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SHORT NOTE

Functional expression of the green fluorescent protein in the ectomycorrhizal model fungus *Hebeloma cylindrosporum*

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Abstract *Hebeloma cylindrosporum* is a model fungus for mycorrhizal studies because of its fast growth rate, simple nutritional requirements, and completion of its life cycle in vitro, and because it is amenable to transformation. To advance cell biological research during establishment of symbiosis, a tool that would enable the direct visualisation of fusion proteins in the different symbiotic tissues [namely, the expression of reporter genes such as Green Fluorescent Protein (GFP)] was still a missing tool. In the present study, *H. cylindrosporum* was transformed using *Agrobacterium* carrying the binary plasmid pBGgHg containing the *Escherichia coli* hygromycin B phosphotransferase (*hph*) and the EGFP genes, both under the control of the *Agaricus bisporus* glyceraldehyde-3-phosphate dehydrogenase promoter. EGFP expression was successfully detected in transformants. The fluorescence was uniformly distributed in the hyphae, while no significant background signal was detected in control hyphae. The suitability of EGFP for reporter gene studies in *Hebeloma cylindrosporum* was demonstrated opening up new perspectives in the *Hebeloma* genetics.

Keywords EGFP · GFP expression · Ectomycorrhiza · *Hebeloma cylindrosporum*

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Introduction

Hebeloma cylindrosporum is a model fungus for mycorrhizal studies, as this organism combines different advantages distinguishing it from other mycorrhizal fungi, such as a fast growth rate, simple nutritional requirements, completion of its life cycles under laboratory conditions, and the amenability to genetic transformation (Marmeisse et al. 2004). Moreover, a fast in vitro mycorrhiza formation with *Pinus pinaster* can be established for both monokaryotic and dikaryotic strains (Marmeisse et al. 2004). Furthermore, two expressed sequence tag programs have been conducted with *Hebeloma* grown on different nitrogen sources (Wipf et al. 2003) and on different conditions regarding potassium and phosphate availability (Lambilliotte et al. 2004). *Pinus pinaster*, due to its high economic value, is one of the most studied gymnosperms, thus a better understanding of the fungal symbiont may provide a way to improve plant

properties. About 1,500 expressed sequenced tags (ESTs) exist from different parts of the tree and have been combined with approximately 10,000 genes from *Pinus taeda* (Kirst et al. 2003) for the genetic study of the mycorrhizal interactions (Marmeisse et al. 2004).

In recent years, new insights at a molecular level in the nutrient exchange mechanisms involved in the ectomycorrhizal symbiosis have been found in the *H. cylindrosporum–P. pinaster* association. Especially nitrogen uptake and metabolism have been extensively studied, and several key genes have been isolated from *Hebeloma cylindrosporum*; three ammonium transporters (HcAMT1-3) (Javelle et al. 2003), one nitrate transporter (HcNRT2) (Jargeat et al. 2003), a general amino acid permease (Wipf et al. 2002), two peptide transporters (HcPTR2A and 2B) (Benjdia et al. 2006), nitrate reductase (HcNAR1) and nitrite reductase (HcNIR1) (Jargeat et al. 2003), glutamine synthetase (GLN1) and glutamate dehydrogenase (GDHA) (Javelle et al. 2003). Expression analysis by RNA gel blot or RT-PCR analyses were performed for all these genes.

Nevertheless, an essential tool [namely, the expression of reporter genes such as Green Fluorescent Protein (GFP)] that will allow direct visualization of their pattern of transcription (e.g., subcellular distribution, in situ expression) in the different symbiotic tissues and the localization of the transporters at the different interfaces was still missing. Such a tool is necessary to clarify the role of those different genes in the symbiosis (e.g., are new transport events switched on as a result of interactions between the symbiotic partners?). Recently, a transformation protocol for ectomycorrhizal fungi based on the capacity of *Agrobacterium tumefaciens* to transfer its T-DNA to fungal cells as it does to plant cells (Bundock et al. 1995) was developed by Pardo et al. (2002). This method represents an interesting alternative to the traditional protoplast-mediated transformation, as this protocol can be implemented on intact fungal hyphae, thus circumventing the need for making protoplasts, which is tedious, time-consuming, and restricted to a limited number of ectomycorrhizal fungal species. The development of such a transformation method was the first essential step in the establishment of the expression of reporter genes in *H. cylindrosporum*.

In this study, we report the use of the *Agrobacterium tumefaciens*-mediated transformation method for a successful expression of EGFP in *Hebeloma cylindrosporum*. Furthermore, the expression was stable after several rounds of subculturing.

Materials and methods

Strains, media, and plasmids

The *H. cylindrosporum* haploid strain h7 isolated from single spore germination (Debaud and Gay 1987) was cultivated on YMG agar medium at 23°C in the dark. The binary plasmid pBGgHg (Chen et al. 2000) was kindly provided by C. Peter Romaine (The Pennsylvania State University). This vector consists of a pCAMBIA1300 backbone containing the *Escherichia coli* hygromycin B phosphotransferase (*hph*) and the EGFP genes, both under the control of the *Agaricus bisporus* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and the cauliflower mosaic virus (CaMV) 35S terminator.

The pBGgHg plasmid was propagated in the hypervirulent *A. tumefaciens* AGL-1 strain then used for *H. cylindrosporum* transformation. *Agrobacterium tumefaciens* was grown as described by Combier et al. (2003).

Agrobacterium tumefaciens-mediated transformation

The transformation was carried out as described by Combier et al. (2003). Briefly, 2-week-old mycelia grown on YMG agar medium were macerated using a razor blade and the hyphal fragments were transferred to YMG liquid medium and grown for 1 week. Maceration with an Ultra-turrax homogenizer and sub-cultivation in fresh liquid medium was repeated every 2 days to obtain fast growing mycelium. Two thalli obtained in 90-mm Petri dishes were finally macerated and resuspended in 50-ml YMG medium. Fifty microliters of macerated mycelium was adsorbed on a 1-cm-diameter glass microfibre disc (GF/D Whatman, UK).

A. tumefaciens was grown at 28°C for 2 days in LB medium supplemented with 50 μg ml^{-1} carbenicillin and 50 μg ml^{-1} kanamycin. Bacterial cell suspensions were subsequently diluted to an OD_{600} of 0.15 in Induction Medium [IM: l^{-1}:10.5 g K_2HPO_4; 4.5 g KH_2PO_4; 1 g $(NH_4)_2SO_4$; 0.5 g Na_3-citrate $2H_2O$; 0.2 g $MgSO_4$ $7H_2O$; 1 mg thiamine-HCl; 2 g glucose; 0.5% glycerol; 40 mM MES, pH 5.3; 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} carbenicillin (Hanif et al. 2002)]. The cells were grown for 5 h before *A. tumefaciens* and *H. cylindrosporum* were cocultivated as follows: 100 μl of bacterial culture (~1×10^3 bacteria) was added to each glass microfibre disc containing macerated fungal mycelium. Discs were placed on cocultivation medium (De Groot et al. 1998) (l^{-1}: 5 g KH_2PO_4; 2.5 g $(NH_4)_2SO_4$; 0.5 g $CaCl_2$ $2H_2O$; 1.5 g $MgSO_4$ $7H_2O$; 0.25 g NaCl; 0.01 mg thiamine-HCl; 2 g glucose; 10 mg $FeCl_3$; 0.5% glycerol; 40 mM MES-KOH pH 5.3) supplemented with 200 μM acetosyringone. The plates were incubated at 23°C for 96 h. After cocultivation, glass microfibre discs were transferred to YMG medium supplemented with 200 μM claforan (Aventis, Germany) to counter select *Agrobacterium* cells and 100 μg ml^{-1} hygromycin B to select for fungal transformants. Each transformant was subsequently transferred to YMG agar medium containing 200 μg ml^{-1} hygromycin B.

Molecular analysis

The integration of the transformed DNA into the *H. cylindrosporum* genome was tested by DNA gel blot analysis. Genomic DNA was isolated from the transformants grown in liquid YMG medium (van Kan et al. 1991). *H. cylindrosporum* mycelia were grown on cellophane-covered YMG plates, harvested, frozen in liquid nitrogen and ground to a fine powder. Extraction buffer (NaCl 0.5 M, SDS 1%, Na_2EDTA 10 mM, Tris–HCl 10 mM, pH 7.5) was heated to 65°C and 2 ml were added to fungal material (1 g). After incubation for 10 min at room temperature (RT), 2 ml phenol (pH 8) was added and phases were separated by centrifugation at 13,000 rpm for 15 min. The upper phase was extracted a second time with 2 ml chloroform/isoamylic alcohol (24:1) and centrifuged at 13,000 rpm for 15 min. Between each step, extracts were incubated for 10 min at RT. The supernatant was transferred to a new Eppendorf tube containing 10 µl RNase A (10 mg ml^{-1}) and incubated for 15 min at 37°C. RNase A was then extracted with 400 µl phenol and the samples were centrifuged at 15,000 rpm for 10 min. DNA was precipitated with 400 µl isopropanol at RT for 15 min. After centrifugation at 13,000 rpm for 10 min, sediments were washed twice with 400 µl 70% ethanol, air dried, and dissolved in 20 µl MilliQ water.

Southern blot analysis

Genomic DNA (10 µg) from control and putative transformants was restricted with *Eco*RV and then fractionated on a 0.6% agarose gel and blotted onto positively charged nylon membranes (Amersham) by using standard methods (Sambrook et al. 1989). For the detection of genomic insertions, we used the ^{32}P-*hph* as a probe constructed with a random priming kit (Fermentas). High stringency hybridization was accomplished with standard protocols (Sambrook et al. 1989).

RT- PCR analysis of *H. cylindrosporum* transformants

Total RNA was extracted from fresh material frozen using Invitrogen's (Karlsbad, California, USA) ChargeSwitch Total RNA Cell Kit. The extraction was done following the protocol (including the DNase treatment) with the following modifications: after the incubation with the lysis buffer, samples were centrifuged at 11,000 rpm at 4°C for 3 min. The supernatant was removed and transferred to a clean tube. cDNA synthesis was performed with Invitrogen's Thermo Script RT-PCR system kit, following the standard protocol. Three to 5 µl of the resulting cDNA was used as a template for PCR amplification using Genaxxon's DF Taq DNA polymerase with the following oligonucleotide primers: 5′ CAC ATG AAG CAG CAC GAC TT 3′(EGFP_forward) and 5′ TGC TCA GGT AGT GGT TGT CG 3′(EGFP_reverse) (Grimaldi et al. 2005).

Fluorescence and confocal microscopy

H. cylindrosporum mycelium was grown for 1 week in liquid YMG medium and then macerated with an Ultra-thurrax as described before and transferred in new liquid YMG. Three- to 5-day-old mycelium was used for microscopic analysis. Microscopic analyses were done with a LEICA TCS 4D confocal microscope (Leica, Wetzlar, Germany) using a ×40 and a ×100 oil immersion lens under the confocal microscope equipped with an argon/krypton laser. GFP fluorescence was imaged using excitation with the 488-nm line of the argon/krypton laser and a 515-nm long pass or 530-band pass emission filter. Merging of images was performed with the Adobe Photoshop (Adobe Systems, Mountain View, CA, USA) image processing software.

Results and discussion

Molecular transformation analysis

Agrobacterium tumefaciens-mediated transformation of filamentous fungi has become an essential tool for functional genomics (Michielse et al. 2005). The homo-basidiomycete ectomycorrhizal fungi *Paxillus involutus*, *Suillus bovinus*, *Hebeloma cylindrosporum*, *Pisolithus* spp., and *Laccaria bicolor*, and the ascomycete *Tuber borchii* (Grimaldi et al. 2005) besides several other non-mycorrhizal fungi (Michielse et al. 2005), have successfully been transformed with *Agrobacterium*. Transformation of *H. cylindrosporum* was carried out using the binary vector pBGgHg, as it had already been shown to be efficient for T-DNA transfer and integration into the *Hebeloma* genome (Combier et al. 2003). Out of 100 fungal colonies cocultivated with *Agrobacterium*, six transformants could be isolated on the selection medium, similar to the efficiency of 12 to 18% described by Combier et al. (2003). The integration of the T-DNA was tested by Southern blot analysis with a ^{32}P-*hph* probe. Out of the six transformants tested, five seemed to have a single insertion of the T-DNA, and in one case the transferred T-DNA was integrated three times into the genome (Fig. 1). The frequency of single insertions of 83% observed in this study is in the same order as the 61% observed by Combier et al. (2003).

Microscopic fluorescence analysis

In the present study, DNA gel blot analysis (Fig. 1) showed the correct integration of the pBGgHg plasmid into the *H. cylindrosporum* genome. To investigate the correct functional expression of the EGFP-gene, transformants were grown in

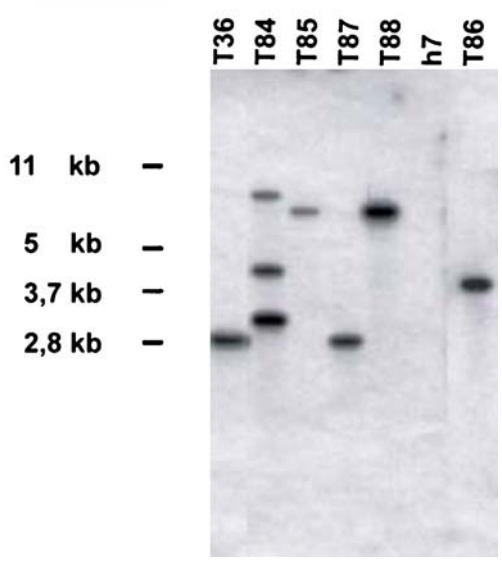

Fig. 1 Southern blot analysis of transformants of *Hebeloma cylindrosporum*. The genomic DNA of control (h7 wild-type untransformed strain) and transformants was restricted with *Eco*RV, gel-size fragmented, transferred onto a nylon membrane and probed with the radiolabelled *hph* gene. Molecular weights are indicated on the *left*

liquid YMG medium. In comparison with the wild type, for which no significant background fluorescence was observed, a weak EGFP expression was detected in the multiple insertion transformants and a clear EGFP expression was detected in five of the transformants (Fig. 2). EGFP expression in these five transformants was confirmed by RT-PCR, whereas no expression was detected in *H. cylindrosporum* wild-type strain h7. Uniformly distributed EGFP fluorescence is found in the hyphae, with higher accumulation in specific organelles, probably representing nuclei (Fig. 2). Nuclear accumulation of free GFP is typical for all eukaryotic organisms as GFP is a small protein, which is below the exclusion size for passive transport through the nuclear pore complex, in contrast to proteasomes (Fabre and Hurt 1994). In previous studies, Chen et al. (2000) could efficiently transform *Agaricus bisporus* with the pBGgHg vector that was used in the present study, but were unable to detect any GFP fluorescence. Moreover, using the same vector, Combier et al. (2003) were able to obtain the integration of the EGFP construct as part of the T-DNA into the *H. cylindrosporum* genome but did not observe GFP fluorescence. Recently, Burns et al. (2005) successfully transformed the mushroom *Agaricus bisporus* with a GFP expressing vector and concluded that efficient GFP expression in *A. bisporus* requires introns. Introns were also used for GFP expression in the two previously transformed homobasidiomycetes *Schizophyllum commune* (Lugones et al. 1999) and *Phanerochaete chrysosporium* (Ma et al. 2001). Nevertheless, Rodriguez-Tovar et al. (2005) recently reported GFP expression in *Pisolithus tinctorius* without the requirement of

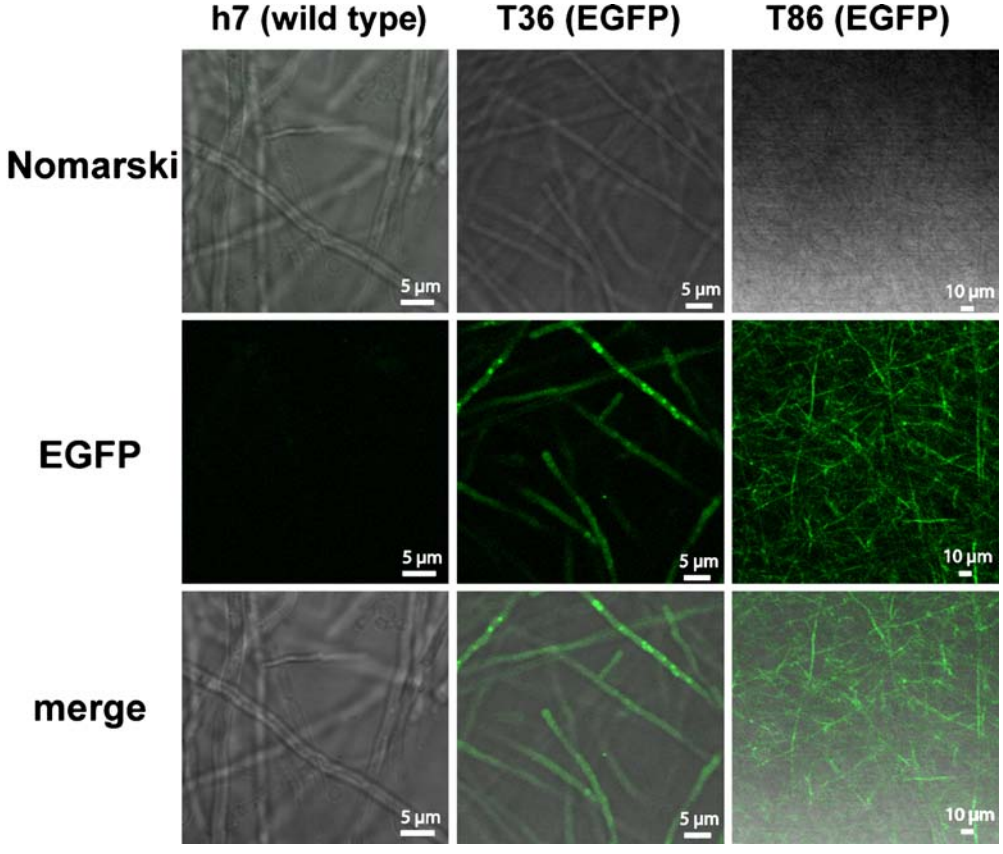

Fig. 2 Fluorescence analysis of *Hebeloma cylindrosporum* transformants T36 and T86 using phase contrast (Nomarski) and fluorescence (EGFP) microscopic images. A merge of the two images is shown in the *third row* (merge). No background fluorescence was detected in the h7 wild-type control strain as shown in the *first column*

introns. Also in this case, introns seem dispensable for the functional expression of the EGFP gene. In the case of *H. cylindrosporum*, an essential factor for successful EGFP detection is the mycelial fitness. Growth of mycelia in liquid medium and direct mounting for microscopy seemed to have avoided stress-induced responses, which usually interfere with GFP observation (for instance, during transfer from solid to mounting media). Stress may lead to an autofluorescence detectable both with excitation at 488 or 568 nm. With the same vector used here, Grimaldi et al. (2005) could show transient EGFP expression in *Tuber borchii*. GFP expression in the *Hebeloma* transformants shown here was stable through multiple rounds of subculturing under standard growth conditions without antibiotics.

These results demonstrate the feasibility of using EGFP as a reporter gene in *H. cylindrosporum*. The successful transformation of *Hebeloma* opens up new possibilities for future research, e.g., to determine the regulation and cellular localization of the nitrogen and sugar transporters involved in nutrient acquisition at the mycelial surface and in the regions of nutrient exchange between host and fungus. Moreover, the reporter gene can be used for many of the yet uncharacterized *H. cylindrosporum* genes whose location in the cell was unknown.

Conclusion

After the development of the stable *Agrobacterium*-mediated transformation of the ectomycorrhizal fungus *H. cylindrosporum* with *hph* as a selection marker by Combier et al. (2003), new perspectives in *H. cylindrosporum* genetics were opened. The present work describes the first application of this method for the expression of a gene in *H. cylindrosporum* mycelia, which lays the ground for the development of further molecular tools such as RNA-mediated interference or overexpression of selected genes. The suitability of EGFP as a reporter gene for *H. cylindrosporum* studies was also demonstrated. With the help of this method, new hints on the expression of previously isolated genes, (e.g., genes involved in nitrogen transport and metabolism) in the free-living *H. cylindrosporum* mycelia or even in the ectomycorrhizal association can be addressed.

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