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Hydrophobins in ectomycorrhizas: heterologous transcription of the *Pisolithus HydPt-1* gene in yeast and *Hebeloma cylindrosporum*

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SUMMARY

Hydrophobins are fungal cell wall proteins involved in aggregation of hyphae. Upon the development of the ectomycorrhizal symbiosis between tree roots and fungal hyphae, the transcripts of hydrophobin genes markedly accumulated. As the precise role of these proteins in symbiosis is not yet known, we develop heterologous expression system of the *Pisolithus* hydrophobin HYDPt-1. This gene has been introduced in *Saccharomyces cerevisiae* and in the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*. Introns were required for *hydPt-1* transcript accumulation in the basidiomycete *H. cylindrosporum*. Heterologous transcript accumulation did not alter the phenotype of either species. The lack of altered phenotype resulted from the absence of HYDPt-1 polypeptide accumulation in transformed strains.

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INTRODUCTION

Ectomycorrhiza is a widespread symbiosis in temperate and boreal forest ecosystems. The mycelium of ectomycorrhizal fungi colonizes tree roots by adhering to their surface to form the ectomycorrhizal mantle. This process is accompanied by changes in gene expression (Burgess *et al.* 1995; Martin *et al.* 1999). In ectomycorrhizas obtained *in vitro* between *Pisolithus* and *Eucalyptus globulus*, the most abundant transcripts correspond to fungal cell wall proteins (Martin *et al.* 1999). These are mostly hydrophobins (Tagu *et al.* 1996) that have been described in various filamentous fungi and are involved in cell morphogenesis, hyphal aggregation, and plant-microbe interactions (Wessels 1997).

We have shown that *Pisolithus* hydrophobin mRNAs are highly accumulated during the initial

stages of root colonization, when hyphae bind to the root surface and aggregate to form a sheath (Tagu *et al.* 1996; Martin *et al.* 1999). The roles of hydrophobins in ectomycorrhizas are still hypothetical. As gene disruption is not feasible with *Pisolithus*, we transferred the *hydPt-1* sequence (one of the hydrophobin gene of *Pisolithus*) in two other fungal species: *Saccharomyces cerevisiae* and *Hebeloma cylindrosporum* as a first step to express this protein and study its roles. No hydrophobin genes is present in *S. saccharomyces* genome making yeast particularly attractive to evaluate the potential phenotype changes resulting from over-expression of these proteins. *H. cylindrosporum* is an ectomycorrhizal basidiomycete for which genetic transformation has been successfully developed (Marmeisse *et al.* 1992). It has hydrophilic hyphae, whereas *Pisolithus* develops extensive rhizomorphs made of aggregated hyphae. Heterologous expression of hydrophobin in *H. cylindrosporum* might thus possibly alter the surface hydrophobicity of the hyphae, and modify ectomycorrhiza formation. In this paper, we report of the heterologous expression of *HydPt-1* in yeast and *H. cylindrosporum*.

MATERIALS AND METHODS

Fungal isolates, culture conditions and genetic transformation

Mycelia of *Pisolithus* isolate 441 - possibly *P. albus* (Bougher and Syme 1998) - were cultivated on a modified Pachlewski's medium (Burgess *et al.* 1995) at 25°C in the dark. The homokaryotic strain h1 of *H. cylindrosporum* (Debaud and Gay 1987) was grown on yeast malt glucose (YMG) at 25°C in the dark (Marmeisse *et al.* 1992). Genetic transformation of *H. cylindrosporum* was performed by direct gene transfer to protoplasts (Marmeisse *et al.* 1992). Resistant colonies were selected with 200 µg of hygromycin-B per ml. *H. cylindrosporum -Pinus pinaster* ectomycorrhizas were synthesized *in vitro* (Gay *et al.* 1994).

The *Saccharomyces cerevisiae* strain Y59 (Mat a, *leu* 2-3, *leu* 2-112, *ura* 3-52, *trp* 1-189, *arg* 4) was grown in a yeast extract - peptone - dextrose medium (Adams *et al.*, 1997).

Bacterial strains and plasmids

Plasmid manipulations were performed in *Escherichia coli* DH5α in LB medium supplemented with 50 µg of ampicillin per ml (Sambrook *et al.* 1989).

Yeast plasmid

A 621 bp cDNA fragment corresponding to the *hydPt-1* cDNA (accession number: U29605) was amplified by PCR using two primers containing an additional *Bam*H1 restriction site (underlined). The first (5'-cgcggatccATGAAGTTCGCCGCCGTCG-3') encompassed the start ATG codon of the *hydPt-1* sequence. The reverse primer (5'-cgcggatccAGACGTCAACGCATTGCACGG-3') matched the 3'-untranslated sequence. PCR reactions were performed in standard conditions in a Perkin Elmer GeneAmp PCR System 9600. Amplified DNA fragments were digested with *Bam*HI and cloned in the yeast centromeric plasmid pG-1 (Hitzeman *et al.* 1982) containing the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and the phosphoglycerate kinase terminator. Recombinant plasmids were propagated in *E. coli* and checked by restriction analyses and DNA sequencing. Transformed cells of *S. cerevisiae* (Ito *et al.* 1983) were selected on a medium without tryptophane (pG-1 contains the *TRP1* gene).

H. cylindrosporum plasmids

The pGHPHT and a recombinant pSGFT plasmids (see below) were used in co-transformation experiments of *H. cylindrosporum* protoplasts. pGHPHT confers hygromycin resistance to transgenic mycelia (Schuren and Wessels 1998). pSGFT was used to clone the *hydPt-1* cDNA or gene fragment between the *gpd* promoter and terminator of *Schizophyllum commune* (Schuren and Wessels 1998). For this, pSGFT was digested with *Nco*I and *Bam*H1 to eliminate a 544 bp fragment containing a phleomycin resistance gene. The *hydPt-1* cDNA was amplified by PCR (see above). The *HydPt-1* genomic copy was amplified by PCR from a recombinant phage DNA containing the *HydPt-1* genomic fragment of *Pisolithus* (Martin, Voiblet, Murphy and Tagu unpublished data). The sequences of the primers were 5'-catgccATGGAGTTCGCCGCCGTCGTCGCTTA-3', and 5'-cgcggatccAGACGTCAACGCATTGCACGG-3'. They allowed the amplification of the coding and the 3'-untranslated sequences of *hydPt-1*. The first primer was bordered by a *Nco*I site whereas the second by a *Bam*HI site (underlined). These fragments (618 bp for the cDNA and 779 bp for the

genomic copy) were cloned in the pSGFT digested vector. Recombinant plasmids were checked by restriction analyses and sequencing.

Nucleic acid manipulations

Total DNA was extracted from yeast (Adams *et al.* 1997) and *H. cylindrosporum* (Marmeisse *et al.* 1992). For Southern blot analyses, five µg genomic DNA were digested with 200 units of the appropriate restriction enzyme. Electrophoresis, transfer and hybridization were performed in standard conditions (Sambrook *et al.* 1989). Yeast RNA was extracted by using the RNeasy kit from Qiagen. *H. cylindrosporum* and *Pisolithus* RNA were extracted (Logeman *et al.* 1987) and northern hybridization were performed (Sambrook *et al.* 1989). The probes used for hybridization were either the *hydPt-1* cDNA, or the Internal Transcribed Spacer (ITS) of the nuclear ribosomal DNA from *Pisolithus* (Carnero Diaz *et al.* 1997) containing the 5.8*S* rDNA. They were labelled by random priming using ^{32}P radiolabelled dCTP. Hybridization signals were numerized by the Personal Molecular Imager fx and quantified by the Quantity One program (Bio-Rad).

Protein manipulations

Yeast soluble proteins were extracted (Ruiz-Herrera *et al.* 1994). Briefly, 6 x 10^7 yeast cells were pelleted and boiled in a 2% SDS containing buffer during three min and vortexed in presence of glass beads. After centrifugation, the pellet containing insoluble material was kept for cell wall protein extraction. Supernatant was filtrated and concentrated by slight evaporation. Total protein content was estimated by SDS-polyacrylamide gel electrophoresis (PAGE). The cell wall pellet was washed three times in 1 M NaCl and water, resuspended in 10% SDS and boiled for 10 min. After centrifugation, the supernatant containing SDS-soluble polypeptides was separated from the SDS-insoluble pellet kept for hydrophobin extraction (de Vries *et al.* 1993) by sonication in pure trifluoracetic acid (TFA). The TFA-soluble fraction was recovered, evaporated under N_2 and resuspended in 10 mM Tris, pH 7, before SDS-PAGE analysis.

H. cylindrosporum soluble proteins (Burgess *et al.* 1995), cell walls and cell wall proteins (Laurent *et al.* 1999), and hydrophobins (de Vries *et al.* 1993; Tagu *et al.* 2001) were extracted and purified following published protocols. Excretion of the HYDPt-1 polypeptide was checked on filtrated culture media by air bubbling for the induction of hydrophobin self-assembly at the air - liquid interface (Wösten *et al.* 1993).

Transmission electron microscopy and immunolabelling

Yeast cells and the mycelium of *H. cylindrosporum* and *Pisolithus* were fixed in 2.5% (v/v) glutaraldehyde in 10 mM Na-phosphate buffer (pH 7.2) overnight at 4°C, and embedded in LRWhite resin (Tagu *et al.* 2001). Immunogold labelling was performed on thin sections (Balestrini *et al.* 1996) and observed with a Philips CM10 transmission electron microscope. The antibodies raised against HYDPt-1 were used at the dilution of 1:500.

RESULTS

Expression of HYDPt-1 in *S. cerevisiae*

To express the *hydPt-1* sequence in yeast, the corresponding cDNA was cloned under the control of the yeast *gpd* gene. No transformed yeast cells could be recovered when the *hydPt-1* cDNA was cloned into a multicopy non-integrative 2 µm plasmid (not shown). In contrast, several transformed yeast cells were selected when the unique copy centromeric plasmid pG-1 was used. Selected colonies, growing on the minimal medium, were analysed by Southern and northern blotting. The presence of the *hydPt-1* sequence in their genome (data not shown) together with the detection of transcripts in the total RNA fraction (Fig. 1) confirm the integration and heterologous transcription of the hydrophobin gene. However, no differences in growth or aggregation were detected in any of these transformants when compared to the wild type. The presence of the HYDPt-1 polypeptide was therefore looked for in i) the soluble protein fraction, ii) the SDS-soluble cell wall polypeptide fraction, iii) the SDS-insoluble cell wall fraction treated with TFA, and iv) the culture medium. HYDPt-1 polypeptide was not detected in either fraction by using western blot analysis with polyclonal antibodies raised against the recombinant HYDPt-1 polypeptide (Tagu *et al.* 2001). Immunocytolocalization did not reveal any specific labelling in the transformed yeast cells (Fig. 2B).

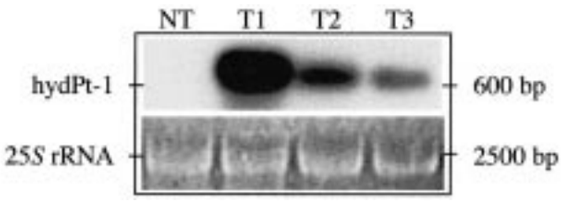

Fig. 1 - Steady state level of *hydPt-1* transcripts in transformed yeast cells. Total RNAs (5 µg) were separated by electrophoresis in a denaturing agarose gel, transferred to a Nylon filter, and hybridized with a the ^{32}P-labelled *hydPt-1* cDNA (upper panel). NT: non transformed yeast cells. T1, T2, T3: three different transformed yeast cells. Lower panel: 25*S* rRNA loaded in each lane (ethidium bromide staining).

These observations indicated that the HYDPt-1 polypeptide did not accumulate in these cells.

Expression of HYDPt-1 in *H. cylindrosporum*

A chimeric construct containing the hydrophobin cDNA *hydPt-1* driven by the *gpd* promoter of *S. commune* was introduced in *H. cylindrosporum*. Co-transformation experiments allowed the selection of 14 resistant colonies. Southern blot analyses revealed that five of them had integrated the *hydPt-1* cDNA in their genome (Fig. 3A) at one to five copies. The hydrophobicity of these mycelia was checked by the absorption of water droplets on their surface (Talbot, Ebbole & Hamer 1993). In all transformants, the droplets were absorbed by the mycelium at a rate identical to that observed with the non transformed hydrophilic mycelium. Thus, the surface hydrophobicity of *H.*

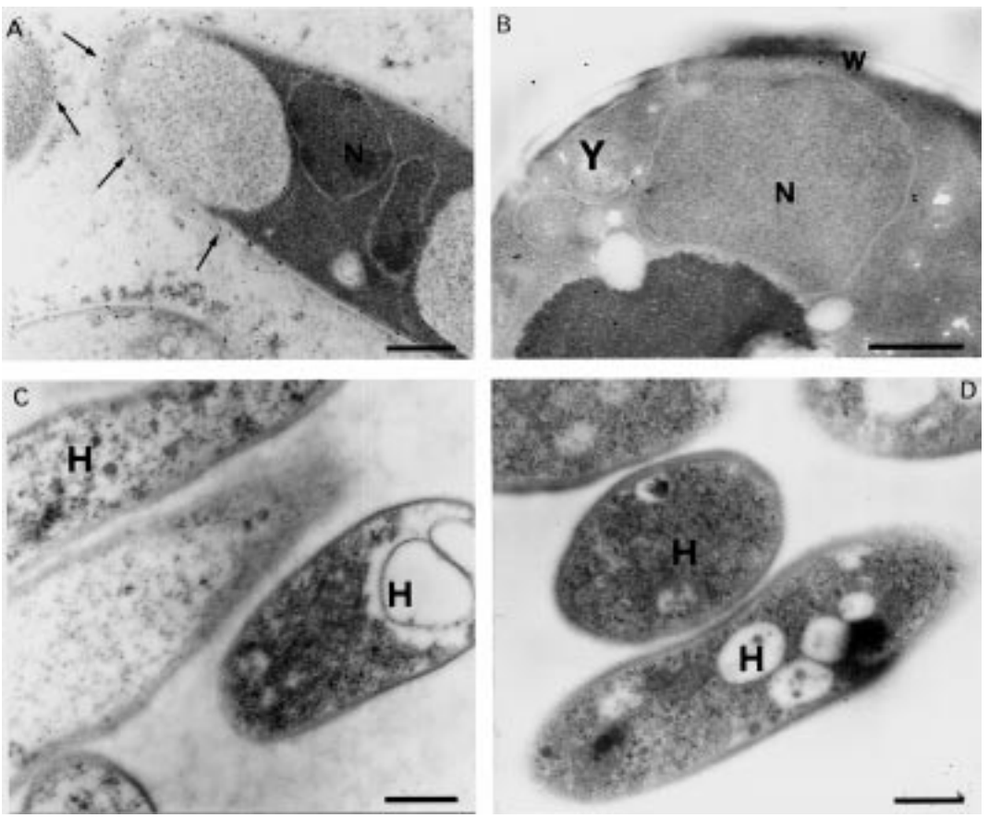

Fig. 2 - Immunogold localization of HYDPt-1 hydrophobins on thin sections of different fungal cells. **A**: Specific labelling (arrows) of cell walls in pure culture grown hyphae of *Pisolithus* after treatment with the anti-HydPt-1 antibodies. **B**: No specific labelling could be observed on transformed yeast cells. **C**: Absence of labelling on the IF1 transgenic *H. cylindrosporum* mycelium transcribing the *HydPt-1* gene. **D:** The non transformed wild type mycelium of *H. cylindrosporum* does not show any labelling. Bars correspond to 0.4 µm. H, hyphae; N, nucleus; W, wall; Y, yeast cell.

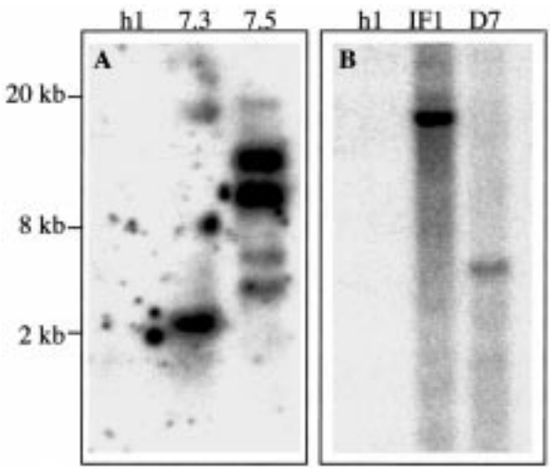

Fig. 3 - Southern analysis of *H. cylindrosporum* mycelia transformed with the *hydPt-1* cDNA without introns (A) or with introns (B). Total DNA (10 µg) was digested with *Bam*H1 (A) or *Eco* RI (B) and hybridized with a ^{32}P-labelled *hydPt-1* cDNA. h1: untransformed monokaryotic mycelium. 7.3, 7.5: monokaryotic mycelium transformed with the *hydPt-1* cDNA. IF1, D7: monokaryotic mycelium transformed with the genomic copy of *HydPt-1* gene containing three introns.

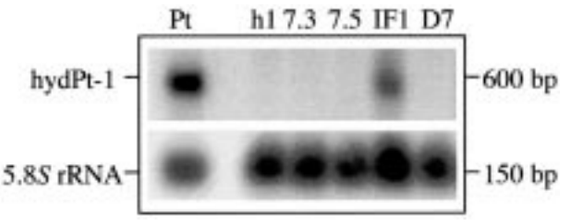

Fig. 4 - Level of *hydPt-1* transcripts in transformed *H. cylindrosporum* mycelia. Total RNAs (10 µg) were separated by electrophoresis in a denaturing agarose gel, transferred to a Nylon filter, and hybridized with a ^{32}P-labelled *hydPt-1* cDNA (upper panel) or an ITS sequence containing the 5.8*S* rRNA (lower panel). Pt: non transformed *Pisolithus* mycelium. h1: untransformed monokaryotic mycelium. 7.3, 7.5: monokaryotic mycelia transformed with the *hydPt-1* cDNA. IF1, D7: monokaryotic mycelium transformed with the *HydPt-1* gene containing three introns.

cylindrosporum was not altered by the heterologous expression of *hydPt-1* cDNA. Furthermore, *hydPt-1* mRNA were not detected in any of these mycelia either by northern blot analysis (Fig. 4) or by RT-PCR (data not shown). The *gpd* promoter of *S. commune* is known to be functional in *H. cylindrosporum* (Jargeat *et al.* 2000) and cannot account for the absence of *hydPt-1* expression.

As recently shown (Lugones *et al.* 1999; Scholtmeijer *et al.* 2001), the presence of introns can be required for heterologous gene transcription in filamentous fungi. For this reason, the *HydPt-1* genomic sequence, which contains three introns, was tested in the chimeric construct. Southern blot analysis demonstrated that two (D7 ad IF1) out of the seven transformants contained the *HydPt-1* gene sequence (Fig. 3B) at low copy. For IF1, *hydPt-1* mRNAs of the expected size were detected (Fig. 4). Their accumulation was five times lower than in *Pisolithus* hyphae. No *hydPt-1* transcript could be detected in D7.

The water droplet test showed that overexpression of *hydPt-1* in the IF1 mycelium did not affect the hydrophobicity of the fungal surface. This mycelium was used to obtain synthetic mycorrhizas with *Pinus pinaster*, but no difference in the number of mycorrhizas formed or in their morphology was observed when compared with the wild type. The presence of the HYDPt-1 polypeptide was checked in the different polypeptide fractions as described for yeast cells: the protein was not detected in any of the sample. Similarly, immunocytolocalization did not show any specific HYDPt-1 accumulation in the IF1 transformed mycelium (Fig. 2C-D). Thus, the lack of altered phenotypes is likely to result from the absence of the HYDPt-1 polypeptide in the transformed mycelium.

DISCUSSION

This paper showed the heterologous expression of a hydrophobin gene in yeast and in *H. cylindrosporum*. We demonstrated that the *hydPt-1* cDNA was correctly transcribed in *S. cerevisiae*. However, introns had to be included in the *hydPt-1* sequence in order to get *hydPt-1* mRNA accumulation in hyphae of the basidiomycete *H. cylindrosporum*. This is in agreement with Lugones' work (Lugones *et al.* 1999) who reported that the heterologous expression in the basidiomycete *S. commune* of the ABH1 hydrophobin from *Agaricus bisporus* was dependent on the presence of introns in the transgene. In the present study, we thus obtained *H. cylindrosporum* hyphae accumulating the *hydPt-1* transcripts. The level of accumulation was about five times lower than in the native *Pisolithus* hyphae. This indicates that either

the *gpd* promoter is weaker than the *hydPt-1* promoter, or that the transgene was inserted in a low transcribed region of the genome of the recipient species.

The HYDPt-1 polypeptide was neither detected in yeast nor in *H. cylindrosporum* transformed cells. The synthesis rate of the polypeptide was so low that it was may be not detectable. Hydrophobins are encoded as precursors containing a signal peptide for their extracellular localization. It could be that the signal peptide of HYDPt-1 was not recognized as such in yeast and *H. cylindrosporum*. The foreign protein may not be synthesized and/or be unstable in yeast and *H. cylindrosporum* cells. In yeast, this hypothesis may explain why cells transformed by the multicopy non-integrative 2 µm plasmid, which leads to high levels of foreign protein expression, were not recovered, as a result of the potential toxicity of HYDPt-1 in yeast when expressed in large amount. Finally, codon usage in yeast (http://genome-www.stanford.edu/Saccharomyces/codon_usage.html) could restrict the translation of the *HydPt-1* sequence, as several adjacent unusual codons are found in this sequence (e.g.: the TGC cysteine codon which is very rare in *S. cerevisiae*).

Kershaw *et al.* (1998) were able to complement a mutant of the ascomycete *Magnaporthe grisea* (deficient in *MPG1* hydrophobin production) by heterologous expression of various hydrophobin sequences. However, the *hydPt-1* sequence was not included in their analyses. Three main differences exist between these studies: i) introns were not necessary for the accumulation of heterologous hydrophobin RNA in *M. grisea*, ii) the majority of the heterologous sequences were expressed under the *M. grisea* hydrophobin gene promoter, and iii) a mutant deficient in hydrophobin production was used for functional complementation of *M. grisea*.

Our investigation confirms the possibility of heterologous transcription of ectomycorrhizal fungi genes in yeast cells. It is the first report of a gene being transfered from one ectomycorrhizal fungus to another. In this respect, our results emphasized the importance of introns in the accumulation of foreign transcripts. They also stress the difficulties in controlling the heterologous expression of the hydrophobin protein of *Pisolithus*. Homologous promoters will problably facilitate the production of the foreign protein in a timely and spatially regulated pattern.

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