Green fluorescent protein expression in the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*

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Abstract

The symbiotic basidiomycete *Hebeloma cylindrosporum* is a model fungal species used to study ectomycorrhizal symbiosis at the molecular level. In order to have a vital marker, we developed a green fluorescent protein (GFP) reporter system efficiently expressed in *H. cylindrosporum* using the *sgfp* coding region bordered by two introns fused to the saprophytic basidiomycete *Coprinopsis cinerea cgl1* promoter. Expression of this reporter system was tested under different environmental conditions in two transformants, and glucose was shown to repress *gfp* expression. Such a reporter system will be used in plant–fungus interaction to evaluate sugar supply by the plant to the compatible mycorrhizal symbiont and to compare the expression of various genes of interest in the free-living mycelia, in the symbiotic (mycorrhizas) and the reproductive (fruit bodies) structures formed by *H. cylindrosporum*.

Introduction

The basidiomycete fungus *Hebeloma cylindrosporum* is used as a model species to study different characteristics of the ectomycorrhizal symbiosis, from gene to ecosystem (Marmeisse *et al*., 2004). Its entire life cycle can be obtained *in vitro*, it can be genetically transformed, and different mutants affected in nutrient uptake (Marmeisse *et al*., 1998) or mycorrhiza formation (Gay *et al*., 1994, Combier *et al*., 2004) have been characterized. Many of the genes involved in nitrogen assimilation and absorption pathways have been cloned and their regulation patterns have been characterized (Jargeat *et al*., 2000, 2003; Javelle *et al*., 2001, 2003; Wipf *et al*., 2002). Furthermore, many other functional genes have been partially sequenced as expressed sequence tags (Wipf *et al*., 2003, Lambilliotte *et al*., 2004).

Obtaining a reporter gene system for this fungus should increase its interest as a model species by allowing *in situ* gene expression studies conducted during ectomycorrhiza differentiation and functioning. As it requires only blue light and oxygen to induce fluorescence, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a highly convenient marker for the specific labelling of living cells and spatio-temporal expression studies (Cubitt *et al*., 1995). The GFP is now widely used in several fungi, including saprophytic basidiomycete species (Lugones *et al*., 1999; Ma *et al*., 2001; Burns *et al*., 2005) and recently ectomycorrhizal ones such as *Tuber borchii* (Grimaldi *et al*., 2005) and *Pisolithus tinctorius* (Rodrìguez-Tovar *et al*., 2005). Several improved GFP-mutated proteins showing various spectral properties, higher protein stability or enhanced fluorescence intensity are available (Cubitt *et al*., 1995; Yang *et al*., 1996; Patterson *et al*., 1997; Cormack, 1998).

The objective of this study was to determine whether a functional GFP could be produced in the symbiotic basidiomycete fungus *H. cylindrosporum* and whether its expression could be modulated under different environmental

conditions. For this purpose, we used the *sgfp* coding region bordered by two introns fused to the promoter of the galectin-encoding gene *cgl1* from *Coprinopsis cinerea* (Boulianne *et al.*, 2000).

Materials and methods

Fungal strains and culture conditions

The h1.7 strain used for the PEG-mediated protoplast transformation is a haploid nitrate reductase-deficient strain derived from the wild-type haploid h1 strain (mating-type *A1 B2*) of *H. cylindrosporum* (Marmeisse *et al.*, 1998). Mycelia were grown on solid media in Petri dishes at 22 °C in the dark. The standard medium was the modified N2P3 medium (Marmeisse *et al.*, 1998) supplemented with 3.78 mM ammonium and 12.6 mM glucose, which was overlaid with a cellophane membrane to allow recovery of the mycelia for further analyses. To study the effect of different environmental variables on the expression system, transformants were cultivated 10 days under different environments that differed with respect to photoperiodic illumination (8 h light/16 h dark day^{-1} versus constant dark) and glucose concentration (100 versus 12.6 mM). To further analyse the effect of external glucose concentrations on the expression system, transformants were cultivated 10 days on standard media containing a range of glucose concentrations (1, 10 and 100 mM) and then transferred for the 2 last days on an identical fresh medium in order to be sure of the sugar concentration acting on *gfp* expression.

Plasmid construction and *H. cylindrosporum* protoplast transformation

Plasmid pP1 was constructed for expression of the SGFP-TYG gene (Spellig *et al.*, 1996) under the control of *C. cinerea cgl1* promoter and terminator sequences (GenBank accession no. AAF34731.1). The *sgfp* coding region (GenBank accession no. AAT34982.1) was amplified from the pblue-SGFP-TYG-nos SK plasmid (Spellig *et al.*, 1996) using primers AgeI-5′egfp (CTACCGGTAGTTCAACATCATGGTGAGCA AGGGCGAG; the AgeI restriction site and the start codon of the *sgfp* coding sequence are underlined) and 3′egfpTYG-nos-NotI (CGCGGCCGCTTTACTTGTACAGCTC; the NotI restriction site is underlined). The corresponding PCR-fragment was digested by AgeI and NotI and was subcloned into the *cgl1* containing plasmid pCG1 (P. Walser, unpublished) digested by AgeI and EcoRV. This plasmid construct was verified by sequencing. The HpaI/BamHI restriction fragment from pCG1 containing 1286 bp *C. cinerea cgl1* promoter, *cgl1* intron 1 (naturally positioned in the 5′ untranslated region of the *cgl1* gene; Boulianne *et al.*, 2000), the SGFP-TYG gene, *cgl1* intron 2 (naturally positioned in the 3′ untranslated region of the *cgl1* gene; Boulianne *et al.*, 2000) and 761 bp *cgl1* terminator was further subcloned into pBluescript II SK (+) digested with EcoRV and BamHI. Before transformation, protoplasts were released from ground mycelium (grown 1 day in 10 mL of liquid medium in Petri dishes) using 20 mg mL^{-1} Cellulase Onozuka (Serva), 5 mg mL^{-1} Driselase (Sigma) and 1 mg mL^{-1} Chitinase (Sigma) in 0.7 M mannitol for 4 h at 30 °C and filtered through bolting cloths. Cotransformation of *H. cylindrosporum* protoplasts was performed as described in Marmeisse *et al.* (1992) using 10^7 protoplasts, 5 μg of pGHPHT conferring hygromycin B resistance (Schuren & Wessels, 1998) and 5 μg of the pP1 plasmid.

Nucleic acid extractions and analyses

Hebeloma cylindrosporum DNA was extracted using the protocol of van Kan *et al.* (1991). For Southern blots, restriction enzyme-digested genomic DNA was fractionated on 0.7% agarose gels. Hybridizations were carried out under stringent conditions at 65 °C (Sambrook & Russell, 2001). Probes corresponding to the *sgfp* coding region and to the *cgl1* promoter were made from gel purified restriction enzyme digested-DNA fragments from the pP1 plasmid and labelled with [α-^{32}P]dCTP using the random labelling kit from Boehringer. PCR reactions using *Taq* DNA polymerase (Invitrogen) were carried out following standard protocols (Gryta *et al.*, 1997) and using specific oligonucleotide pairs deduced, respectively, from *cgl1* promoter and *sgfp* coding region sequences (Table 1).

Protein extraction and fluorometric determination of fluorescence intensity

Frozen mycelia (*c.* 1 g fresh weight) were ground in 2 mL extraction buffer (100 mM Tris pH 7.5; Triton × 100 1%; EDTA 2.5 mM; DTT 5 mM; PMSF 1 mM; pepstatin 5 μg mL^{-1}). After a 10-min centrifugation at 15 500 ***g*** at 4 °C, the supernatant was assayed for protein content and fluorescence. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The intensity of GFP fluorescence was determined on 2 mL supernatant using an SFM 23/B spectrofluorometer (Kontron Instruments). The excitation and emission wavelengths were, respectively, 489 and 511 nm.

Table 1. Primer nucleotide sequences specific to the *cgl1* promoter and to the *gfp*-coding region

Primer name*	Nucleotide sequence
Cgl1F	5′-GGGTGTCAGTCGGGCGTCAG-3′
Cgl1R	5′-GCTTGGGCCAGATATGACCAG-3′
gfpF	5′-GGACGGCGACGTAAACGGCC-3′
gfpR	5′-GATCGCGCTTCTCGTTGGGG-3′

*In the pP1 plasmid, cgl1F and cgl1R primers amplify a 362-bp fragment, gfpF and gfpR a 595-bp fragment and cgl1F and gfpR a fragment of 1278 bp.

The fluorescence measurements were normalized against protein extracts from h1.7 control strain grown for 10 days under standard conditions (in the dark at 22 °C on a medium containing 3.78 mM ammonium and 12.6 mM glucose), and specific fluorescence was calculated with respect to protein content.

Microscopy analysis

Microscopic observations were performed using a Zeiss Axioskop microscope coupled to a Zeiss AxioCamMRc5 digital camera. Differential interference contrast (DIC) was used for light microscopy. For fluorescence microscopy, Zeiss standard fluorescein-isothiocyanate filters were used. The images were recorded on Kodak Elite Chrome 400 films and converted into computer files using a SnapScan E50 scanner (Agfa) or acquired directly using a digital camera coupled with the AXIOVISION software (Carl Zeiss Vision GmbH, München, Germany). The images were converted using ADOBE PHOTOSHOP 4.0 software to generate composite images for publication.

Results and discussion

GFP expression in *H. cylindrosporum*

Protoplasts from the monokaryotic strain h1.7 of *H. cylindrosporum* were cotransformed with the pGHPHT plasmid conferring a hygromycin resistance and the pP1 plasmid containing the *sgfp* gene bordered by two introns under the control of the *C. cinerea cgl1* promoter. Southern blot genomic DNA analysis using a probe specific of the *sgfp* gene showed that 52 out of the 71 hygromycin-resistant transformants had integrated the pP1 plasmid in their genomes (as illustrated in Fig. 1a for nine of them). The number of plasmid integration sites per genome ranged from one to four in 87% of cases. Fluorescence microscopy observations failed to detect a fluorescence signal for most of the cotransformants, revealing that the *C. cinerea cgl1* promoter may not be strong enough for an efficient expression of the *sgfp* gene in *H. cylindrosporum*. Only two transformants (B17 and F67) displayed a significant green fluorescence as illustrated in Fig. 2 for the B17 transformant. This result demonstrates that the GFP protein can be synthesized in its active form in *H. cylindrosporum* cells. Fluorescence measurements on cellular extracts (illustrated in Fig. 3 for five transformants) confirmed the high fluorescence levels of the B17 and F67 transformants. Extracts from the F67 transformant were three to fourfold more fluorescent than those from B17. This could have resulted from the fact that B17 had only one copy while F67 had two plasmid copies integrated in its genome (Fig. 1a). However, as one nonfluorescent transformant had up to eight plasmid copies integrated in its genome, the strong fluorescence detected in

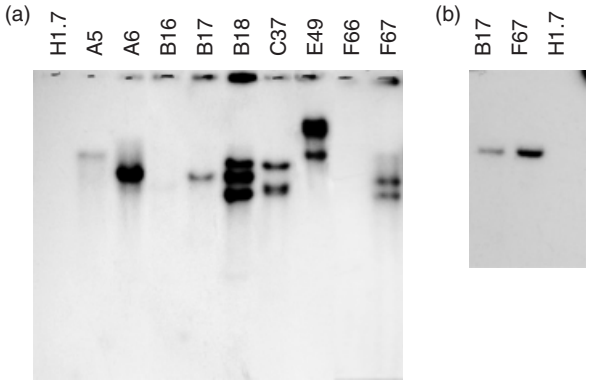

Fig. 1. Southern blot analyses of *Hebeloma cylindrosporum* strains obtained after transformation with the pP1 plasmid. Genomic DNA was digested (a) with EcoRV, which does not cut the pP1 plasmid and (b) with KpnI, which cuts twice in the pP1 plasmid. The hybridization was carried out under stringent conditions with probes corresponding to the *sgfp* coding region (a) and to the entire *cgl1-sgfp* chimeric region (b) of pP1. A5 to F67: hygromycin-resistant cotransformants. H1.7: untransformed control strain.

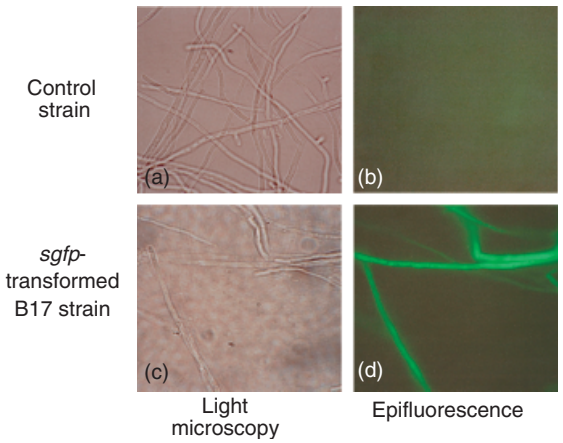

Fig. 2. *In vivo* fluorescence in *sgfp* transformant mycelia of *Hebeloma cylindrosporum*. Freshly grown mycelia of the h1.7 control strain (a, b) and of the B17 transformant (c, d) were observed under DIC light microscopy (a, c) and epifluorescence (b, d).

the B17 and F67 transformants is likely a consequence of the genomic environment in which plasmid integrations took place.

In both B17 and F67 transformants, the hybridization of KpnI-digested genomic DNA with a probe encompassing the entire *cgl1* promoter and the *sgfp* coding regions revealed a unique band of 2160 bp (Fig. 1b). This band corresponds to the chimeric DNA fragment present in the pP1 plasmid, showing that this part of the plasmid is not truncated in both transformants. To further detect whether no accidental deletions occurred in these two transformants, PCR amplifications of the *cgl1-sgfp* region were also carried out (data

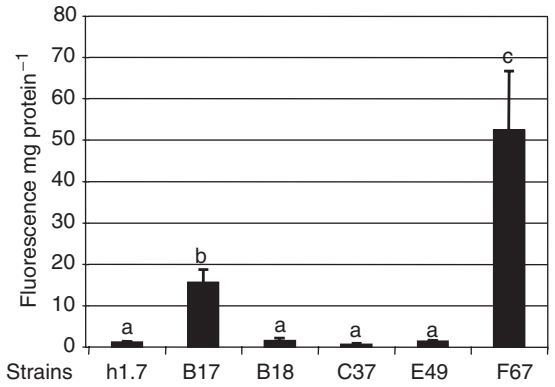

Fig. 3. Fluorescence intensity of protein extracts from different *sgfp* transformants of *Hebeloma cylindrosporum*. Soluble proteins were extracted from mycelia grown in the dark on 12.6 mM glucose containing medium. Fluorescence emission of the protein extracts was measured at 511 nm and expressed as arbitrary units (fluorescence mg protein^{-1}). The B17, B18, C37, E49 and F67 strains are *sgfp* transformants. The wild-type strain h1.7 has been used as a control. Values are the mean of three independent experiments ± SE. Values with the same letter (a, b or c) are not statistically different ($P < 0.05$).

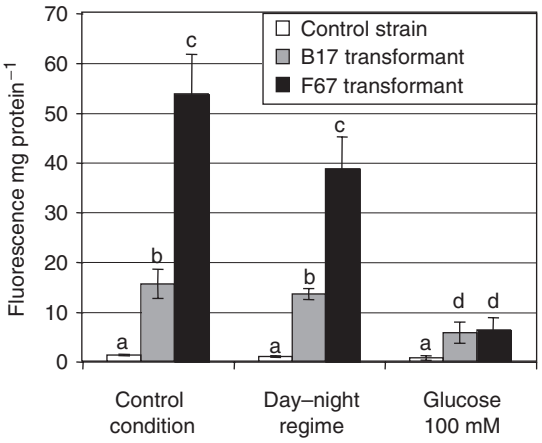

Fig. 4. Fluorescence intensity of protein extracts from two *sgfp*-expressing transformants (B17 and F67) of *Hebeloma cylindrosporum*. Soluble proteins were extracted from mycelia grown under two different environments: (i) in the dark (control condition) versus under a day–night regime and (ii) on 12.6 mM (control) versus 100 mM glucose. Fluorescence emission of the protein extracts was measured at 511 nm and expressed as arbitrary units (fluorescence mg protein^{-1}). The B17 and F67 *sgfp*-expressing transformants are indicated by grey and black bars, respectively. The nonfluorescent wild-type strain h1.7 (white bars) has been used as a control. Values are the mean of three independent experiments ± SE. Values with the same letter (a, b, c or d) are not statistically different ($P < 0.05$).

not shown). This analysis confirmed that the *cgl1* promoter was correctly fused to the *sgfp* coding region and not truncated in both B17 and F67 transformants.

Modulation of *gfp* expression

We tested the possibility of modulating the *sgfp* level in the mycelia of B17 and F67 transformants by growing them under different environmental conditions known or supposed to affect *cgl1* expression in *C. cinerea*. The CGL1 protein is produced in the young developing fruiting bodies (primordia) of *C. cinerea* but the transcriptional regulation of the *cgl1* gene is not known as opposed to the regulation of the second *C. cinerea cgl2* galectin-encoding gene that has been extensively studied (Boulianne *et al*., 2000; Bertossa *et al*., 2004). As the different *cgl* genes most likely arise from a gene duplication event (Boulianne *et al*., 2000), it is probable that light-regulated factors and nutritional cues (such as glucose and ammonium) controlling *cgl2* expression may also regulate *cgl1* transcription. The fluorescence intensity of B17 and F67 was then assayed in mycelia grown (1) under light–dark regimes, and (2) under a high glucose concentration (Fig. 4) and compared with the values measured under the control condition (in darkness on 12.6 mM glucose concentration).

We first compared expression under complete darkness (control condition) versus light–dark regimes that mimicked the natural day–night rhythm (Fig. 4). In *C. cinerea*, *cgl1* expression is first detected in the fruiting body primordium, which arises from the dikaryon under control of *A* mating-type genes after a light stimulation (Kües *et al*., 1998, 2002; Boulianne *et al*., 2000). In the *H. cylindrosporum* transformants, a light–dark regime did not affect SGFP protein accumulation (Fig. 4), thus suggesting that the *C. cinerea* consensus sequences for light regulation may not be functional in *H. cylindrosporum*.

The second tested factor was glucose concentration in the culture medium. High glucose concentration is known to repress the transcription of *cgl2* (Bertossa *et al*., 2004). This repression could result from the presence of a GC box in the promoter region of the *cgl2* gene, which is also present in the promoter of *cgl1*. This SYGGRG motif is involved in carbon catabolite repression in other fungi and is recognized by a highly conserved Cys2His2 zinc finger class transcription factors (Lundin *et al*., 1994; Takashima *et al*., 1996; Scazzocchio, 2000). In both B17 and F67 transformants, a high glucose concentration drastically decreased the level of fluorescence (Fig. 4). This suggests that *H. cylindrosporum*-regulatory proteins could exert a carbon catabolite repression on this heterologous promoter region. The exact role of the regulatory consensus sequences present in *cgl1* promoter remains to be demonstrated. However, this glucose catabolite repression effect is of some interest as it could be used to monitor *in situ* the bioavailability of sugars in the apoplastic spaces of ectomycorrhizas formed between *Pinus pinaster* and *H. cylindrosporum*, especially in the Hartig net and the mantle compartments. In order to know whether or not the *sgfp* construct can be used to appreciate apoplastic sugar concentration in symbiotic plants, we also tested the effect

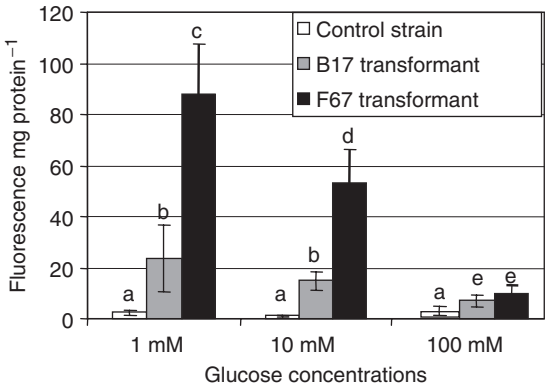

Fig. 5. Effect of external glucose concentrations on the fluorescence intensity of protein extracts from two *sgfp*-expressing transformants (B17 and F67) of *Hebeloma cylindrosporum*. Soluble proteins were extracted from mycelia grown in the dark under a range of external glucose concentrations (1, 10 and 100 mM). Fluorescence emission of the protein extracts was measured at 511 nm and expressed as arbitrary units (fluorescence mg protein^{-1}). The B17 and F67 *sgfp*-expressing transformants are indicated by grey and black bars, respectively. The nonfluorescent wild-type strain h1.7 (white bars) has been used as a control. Values are the mean of three independent experiments ± SE. Values with the same letter (a, b, c, d or e) are not statistically different ($P < 0.05$).

of 1 mM and 10 mM glucose concentrations on fluorescence intensity (Fig. 5). As a control, we used a 100 mM glucose concentration. In the B17 transformant, the fluorescence detected after growth on 1 and 10 mM glucose was not statistically different, whereas in the F67 transformant the very high fluorescence detected after growth on 1 mM glucose was approximately reduced by half on 10 mM glucose, showing that the expression system is glucose-repressed even at low concentrations in this transformant so that it could be used as a biosensor for detecting glucose concentrations in the apoplast of ectomycorhiza. This will be important for understanding gene regulation in the symbiotic tissue as some glucose-regulated fungal genes are known to be differentially expressed in the two ectomycorrhiza fungal compartments. This is the case for example of the genes encoding a monosaccharide transporter and a phenylalanine ammonia lyase from *Amanita muscaria* associated with Norway spruce or *Populus* hybrids (Nehls *et al.*, 1998, 1999, 2001; Wiese *et al.*, 2000).

Conclusions

We have shown that a functional GFP can be produced in *H. cylindrosporum* and that the *sgfp* gene is a suitable reporter gene in this symbiotic basidiomycete fungus. The *H. cylindrosporum* F67 transformant whose GFP expression is repressed by glucose even at low concentrations (1–10 mM) will be a potential tool for evaluating sugar supply by the plant to a compatible mycorrhizal symbiont. The *sgfp* reporter gene will also be used to study the expression of different genes of interest in this fungus, in particular those putatively involved in mycorrhiza formation and functioning, from the initial steps of plant–fungus interaction to the bidirectional nutrient exchanges.

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