parent fungal cultures grown on YMG using a cork borer. On the 10th day, fungal plugs on cellophane membranes were starved by transferring them to NP2/2 (Benjdia et al. 2006) liquid medium for 24 hours without any C or N sources added. After 24 hours of starvation fungal plugs were transferred to NP2/2 agar plates where C was supplied as glucose and there was one of 6 N sources: ammonium, nitrate, all 20 amino acids found in proteins (termed 'all amino acids'), selected amino acids (glutamic acid, glutamine, asparagine, and proline; termed 'selected amino acids'), diLeucine, or bovine serum albumin (BSA). We chose the selected amino acids because Wipf et al. (2002) demonstrated that they are specifically transported by *HcGAP1*, and glutamic acid, glutamine and asparagine are preferred amino acid N sources (Wipf et al. 2002; Guidot et al. 2005). After transfer to NP2/2 agar plates, fungi were harvested at two times after transfer: 12 hours (for gene expression analysis), or 14-16 days (for biomass analysis). To harvest the fungal cultures the layer of growing hyphal tissue on the cellophane membrane was cut away from the agar plug using a scalpel, tissue was placed in microcentrifuge tubes using tweezers and immediately flash frozen in liquid N. Frozen fungal tissue was stored in a -80°C freezer until they were freeze dried in a Liophilizer (Beta 1-8 K, Christ, Osterode am Harz, Germany) to fully dehydrate fungal tissue. Dried fungal tissues were weighed and stored in a desiccator until they were used for RNA extractions. Three fungal plugs were transferred back onto YMG to serve as a control for the 14-day growth experiment.

The experiment was a 3×6 factorial experiment, with three C/N ratios and six N treatments. There were three replicates for each treatment. Each nitrogen source was made in three C/N ratios, calculated by weight. We chose to manipulate C/N ratio by holding C constant and modifying N because we wanted to simulate the process of N deposition on the forest floor. In order to accurately manipulate the C/N ratio, for all treatments, except BSA, the numbers of carbon and nitrogen atoms for each N source were accounted for. Three C/N ratios were used: 7-10, 35-40, 59-62. The C/N ratios were not exact because of the excess C associated with the organic N sources. The low C/N ratio was 9 with 9 mM N L^{-1}, mid C/N ratio was 38 with 2 mM N L^{-1}, and the high C/N ratio was 60 with 1.2 mM N L^{-1}. The amount of carbon remained the same for all C/N treatments, 2.5 gL^{-1}.

Primer design and sequencing

All primers were designed to anneal with regions of the mRNA that encoded loops in the proteins (not transmembrane regions) for the 11 genes of interest in this study (Table 1). Primers were designed using AmplifX v1.4.0 by N. Jullien (http://ifrir.nord.univ-mrs.fr/AmplifX) and were designed to all be of similar annealing temperature and amplify approximately 500 bp products. Primers were made by Metabion (Germany) and reconstituted to 10 pmol μL^{-1}. All primer pairs were tested on both DNA and cDNA (from RNA) templates for the D2 strain. DNA and cDNA PCR products were sequenced (Macrogen, Korea) to check their accuracy (Table 1).

RNA Extractions and cDNA synthesis

mRNA was extracted from 5-7 mg of dried fungal hyphal tissue using Invitrogen's Dynalbeads mRNA Direct Micro Kit (Carlsbad, CA, USA). mRNA was extracted according to manufacture's recommendations for mRNA to be used for cDNA synthesis. mRNA purity and quantity were checked with a spectrophotometer (BioPhotometer, Eppendorf, Germany). cDNA was made using Invitrogen's Superscript III First-Strand Synthesis System for RT-PCR kit. cDNA synthesis was done according to manufacturer's recommendations in 10 μL reactions with the following modifications: 6 μL of RNase free water containing all the microbeads were used in each reaction; the mRNA, dTTT primers, and dNTP mix was incubated first at 50°C for 5 min and then the protocol was followed. Once the tubes were removed from the thermocycler (GeneAmp 9700, Perkin-Elmer), 40 μL of dH_2O were added to give a final volume of 50 μL and stored at -20°C until further use.

Polymerase chain reaction

PCR of the cDNA reactions were carried out for each replicate amplifying all 14 genes at once in separate PCR reactions, including a positive control (tubulin primers (Corratge et al. 2007) with template) and a negative control (tubulin primers and no template). PCR reactions were as follows: 94°C for 3 min; then for 36 cycles 94°C for 30 sec, 58°C for 30 sec, 72°C for 45 sec; then 72°C for 10 min and

10°C for 1-12 hours. PCR concentrations were 1X of Genaxxon (Biberach, DE) Amplification Buffer, 200 μM of each dNTP, 10 pmol of each primer, 1.5 units of Genaxxon's DF Taq Polymerase, and 2 μL of the cDNA template in 20 μL reactions. The PCR reaction was optimized to consistently be able to quantify amplified products while maintaining the ability to detect differences between PCR-product quantities; the PCR products never reached a plateau.

Gel quantification

The relative quantities of each PCR product were assessed on agarose gels. To each PCR product 3.5 μL of loading dye was added, and 4.5 μL was loaded into a 0.8% agarose gel. 10 μL of Invitrogen's 1 KB + ladder was used. Gels were run until there was a clear separation of the ladder (up to one hour). Gels were then put on a UV-Table and photographed using an Olympus C770 digital camera. Gel images were opened in Adobe Photoshop (Adobe, San Jose, USA) and were modified to optimize visualization of the bands by varying brightness and contrast levels. The intensity of the PCR products in the optimized images was quantified using ImageJ v.1.37 software (http://rsb.info.nih.goc/ij/). In order to normalize the data across gels and replicates, all products were quantified as a percentage of tubulin expression within a replicate. Tubulin is a housekeeping gene that is constitutively expressed and often used as a reference when studying *H. cylindrosporum* (Corratge et al. 2007; Tatry et al. 2009).

Statistical analyses

Data were statistically analyzed using SAS software (version 9.1.3, 2003, SAS Institute, Cary, NC, USA). Treatments were considered significant at $\alpha < 0.05$. Growth biomass data were $\log_{10}$ transformed to meet the assumptions of ANOVA of normality and homogeneity of variance. In cases of significance, Tukey's Honestly Significant Difference test was used for pairwise comparisons of means. Gene expression data were normalized to tubulin expression within each replicate and then we added 1 to each value to remove zero's from the dataset. All gene expression data was then $\log_2$ transformed for normality, henceforth referred to as relative gene expression. To assess whether there were significant changes in expression levels for each gene, t-tests were performed between the relative gene expression levels of each treatment combination of N source and C/N ratio with the relative gene expression levels for the all amino acid N source on the mid C/N ratio treatment. Thus, the mean of the all amino acids on the mid C/N ratio treatment was used as the base-line condition. This analysis was not performed for the *HcOPT* gene because there were not enough replicates of the baseline.

Multivariate tests were performed on the $\log_2$ percent tubulin data. For all multivariate tests the OPT gene was dropped from the analysis to meet the assumptions of no missing data. Ward's method with Euclidian distance was used for the cluster analysis (D'haeseleer 2005). MANOVAs were computed on the gene clusters revealed from the cluster analysis to determine whether the N source, C/N ratio or their interaction affected the regulation of the clusters.

Lastly, the effects of C/N of the media (high or low), inorganic (*HcAMT1, HcAMT2, HcAMT3*, and *HcNRT2*) or organic (*HcGAP1, HcOPT, HcPTR2A, and HcPTR2B*) N transporter proteins, and N source in the media on gene expression patterns were studied. N source in the media was classified differently for each of the three analyses. 1) N source was classified as either preferred (ammonium, all amino acids, selected amino acids) or un-preferred (nitrate, diLeucine, and BSA), 3) N source was classified as either the substrate of the transporter (i.e., for *HcNRT2* when nitrate was in the media) or not, and 3) and N source was classified by the type of N, either inorganic of organic. Three 3-way ANOVAs were performed testing the effects of C/N, N transporter type and either preferred N source, substrate of N transporter protein, or N type. For these analyses the mid C/N was dropped due to missing data for the BSA and diLeucine N sources.

Results

Growth results

The C/N ratio significantly affected the biomass of the cultures after 14 days (Table 2; $p<0.0001$) however, the different N treatments did not ($p=0.2801$) and there was not a significant interaction between the two ($p=0.4045$).

Effects of C/N ratio and N source on N utilization genes

Overall, the expression of all N genes studied varied between the different treatments 12 hours after being transferred back to a media with N sources (Fig. 2).

Inorganic N utilization genes

Of the ammonium transporters, the expression of *HcAMT1* was generally lower than the other transporters, and all three transporters showed regulation. *HcAMT1* was turned off on BSA with low C/N, nitrate with mid C/N, and ammonium with high C/N. However, *HcAMT1* expression was never statistically different from all amino acids on mid C/N, our baseline. *HcAMT2* was up-regulated on all amino acids with low C/N and down-regulated on ammonium with high C/N. *HcAMT3* was up-regulated on BSA with high C/N, and nitrate

Table 2 Mean (± S.E.M.) percent of biomass when grown on N sources than when grown on YMG after 14 days. Different letters indicate means that are significantly different at $p<0.05$ based on Tukey's HSD test for pairwise comparisons

	Ammonium	Nitrate	All Amino Acids	Selected Amino Acids	DiLeucine	BSA
Low C/N^{B}	0.322 (0.04)	0.365 (0.01)	0.381 (0.02)	0.379 (0.01)	0.339 (0.02)	0.428 (0.03)
Mid C/N^{B}	0.311 (0.01)	0.326 (0.01)	0.355 (0.02)	0.366 (0.01)	0.320 (0.01)	0.357 (0.01)
High C/N^{A}	0.577 (0.02)	0.629 (0.11)	0.548 (0.02)	0.615 (0.01)	0.544 (0.03)	0.515 (0.004)

with low C/N. Of the nitrate genes, *HcNRT2* was up-regulated on BSA with the high C/N and on nitrate with low C/N. *HcNAR1* was up-regulated on selected amino acids and BSA with high C/N and on nitrate with both low and mid C/N.

Internal N processing genes were consistently expressed. *HcGDHA* was up-regulated on BSA with high C/N, and selected amino acids with low C/N. *HcGLNA* expression levels were never statistically different from the baseline, all amino acids on mid C/N.

Organic N utilization genes

HcGAP1 was up-regulated on all amino acids, diLeucine and nitrate with low C/N, and BSA with high C/N. *HcPTR2A* did not differ in relative gene expression levels among the treatments. *HcPTR2B*, however, was down-regulated on nitrate with high C/N, and both ammonium and nitrate on mid C/N. Lastly, *HcOPT1*, an oligo-peptide transporter, was unregulated and expression levels remained the same on all treatments.

Gene clusters

We performed a cluster analyses on the gene expression data to see if groups of genes were regulated in similar patterns. We chose three clusters because the low root mean square standard deviation associated with that number of clusters. The first cluster included *HcAMT1*, *HcNAR1*, *HcPTR2A*,

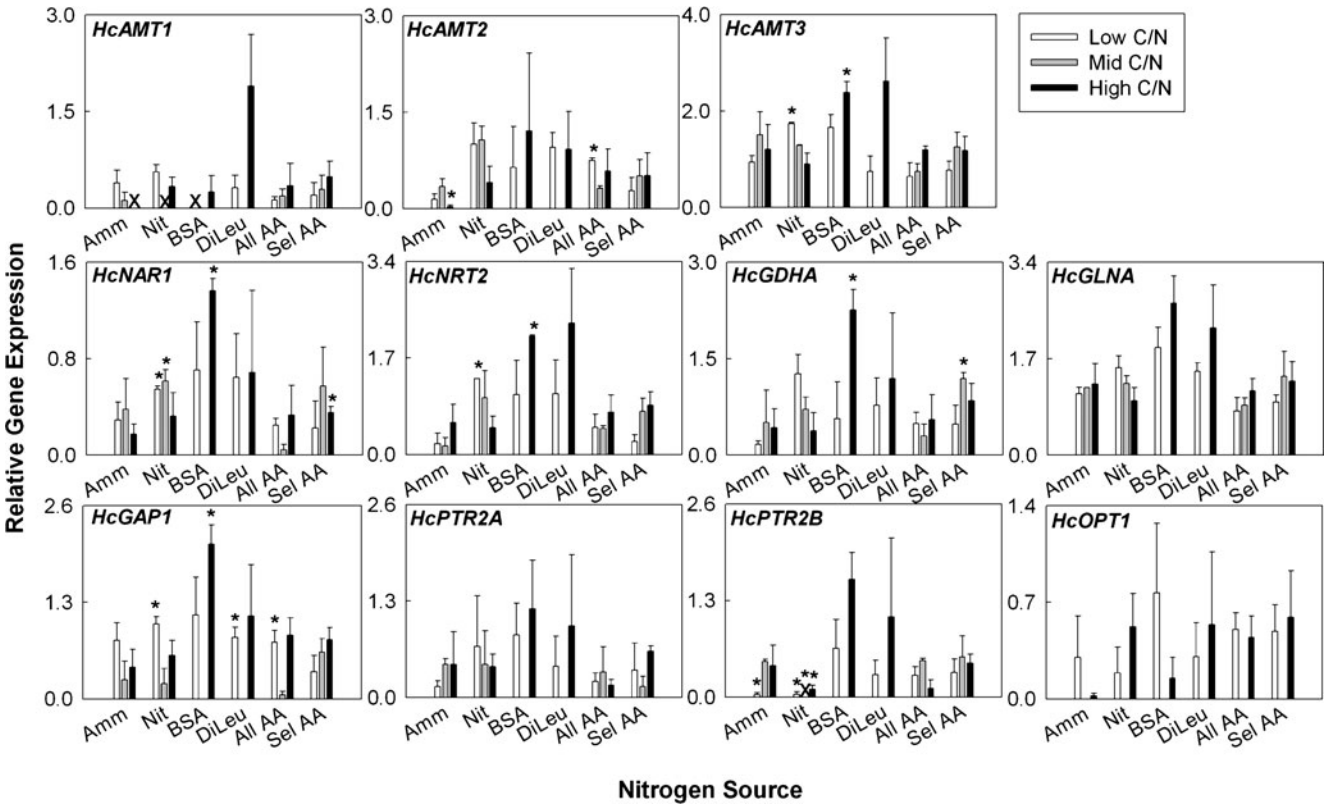

Fig. 2 Relative expression (mean ± S.E.M.) patterns of the *H. cylindrosporum* genes when grown on different nitrogen sources and C/N ratios. An asterisk denotes a significant difference for that condition from the expression of that particular gene on the all amino acid N source and mid C/N ratio treatment. An "X" denotes that the gene was turned off on that particular treatment. We did not perform the regulation analysis for *HcOPT* and there are no observations of BSA and diLeucine in the mid C/N ratio. Please note the range of the y-axis is not uniform

and *HcPTR2B*. The second cluster contained *HcAMT2*, *HcGAP1*, *HcGDHA*, and *HcNRT2*. Lastly, the third cluster contained *HcAMT3* and *HcGLNA*.

MANOVAs were performed for each gene cluster to determine whether the C/N and N source treatments affected the regulation of gene pathways. Of the three gene clusters only the second was significantly affected by the C/N ratio (Table 3). Expression was highest on the high C/N and lowest on the mid C/N. Gene expression patterns within a cluster were never affected by the N source, and there was never an interaction between N source and C/N ratio.

The effect of C/N ratio, N transporter type, and N source on relative gene expression levels

We found that both C/N ratio and N transporter types (inorganic or organic) significantly affected levels of gene expression (Table 4a-c). Relative gene expression levels were 38% higher on the high C/N compared with the low C/N, and were 44% higher for inorganic N transporters than organic N transporters. We found relative gene expression levels were 89% higher in the absence of a preferred N source than in the presence of a preferred N source (Table 4a). Conversely, we found no evidence of pathway specific regulation; there was no effect of the correct substrate being in the media on relative expression of N transporter genes (Table 4b). There was, however, an interaction between N transporter type and substrate present where inorganic N transporters were expressed at higher levels when their substrate was not in the media compared with organic N transporters. There was also an interaction between C/N ratio, N transporter type, and substrate, where inorganic N transporters had higher relative gene expression on the high C/N ratio when their substrate was not in the media. Lastly, we found a strong effect of N source type, where relative gene expression was 60% higher on organic N as sole N source, as compared to an inorganic N source (Table 4c). There was also an interaction between N source types with C/N ratio of the substrate, where relative gene expression was higher on the high C/N ratio when organic N was in the media relative to when inorganic N was in the media (Fig. 3).

Discussion

We investigated the regulation of all characterized N uptake and metabolism genes in the model fungus *H. cylindrosporum* across a range of conditions, manipulating N source and C/N ratio of the substrate. We found groups of genes that have similar expression patterns across a range of conditions, suggesting that these groups of genes are regulated similarly. As hypothesized C/N ratio, N transporter type, and N source of the media strongly affected gene expression levels, with interactions between all three. This demonstrates the need to continue to understand how environmental conditions affect N gene regulation.

Fungal N genes can be regulated by NCR or pathway specific regulation. Consistent with NCR, previous studies have shown that ammonium and glutamine are preferred N sources for *H. cylindrosporum* (Javelle et al. 2003), and our results support this. Overall expression levels were lower when a preferred N source was present in the media. Relative expression levels were lowest on the ammonium, selected amino acids, and all amino acids as N sources; and were highest on BSA and diLeucine, suggesting they were poor N sources. We also found that N source strongly affected expression levels of N transporter genes, however we did not see consistent evidence of pathway specific regulation, (e.g. when nitrate was the N source *HcNRT2* was not always correspondingly up-regulated), suggesting that when a preferred N source is not available, other factors, beyond simply N source in media, affect the preferred N source of the fungus.

Environmental factors—N source and C/N ratio—affected regulation of N transporter genes, which presumably cascades to which forms of N the fungus is utilizing from its external environment. C/N ratio strongly affected N transporter regulation, both for inorganic and organic N sources. For most genes the highest expression levels were found in the high C/N ratio on either BSA or diLeucine, indicating that the fungus was very N-stressed under those conditions. BSA has previously been found to be a poor N source for *H. cylindrosporum* on a mid C/N ratio (Wipf et al. 2002; Guidot et al. 2005). Also, the colonies had the most biomass on the high C/N ratio. It is probable that the fungi were actively growing and seeking other N sources not present in the media. Chalot et al. (1995) found the C/N

Table 3 Results of MANOVA for each gene cluster. All reported p-values are for Wilk's Lambda. Significant results are bolded

Cluster	C/N		N Source		Interaction	
	F-ratio	p-value	F-ratio	p-value	F-ratio	p-value
1 - HcAMT1, HcNAR1, HcPTR2A, HcPTR2B	0.74	0.6569	1.44	0.1305	1.05	0.4158
2 - HcAMT2, HcGDHA, HcGAP1, HcNRT2	**2.41**	**0.0398**	1.49	0.1330	0.81	0.7305
3 - HcAMT3, HcGLNA	1.67	0.1712	1.18	0.3256	1.44	0.1509

Table 4 Results of three separate three-way ANOVAs analyzing the expression levels of genes for N transporter proteins. We tested the effect of C/N (low or high), N transporter type (inorganic or organic) and either a) preferred N source (yes: ammonium, all amino acids, selected amino acids or no: nitrate, diLeucine, BSA), b) substrate present (yes: when the transporter protein transported the substrate in the media, i.e., yes for NRT2 when nitrate was in the media), and c) N source type (inorganic or organic N). The overall model was significant for all three ANOVAs (a: $F=6.44$, $p<0.0001$; b: $F=3.63$, $p=0.0009$; c: $F=5.369$, $p<0.0001$). Significant results are bolded

A	F- ratio	p-value
C/N	**6.51**	**0.0113**
N Transporter Type	**8.40**	**0.0041**
Preferred N Source	**25.83**	**<0.0001**
C/N×N Transporter Type	1.06	0.3044
C/N×Preferred N Source	0.91	0.3415
N Transporter Type×Preferred N Source	2.36	0.1260
C/N×N Transporter Type×Preferred N Source	0.02	0.8904
B	F- ratio	p-value
C/N	**6.10**	**0.0142**
N Transporter Type	**7.86**	**0.0054**
Substrate Present	0.44	0.5071
C/N×N Transporter Type	0.99	0.3202
C/N×Substrate Present	0.25	0.6194
N Transporter Type×Substrate Present	**4.64**	**0.0322**
C/N×N Transporter Type×Substrate Present	**5.14**	**0.0242**
C	F- ratio	p-value
C/N	**6.36**	**0.0097**
N Transporter Type	**8.20**	**0.0031**
N Source Type	**11.18**	**<0.0001**
C/N×N Transporter Type	1.03	0.3014
C/N×N Source Type	**8.31**	**0.0043**
N Transporter Type×N Source Type	0.09	0.7637
C/N×N Transporter Type×N Source Type	2.54	0.1124

ratio of the substrate did not affect the ability of *Paxillus involutus* to take up amino acids. While we did not directly measure N uptake and translocation from media into the fungus, the strong regulation response probably resulted in either increased or decreased N uptake. *H. cylindrosporum* is typically found in sandy heterogeneous soils with little organic matter (Marmeisse et al. 2004), while *P. involutus* are more typical of soils with a large organic layer. The patchy environment of *H. cylindrosporum* might select for the ability to adjust N source according to subtle changes in the environment, such as C/N ratio of the substrate. We did not find evidence of *H. cylindrosporum* focusing only on inorganic or organic N sources when they were present in the media, because we found no interaction between N type and N transporter type. Thus, it appears that *H. cylindrosporum* does not solely import organic or inorganic N sources at a time, but is able to utilize both N sources simultaneously. These results are similar to a study of *L. bicolor* where nitrate uptake and processing genes were not turned off on organic N sources, but co-expressed (Kemppainen et al. 2010). Similar results were obtained for *P. involutus* (Chalot et al. 1995), where a moderate presence of ammonium did not affect amino acid uptake. Together, this suggests that for ectomycorrhizal fungi, being able to utilize a range of N sources simultaneously is advantageous because forest soils are highly heterogeneous and there is always a mixed pool of organic and inorganic N sources.

Our study also sheds light on *H. cylindrosporum* N utilization. For *H. cylindrosporum* the GS-GOGAT pathway was shown to be the predominant pathway of ammonium assimilation over the GDH-GS pathway (Chalot et al. 1991). Our results confirm this, as *HcGLNA* (involved in GS-GOGAT) was always expressed at higher levels than *HcGDHA* (involved in GDH-GS). Further, the clustering of *HcAMT3* and *HcGLNA* and lack of a significant response of this cluster to the C/N ratio of the media or N source suggest that these two genes are involved in basal N assimilation pathways in *H. cylindrosporum*. Javelle et al. (2003) also found that *HcAMT3* and *GLNA* are highly expressed and often poorly regulated. We, however, found *HcAMT3* to undergo regulation, being up-regulated on BSA and nitrate. The second cluster was expressed at higher levels on the high C/N ratio, suggesting that these genes are up-regulated in N-stressed conditions. Our findings suggest that the transporter genes, *HcAMT2*, *HcGAP1*, and *HcNRT2* are primary N uptake pathways utilized during N-stressed conditions, actively searching for ammonium, amino acids, and nitrate, respectively. Lastly, Benjdia et al. (2006) demonstrated that *HcPTR2B* was constitutively expressed at basal levels, while we found that it was down-regulated on nitrate and

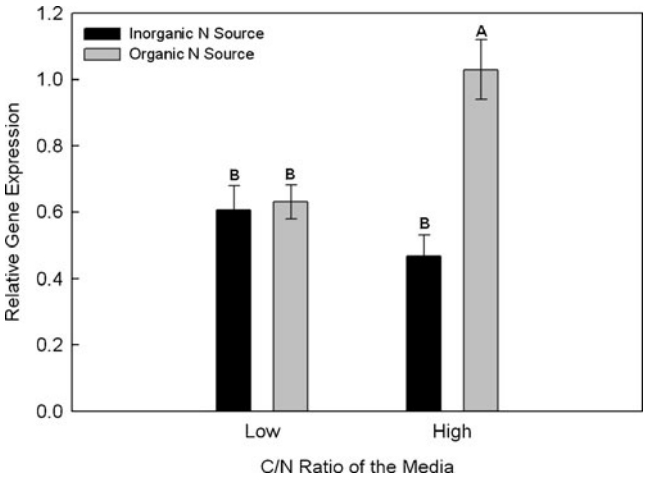

Fig. 3 Comparing relative expression levels (mean ± S.E.M.) of all N transporter proteins on low and high C/N ratios with type of nitrogen in the media. All N sources in the media were classified as either inorganic (ammonium and nitrate) or organic (selected amino acids, all amino acids, diLeucine, and BSA). Different letters indicate means are significantly different at $p<0.05$ based on Tukey's HSD

ammonium. An experiment conducted by Lucic et al. (2008) studying *L. bicolor* grown on ammonium near our mid C/N found that *AMT3* was not expressed, while *AMT1* and *AMT2* were, which is in contrast to our findings, where we found higher expression levels of *AMT3* than the other ammonium transporters.

Decreased soil C/N ratios with N deposition probably results in a reduction in root C allocation by ectomycorrhizal host trees, which drives the reduction in fungal biomass under N deposition (Nilsson and Wallander 2003; Hogberg et al. 2007). Talbot et al. (2008) argue that N deposition may cause ectomycorrhizal fungi to actively scavenge C from soil compounds by acting as decomposers. Lucas and Casper (2008) found N deposition resulted in greater proteolytic activity suggesting higher protein degradation in soils surrounding ectomycorrhizal fungus. We did not find evidence of this in our results. At low C/N ratios there was not an increase in the expression levels of organic N transporters. Instead, at high C/N levels both inorganic and organic N transporters were up-regulated on organic N sources, indicative of overall N limitation. Inorganic N transporter genes were always expressed at higher levels than organic N transporter genes, suggesting that regardless of the C/N ratio *H. cylindrosporum* prefers inorganic N sources. It is probable that a shift would have been more evident if C were not always present in the media, because, as Geisseler et al. (2010) note, use of organic N by microorganisms for a C source should depend on the presence of other C sources. Another possible explanation is that although we studied all known N utilization genes, there are probably other organic N transporters that have not yet been characterized in *H. cylindrosporum*. Perhaps if we had included more organic N transporter proteins we may have observed switching N source preference to supplement C.

Taken together, our results may be of ecological relevance, because we demonstrate that for the N transporter genes we studied, we did not observe changes in the preferred N source by *H. cylindrosporum*. If the C/N ratios of soils continue to decrease with increasing levels of inorganic N deposition ectomycorrhizal (Aber et al. 2003), our results suggests that fungi may not change the N source they preferentially take up. Although extrapolation of our results is limited, again because of the limited number of genes studied, they are still of interest because we did not observe the pattern of switching N sources to supplement C in the low C/N ratio that we expected. This finding reflects the observation that N deposition results in fungal decline (Treseder 2004), and although fungi may have the ability to take up and use organic N (Chalot and Brun 1998), they may not switch to an organic N source to supplement environmentally reduced C/N ratios.

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References

Aber JD, Goodale CL, Ollinger SV, Smith ML, Magill AH, Martin ME, Hallett RA, Stoddard JL (2003) Is nitrogen deposition altering the nitrogen status of northeastern forests? Bioscience 53:375–389

Benjdia M, Rikirsch E, Müller T, Morel M, Corratge C, Zimmermann S, Chalot M, Frommer WB, Wipf D (2006) Peptide uptake in the ectomycorrhizal fungus *Hebeloma cylindrosporum*: characterization of two di- and tripeptide transporters (HcPTR2A and B). New Phytol 170:401–410

Buscot F, Munch JC, Charcosset JY, Gardes M, Nehls U, Hampp R (2000) Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems. Fems Microbio Rev 24:601–614

Caddick MX, Peters D, Platt A (1994) Nitrogen Regulation in Fungi. Anton Leeuw Int J G 65:169–177

Chalot M, Brun A (1998) Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. Fems Microbio Rev 22:21–44

Chalot M, Brun A, Debaud JC, Botton B (1991) Ammonium-Assimilating Enzymes and Their Regulation in Wild and Nadp-Glutamate Dehydrogenase-Deficient Strains of the Ectomycorrhizal Fungus *Hebeloma Cylindrosporum*. Physiol Plant 83:122–128

Chalot M, Brun A, Finlay RD, Soderstrom B (1994) Metabolism of [C-14] Glutamate and [C-14] Glutamine by the Ectomycorrhizal Fungus *Paxillus Involutus*. Microbiology 140:1641–1649

Chalot M, Javelle A, Blaudez D, Lambilliote R, Cooke R, Sentenac H, Wipf D, Botton B (2002) An update on nutrient transport processes in ectomycorrhizas. Plant Soil 244:165–175

Chalot M, Kytoviita MM, Brun A, Finlay RD, Soderstrom B (1995) Factors Affecting Amino-Acid-Uptake by the Ectomycorrhizal Fungus *Paxillus Involutus*. Mycol Res 99:1131–1138

Chalot M, Plassard C (2011) Ectomycorrhiza and Nitrogen Provision to the Host Tree. In: Polacco J, Todd C (eds) Ecological Aspects of Nitrogen Metabolism in Plants. John Wiley & Sons, Inc, Hoboken, NJ

Corratge C, Zimmermann S, Lambilliotte RRL, Plassard C, Marmeisse R, Thibaud JB, Lacombe B, Sentenac H (2007) Molecular and functional characterization of a Na + -K + transporter from the Trk family in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. J Biol Chem 282:26057–26066

D'haeseleer P (2005) How does gene expression clustering work? Nat Biotechnol 23:1499–1501

Debaud JC, Gay G (1987) Invitro Fruiting under Controlled Conditions of the Ectomycorrhizal Fungus *Hebeloma Cylindrosporum* Associated with *Pinus Pinaster*. New Phytol 105:429–435

Geisseler D, Horwath WR, Joergensen RG, Ludwig B (2010) Pathways of nitrogen utilization by soil microorganisms - A review. Soil Biol Biochem 42:2058–2067

Guescini M, Pierleoni R, Palma F, Zeppa S, Vallorani L, Potenza L, Sacconi C, Giomaro G, Stocchi V (2003) Characterization of the *Tuber borchii* nitrate reductase gene and its role in ectomycorrhizae. Mol Genet Genomics 269:807–816

Guidot A, Verner MC, Debaud JC, Marmeisse R (2005) Intraspecific variation in use of different organic nitrogen sources by the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mycorrhiza 15:167–177

Hofman-Bang J (1999) Nitrogen catabolite repression in *Saccharomyces cerevisiae*. Mol Biotech 12:35–73

Hogberg MN, Baath E, Nordgren A, Arnebrant K, Hogberg P (2003) Contrasting effects of nitrogen availability on plant carbon supply to mycorrhizal fungi and saprotrophs - a hypothesis based on field observations in boreal forest. New Phytol 160:225–238

Hogberg MN, Hogberg P, Myrold DD (2007) Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three? Oecologia 150:590–601

Jargeat P, Gay G, Debaud JC, Marmeisse R (2000) Transcription of a nitrate reductase gene isolated from the symbiotic basidiomycete fungus *Hebeloma cylindrosporum* does not require induction by nitrate. Molec Gen Genet 263:948–956

Jargeat P, Rekangalt D, Verner MC, Gay G, Debaud JC, Marmeisse R, Fraissinet-Tachet L (2003) Characterisation and expression analysis of a nitrate transporter and nitrite reductase genes, two members of a gene cluster for nitrate assimilation from the symbiotic basidiomycete *Hebeloma cylindrosporum*. Curr Genet 43:199–205

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

Javelle A, Rodriguez-Pastrana BR, Jacob C, Botton B, Brun A, Andre B, Marini AM, Chalot M (2001) Molecular characterization of two ammonium transporters from the ectomycorrhizal fungus *Hebeloma cylindrosporum*. FEBS Lett 505:393–398

Kemppainen MJ, Crespo MCA, Pardo AG (2010) fHANT-AC genes of the ectomycorrhizal fungus *Laccaria bicolor* are not repressed by l-glutamine allowing simultaneous utilization of nitrate and organic nitrogen sources. Env Microbiol Rep 2:541–553

Lindahl BD, Ihrmark K, Boberg J, Trumbore SE, Hogberg P, Stenlid J, Finlay RD (2007) Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytol 173:611–620

Lucas RW, Casper BB (2008) Ectomycorrhizal community and extracellular enzyme activity following simulated atmospheric N deposition. Soil Biol Biochem 40:1662–1669

Lucic E, Fourrey C, Kohler A, Martin F, Chalot M, Brun-Jacob A (2008) A gene repertoire for nitrogen transporters in *Laccaria bicolor*. New Phytol 180:343–364

Marmeisse R, Guidot A, Gay G, Lambilliotte R, Sentenac H, Combier JP, Melayah D, Fraissinet-Tachet L, Debaud JC (2004) *Hebeloma cylindrosporum* - a model species to study ectomycorrhizal symbiosis from gene to ecosystem. New Phytol 163:481–498

Martin F et al (2008) The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. Nature 452:88–U87

Marzluf GA (1997) Genetic regulation of nitrogen metabolism in the fungi. Microbiol Mol Biol R 61:17–32

Müller T, Avolio M, Olivi M, Benjdia M, Rikirsch E, Kasaras A, Fitz M, Chalot M, Wipf D (2007) Nitrogen transport in the ectomycorrhiza association: The *Hebeloma cylindrosporum-Pinus pinaster* model. Phytochem 68:41–51

Nilsson LO, Wallander H (2003) Production of external mycelium by ectomycorrhizal fungi in a Norway spruce forest was reduced in response to nitrogen fertilization. New Phytol 158:409–416

Rao PS, Niederpruem DJ (1969) Carbohydrate Metabolism during Morphogenesis of Coprinus Lagopus (Sensu Buller). J Bacteriol 100:1222–1228

Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance? New Phytol 157:475–492

Reay DS, Dentener F, Smith P, Grace J, Feely RA (2008) Global nitrogen deposition and carbon sinks. Nat Geosci 1:430–437

Salsac L, Chaillou S, Morotgaudry JF, Lesaint C (1987) Nitrate and Ammonium Nutrition in Plants. Plant Physiol Bioch 25:805–812

Smith SE, Read DJ, 2008 *Mycorrhizal symbiosis*, 3rd ed. Academic Press, Amsterdam ; Boston

Talbot JM, Allison SD, Treseder KK (2008) Decomposers in disguise: mycorrhizal fungi as regulators of soil C dynamics in ecosystems under global change. Funct Ecol 22:955–963

Talbot JM, Treseder KK (2010) Controls over mycorrhizal uptake of organic nitrogen. Pedobiologia 53:169–179

Tatry MV et al (2009) Two differentially regulated phosphate transporters from the symbiotic fungus *Hebeloma cylindrosporum* and phosphorus acquisition by ectomycorrhizal *Pinus pinaster*. Plant J 57:1092–1102

Treseder KK (2004) A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO2 in field studies. New Phytol 164:347–355

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG (1997) Human alteration of the global nitrogen cycle: Sources and consequences. Ecol App 7:737–750

Wiame JM, Grenson M, Arst HN (1985) Nitrogen Catabolite Repression in Yeasts and Filamentous Fungi. Adv Microb Physiol 26:1–88

Wipf D, Benjdia M, Tegeder M, Frommer WB (2002) Characterization of a general amino acid permease from *Hebeloma cylindrosporum*. FEBS Lett 528:119–124

Wong KH, Hynes MJ, Davis MA (2008) Recent advances in nitrogen regulation: a comparison between *Saccharomyces cerevisiae* and filamentous fungi. Eukaryot Cell 7:917–925