**Original Paper**

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**Transcription of a nitrate reductase gene isolated from the symbiotic basidiomycete fungus *Hebeloma cylindrosporum* does not require induction by nitrate**

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**Abstract** Ectomycorrhizal fungi contribute to the nitrogen nutrition of their host plants, but no information is available on the molecular control of their nitrogen metabolism. The cloning and pattern of transcriptional regulation of two nitrate reductase genes of the symbiotic basidiomycete *Hebeloma cylindrosporum* are presented. The genomic copy of one of these genes (nar1) was entirely sequenced; the coding region is interrupted by 12 introns. The nar1 gene, which is transcribed and codes for a putative 908-amino acid polypeptide complemented nitrate reductase-deficient mutants of *H. cylindrosporum* upon transformation, thus demonstrating that the gene is functional. The second gene (nar2), for which no mRNA transcripts were detected, is considered to be an ancestral, non-functional duplication of nar1. In a 462 nt partial sequence of nar2 two introns were identified at positions identical to those of introns 8 and 9 of nar1, although their respective nucleotide sequences were highly divergent; the exon sequences were much more conserved. In wild-type strains, transcription of nar1 is repressed in the presence of a high concentration of ammonium. High levels of transcription are observed in the presence of either very low nitrogen concentrations or high concentrations of nitrate or organic N sources such as urea, glycine or serine. This indicates that in *H. cylindrosporum*, in contrast to all nitrophilous organisms studied so far, an exogenous supply of nitrate is not required to induce transcription of a nitrate reductase gene. In contrast, repression by ammonium suggests the existence of a wide-domain regulatory gene, as already characterized in ascomycete species.

**Key words** Nitrate reductase · Nitrogen regulation · Mycorrhiza · *Hebeloma* · Basidiomycete

**Introduction**

The ectomycorrhizal symbiosis is a ubiquitous association between the roots of trees and numerous fungal species belonging mainly to the basidiomycetes and the ascomycetes. Among the several beneficial effects of this association is the improved supply of nitrogen compounds to the plant partner (Smith and Read 1997). The contribution of the fungus to plant nutrition results from the capacity of the fungal hyphae connected to the plant to explore a larger volume of soil than can be reached by the root system, and its ability to metabolise compounds which a plant usually cannot assimilate directly – for example, proteins and some amino acids (Abuzinadah and Read 1986, 1989). These nutrients are taken up and metabolized by the fungal cells and are supplied to the plant through the Hartig net, a highly differentiated exchange structure of hyphae surrounding the root cortical cells. The whole process is therefore the result of various metabolic activities – absorption, primary assimilation, hyphal transport, storage and finally transfer to the plant – which occur in different fungal cells (from apices in the soil to hyphae in close contact with the plant root cortical cells). Studies on N metabolism carried out with different plant/fungus associations suggest that several of the enzyme systems involved (e.g. glutamate dehydrogenase, glutamine synthetase, amino transferases) are tightly regulated, and that different patterns of regulation can be observed in the free-living mycelia and in the mycorrhizas (for reviews, see Botton and Chalot 1995; Martin and Lorillou 1997). So far, none of the underlying regulatory mechanisms has been characterized.

To understand better the contribution of the fungal partner to the symbiotic N nutrition of the plant we have undertaken the molecular characterization of the nitrate assimilation pathway of the ectomycorrhizal...
basidiomycete *Helioboma cylindrosporum*. In most organisms, nitrate assimilation is achieved by a single pathway characterized by the sequential reduction of nitrate to nitrite and then to ammonium by the nitrate (NR) and nitrite (NiR) reductases, respectively. The corresponding genes, as well as putative nitrate transport systems, have been cloned from various saprophytic and pathogenic filamentous ascomycetes; all of these genes are single-copy genes and their transcription is subject to ammonium/glutamine repression and nitrate induction (see Kinghorn and Unkles 1994). The genetic and molecular bases of repression and induction have been studied in detail in *Aspergillus nidulans* and *Neurospora crassa* (see Scaccavocchio and Arst 1989; Caddick et al. 1994; Marzluf 1997 for reviews). In both species nitrate induction is mediated by a pathway-specific regulatory gene (*nitA* and *nit-4* in, respectively, *A. nidulans* and *N. crassa*), whose product binds to the promoters of the nitrate pathway genes when NO₃⁻ is present in the culture medium. Similarly, derepression is mediated by a wide-domain regulatory gene (respectively *areA* and *nit-2*), which encodes a GATA DNA-binding protein. Both *areA* and *nit-2* are responsible, at least in part, for the derepression, when ammonium is absent, of several other genes implicated in the use of other nitrogen sources, such as several amino acids or proteins.

The nitrate assimilation pathway is an attractive model system in which to study the contribution of a mycorrhizal fungus to the N nutrition of its host plant. As no other alternative nitrate assimilation pathway exists, cloning of the corresponding genes may be used to produce transgenic fungal strains with modified fluxes through this pathway and to study the consequences of these modifications on the nitrate nutrition of the host plant. Furthermore, careful analysis of the regulation patterns could lead to the identification of major regulatory genes which may control the transcription of additional metabolic genes whose expression has been shown to change in the symbiotic tissues.

The fungus used in this study is the basidiomycete agaric *H. cylindrosporum*. In contrast to many ectomycorrhizal species, this fungus can be easily manipulated in the laboratory. *H. cylindrosporum* is heterothallic with a bifactorial mating system, both haploid homokaryotic and dikaryotic mycelia form mycorrhizas with *Pinus pinaster* plants in the laboratory (see Debaud et al. 1997). It is the only ectomycorrhizal species for which sporulating fruit bodies can be obtained under axenic conditions in association with a host plant (Debaud and Gay 1987) and one of the very few symbiotic fungi for which a functional transformation system is available (Marmesse et al. 1992).

*H. cylindrosporum* efficiently uses ammonium, nitrate, proteins and the most common amino acids as primary nitrogen sources, but not proline or aromatic amino acids, for instance (our unpublished results). Nitrate assimilation in *H. cylindrosporum* is unusual in that significant nitrate reductase activity can be detected in mycelia growing on ammonium (Scheromm et al. 1990a, b). Moreover, ^15^N labelling experiments have confirmed that ammonium does not prevent nitrate assimilation, thus suggesting that regulation in this species may not follow the same rules as those described for saprophytic ascomycetes (Marmesse et al. 1998).

In this paper we report the molecular characterization of the nitrate reductase genes of *H. cylindrosporum* and their transcriptional regulation in response to different nitrogen sources present in the culture medium.

Materials and methods

Fungal strains, media and culture conditions

Five haploid homokaryotic strains of *H. cylindrosporum* were used, the wild-type strains h1 (mating-type A1 B2) and h7 (A2 B1), and two of their nitrate reductase-deficient mutants (NR'), h1.7 and h7.12 (Marmesse et al. 1998). The dikaryotic strain D2 (h1 × h7) was also used. Routine propagation of the mycelia was performed on YMg medium (Rao and Niederpruem 1969). To study gene regulation, the minimal medium N2P3 (Marmesse et al. 1998), buffered at pH 6.0 with 25 mM MES, was used, but phosphorus and potassium concentrations were reduced to 2.7 and 1.8 mM, respectively. Nitrogen sources were prepared and filter-sterilized separately as 100× concentrated stock solutions, and added after autoclaving of the medium. All cultures were grown in Petri dishes at 22 °C in the dark and the media were solidified with either agar or agarose to prevent contamination by nitrate. In order to transfer mycelium from one medium to another and to recover the mycelium for nucleic acid or protein extraction, the solid media were overlaid with a cellophane membrane before inoculation with a small inoculum.

Nucleic acid extraction and hybridizations

Fungal DNA and RNA were extracted from mycelium by the methods described in Van Kan et al. (1991). Total RNA was fractionated on 1% agarose gels containing 2.2 M formaldehyde. Southern and Northern blotting and hybridizations with homologous probes were performed using standard techniques (Sambrook et al. 1989). Heterologous hybridizations using the *Ustilago maydis* narl gene as a probe were performed overnight at 55 °C followed by a 30-min wash in 2× SSC at 60 °C. The *U. maydis* narl probe was a 1-kb BglII fragment isolated from pMH305 (Banks et al. 1993); the 5.8S rDNA gene was obtained by PCR amplification of the ITSI-5.8S-ITS2 region (Sequerra et al. 1997). The narl-specific probe used in Southern and Northern experiments is shown in Fig. 1. All probes were made from gel-purified fragments and labelled with [³²P]dCTP using the random priming kit from Boehringer. Quantification of the hybridization signals was performed by scanning the X-ray films.

Cloning and construction

To construct a genomic library, DNA extracted from strain h1 was partially digested with *Sac*III and the sites partially filled-in with dGTP and dATP, using Klenow polymerase. The fragments were cloned into the partially filled-in *XhoI* cloning site of *XEGM*2, according to the vector manufacturer's instructions (Promega). The library, which had an initial titre of 6.8 × 10⁸ recombinant phages, was propagated in the *E. coli* strain LE392. The average size of the inserts was 14 kb. Among the 6 × 10⁹ phages screened under low-stringency conditions with the *U. maydis* narl probe, seven gave a positive signal. Their inserts were excised with *Not*I, which cuts on both sides of the *XEGM* XhoI cloning site, and cloned in pBlue-script II (Stratagene), which was used for all subcloning steps. The