

Development of a rapid and simple *Agrobacterium tumefaciens*-mediated transformation system for the fungal pathogen *Heterobasidion annosum*

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Keywords

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Abstract

Heterobasidion annosum causes root and butt-rot in trees and is the most serious forest pathogen in the northern hemisphere. We developed a rapid and simple *Agrobacterium*-mediated method of gene delivery into *H. annosum* to be used in functional studies of candidate genes and for visualization of mycelial interactions. *Heterobasidion annosum* TC 32-1 was cocultivated at pH 5.6 and 20 °C in Hagem's medium with *Agrobacterium tumefaciens* C58 carrying plasmids with hygromycin B resistance as the selectable marker and green fluorescent protein as a visual marker. We obtained 18 mitotically stable transformed isolates showing green fluorescence protein activity.

Introduction

Agrobacterium tumefaciens-mediated transformation in various eukaryotic systems relies on fundamental studies that took place between 1970 and 1990 (Escobar & Dandekar, 2003). The first successful transformation of a filamentous fungus using *A. tumefaciens* was reported by de Groot *et al.* (1998). Since then, various fungal species have been transformed using *A. tumefaciens* (Chen *et al.*, 2000; Mikosch *et al.*, 2001; Mullins *et al.*, 2001; Hanif *et al.*, 2002; Pardo *et al.*, 2002; Combi *et al.*, 2003; Rolland *et al.*, 2003; Burns *et al.*, 2005). One of the principal advantages of this technique is that *A. tumefaciens* can transform protoplasts as well as hyphae, spores and even blocks of mycelial tissue from fruiting bodies (de Groot *et al.*, 1998; Mullins *et al.*, 2001).

The basidiomycete fungus *Heterobasidion annosum* (Fr.) Bref. causes root and butt-rot in standing trees in northern temperate regions, and is considered to be the most economically devastating pathogen in forestry (Woodward *et al.*, 1998). Primary infection is initiated by basidiospores

germinating on fresh and exposed woody tissues, with coniferous species (*Pinus*, *Picea* and *Abies*) being the primary hosts in Europe (Korhonen & Stenlid, 1998).

Despite the significant economic impact caused by *H. annosum*, the molecular basis of its interaction with its hosts is still poorly understood. Recently, a substantial amount of knowledge about gene function and expression in *H. annosum* during various biological processes has begun to accumulate (Karlsson *et al.*, 2003; Abu *et al.*, 2004; Iakovlev *et al.*, 2004). However, about 30% of the expressed sequence tags obtained by Karlsson *et al.* (2003) did not show homology with a gene of known function. Gene disruption experiments are therefore of great importance for determining the biological role of the encoded proteins with unknown functions. Analysing gene activation patterns under different conditions is an additional way to elucidate the role and importance of a gene and its corresponding product. In this context, fusing the promoter of a gene of interest to a marker gene, such as the gene encoding green fluorescent protein (GFP) from *Aequorea victoria*, is an efficient way to study gene regulation in basidiomycetes (Ma *et al.*, 2001).

Our aim was to develop a transformation system for *H. annosum* in order to facilitate future interaction studies between *H. annosum* and the biocontrol fungus *Phlebiopsis gigantea*. GFP tagging of *H. annosum* isolates would make it possible to visualize microscopic hyphal–hyphal interactions. Development of a successful gene delivery system for *H. annosum* would have the additional benefit of enabling gene knock-out studies and the analysis of promoters of genes with unknown functions.

Methods and materials

Strains and plasmids

Two different constructs were used in the experiments (Fig. 1). The first construct was based on the plasmid pAN7-1 (Punt *et al.*, 1987). pAN7-1 was digested with *Bgl*II and *Hind*III (Fermentas, Vilnius, Lithuania) and a fragment containing the promoter of the *Aspergillus nidulans* *GPD* gene (*PgpdA*), the coding region of the *Escherichia coli* hygromycin phosphotransferase gene (*hph*) and the terminator of the *A. nidulans* *TRPC* gene (*TrpC*) was cloned into the *Bam*HI/*Hind*III site of pPZP102 (Hajdukiewicz *et al.*, 1994), creating pJF1. In parallel, pJF2 was formed by digesting the plasmid pLUBIGFP13 (Nehlin *et al.*, 2000) with *Bam*HI and *Hind*III and the resulting fragment, carrying the coding sequence of a *GFP* gene (*gfp*), driven by a sunflower ubiquitin promoter (*PUBi*) and terminated with a *nos3'*-sequence, was inserted into the *Bam*HI/*Hind*III site of pPZP102. An *Sma*I/*Hind*III fragment of pJF1 was treated with Kleenow (Pharmacia Amersham, Uppsala, Sweden) and blunted into the *Sma*I site of pJF2 to form pCD61. In the second gene construct, the pGPDGFP plasmid (Sexton & Howlett, 2001) was digested with *Kpn*I (Fermentas). The resulting fragment carrying the coding sequence of the *EGFP* (*egfp*) gene (Clontech, Palo Alto, CA) under control of the *gpdA* promoter (*PgpdA*) (Punt *et al.*, 1987) was ligated into the *Kpn*I site of pPZP102 (Hajdukiewicz *et al.*, 1994) to form pJF3. In a second step, the pGPDGFP plasmid (Sexton & Howlett, 2001) was restricted with *Spe*I/*Not*I (Roche Diagnostics, Penzberg, Germany) to generate a fragment containing

the coding region of the *E. coli* hygromycin phosphotransferase gene (*hph*) flanked by the *TRPC* promoter and terminator sequences (*PtrpC* and *TrpC*, respectively) (Lu *et al.*, 1994). The gene cassette was ligated into the *Sma*I site of pJF3, resulting in pJF4. The orientation of the gene cassettes was assessed by restriction digests and DNA sequencing. Two PCR primers, 5'-AGGCGATTAAGTTGGGTAAC-3' and 5'-GGAATTGTGAG CGGATAAC-3', complementary to sequences flanking the inserted genes in pPZP102, were used in the sequencing reactions. The conditions for PCR and sequencing were the same as described by Fahleson *et al.* (2004).

The binary vectors pCD61 and pJF4 were transferred via electroporation onto the *A. tumefaciens* strain C58C1Rif harbouring the disarmed Ti-plasmid pGV 2260, which carries a carbenicillin resistance gene (Deblaere *et al.*, 1985). The North American *H. annosum* homokaryotic isolate TC 32-1 (P intersterility group) (Chase *et al.*, 1985) was used as the recipient.

Viability of *Agrobacterium tumefaciens* at different pH conditions

Agrobacterium tumefaciens C58 was grown in liquid yeast extract mannitol broth (YMB: K₂HPO₄ 0.5 g L⁻¹; MgSO₄ 2.0 g L⁻¹; NaCl 0.1 g L⁻¹; mannitol 10 g L⁻¹; yeast extract 0.4 g L⁻¹; agar 15 g L⁻¹) supplemented with 50 µg mL⁻¹ rifampicin at 28 °C. The cultures were pelleted by centrifugation, washed and resuspended in Hagem's medium (Stenlid, 1985) diluted to one-tenth nutrient strength (1/10 Hagem's medium), and buffered to pH 5.6 or pH 3.6 with 10 mM 2-*N*-morpholinoethanesulphonic acid (MES). The resuspended bacteria were mixed 1:1 with 1/10 Hagem's medium pH 5.6 or 1/10 Hagem's medium pH 3.6. To evaluate whether the presence of germinating *H. annosum* conidia influenced the viability of the *A. tumefaciens*, a parallel experiment was run with culture filtrate (CF) from conidia (10⁸ conidia mL⁻¹) germinating in Hagem's medium buffered to pH 5.6 or pH 3.6. Bacteria were plated on YMB medium after 24 and 44 h. CFUs were scored after incubation at 28 °C for 48 h.

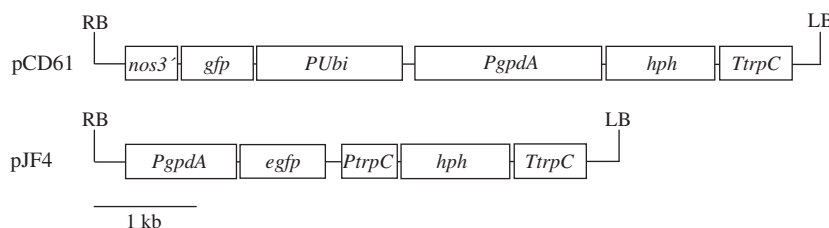


Fig. 1. Schematic drawings of the gene constructs used in the transformation of *Heterobasidion annosum*. Plasmid pCD61 was created by moving the gene cassettes *PgpdA*–*hph*–*TrpC* and *PUBi*–*gfp*–*nos3'* from pAN7-1 (Punt *et al.*, 1987) and pLUBIGFP13 (Nehlin *et al.*, 2000), respectively, to the binary vector pPZP102 (Hajdukiewicz *et al.*, 1994). Plasmid pJF4 was constructed by moving the two gene cassettes *PtrpC*–*hph*–*TrpC* and *PgpdA*–*egfp* from the pGPDGFP plasmid (Sexton & Howlett, 2001) to the binary vector pPZP102 (Hajdukiewicz *et al.*, 1994). RB and LB denotes left and right borders of the constructs in the pPZP102 plasmid. Bar represent 1 kb.

Transformation and media

Agrobacterium tumefaciens carrying plasmid pCD61 and pJF4 was grown in liquid YMB supplemented with 50 µg mL⁻¹ rifampicin and 30 µg mL⁻¹ of chloramphenicol at 28 °C to an optical density (OD_{600 nm}) of c. 0.2. 1.5 mL of culture fluid was centrifuged. The pellet was washed and resuspended in 1/10 Hagem's medium buffered to pH 5.6 with 10 mM MES. *Heterobasidion annosum* was grown on Hagem's medium plates for 10 days. Conidia were harvested from the cultures by pouring cocultivation medium (1/10 Hagem's medium, pH 5.6) on fungal colonies, and then the surface of the colony was gently rubbed using a glass rod. The conidia concentration was adjusted to 10⁶ conidia per millilitre. Prior to the first transformation experiment, the lethal concentration of hygromycin B was determined to be 45 µg mL⁻¹. This was carried out by growing *H. annosum* TC 32-1 for 2 weeks on Hagem's medium plates supplemented with 0, 10, 20, 30, 35, 40, 45 and 50 µg mL⁻¹ of hygromycin B. Petri plates were kept in the dark at room temperature. No hyphal growth was observed at 45 or 50 µg mL⁻¹ of hygromycin B. TC 32-1 was shown to have a normal growth rate at 300 µg mL⁻¹ of cefotaxime, which was added to eliminate residual *A. tumefaciens* from fungal cultures.

Cocultivation was performed in culture tubes; 200 µL⁻¹ of bacterial suspension was added to 150 µL⁻¹ of conidial suspension, and acetosyringone (AS) was added to a final concentration of 200 µM. The cocultivation tubes were then placed on a shaker and incubated for 36 h with gentle agitation at 20 °C. The cocultivation fluid was then spread on Hagem's medium plates supplemented with 50 µg mL⁻¹ of hygromycin B and 300 µg mL⁻¹ of cefotaxime. The plates were incubated at 25 °C in the dark. *Heterobasidion annosum* colonies were transferred to Hagem's medium plates supplemented with hygromycin B (50 µg mL⁻¹). After 10 days, the radial growth of the mycelium was determined by the average value from two transects (max & min growth).

Fluorescence microscopy

Fluorescence microscopy was performed with an Axioplan fluorescence microscope (Carl Zeiss, Göttingen, Germany) using UV-filter blocks (450–490 nm excitation). Photomicrographs were produced using an Axiocam camera and the Axiovision Release 4.2 software (Carl Zeiss). Screening the transformed isolates for GFP fluorescence was achieved by removing small samples (5 × 5 mm) of mycelium at random positions in the plates. These were mounted in water and immediately viewed with UV-light. GFP fluorescence was detected visually, and the isolates were scored on the intensity of the GFP activity and total distribution of activity in the mycelium.

DNA isolation and molecular analyses

DNA was extracted using hexadecyltrimethylammonium bromide (CTAB) and a phenol–chloroform DNA extraction

protocol (Gardes & Bruns, 1993) omitting the β-mercaptoethanol. The primer pair ITS1F and ITS4 (White *et al.*, 1990) was used to amplify the internal transcribed spacer (ITS)-region (ribosomal DNA) to verify whether the quality of the extracted DNA was sufficient for PCR. The absence of contaminating *A. tumefaciens* in the isolate cultures was verified by PCR with primers designed to recognize the *A. tumefaciens* Vir D3 gene (Lindroth *et al.*, 1999). A 10 µL reaction contained 2 µL diluted template DNA (20–40 ng µL⁻¹), PCR buffer with 1.5 mM MgCl₂, 0.4 mM dNTP, 0.5 mM of each primer and 1.2 U RedTaq polymerase (Sigma, Sigma-Aldrich Sweden AB, Stockholm, Sweden). The thermal profile was as follows: 94 °C for 2 min, then 30 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 1 min, followed by a final 10 min extension at 72 °C.

The *egfp* gene was detected by PCR using the Pgpda1 (5'-TACCGAGCTCCCAA TCTGT-3') and TtrpC (5'-TGTGCATTCTGGGTAAACGA-3') primers. A 20 µL PCR reaction contained 2 µL diluted template DNA, PCR buffer with 1.5 mM MgCl₂, 0.5 mM dNTP, 0.5 mM of each primer, 3% dimethyl sulphoxide and 0.8 U Phusion polymerase (Finnzymes, Espoo, Finland). The thermal profile was as follows: 98 °C for 2 min, then 35 cycles of 98 °C for 15 s, 59 °C for 15 s and 72 °C for 1 min, followed by a final 10 min extension at 72 °C. The *gfp* gene was amplified using GFP Fwd1 (5'-CACATGAAGCAGCAGACTT-3') and GFP Bwd1 (5'-TGTTCTGCTGGTAGTGGTCG-3'). The template was denatured at 94 °C for 2 min, followed by 35 cycles: denaturation at 94 °C for 20 s, annealing at 57 °C for 20 s, extension at 72 °C for 1 min and finally a 10 min extension at 72 °C.

The *hph* gene was amplified using the hph F (5'-AAGCCTGAACCTCACC GCGAC-3') and hph R (5'-CTAT TCCTTTGCCCTCGGAC-3') primer pair. A 20 µL PCR reaction contained 5 µL diluted template DNA (20–40 ng µL⁻¹), PCR buffer with 1.5 mM MgCl₂, 0.4 mM dNTP, 0.5 mM of each primer and 1.2 U RedTaq polymerase (Sigma, Sigma-Aldrich Sweden AB). The template was denatured at 94 °C for 2 min, followed by 35 cycles: denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, extension at 72 °C for 1 min, followed by a final 10 min extension at 72 °C. The PCR products were separated on a 1% agarose gel and visualized under UV light after ethidium bromide staining.

Results

Cocultivation and selection of transformed isolates

Cocultivation of *Agrobacterium tumefaciens* and *Heterobasidion annosum* conidiospores in the induction medium described by Bundock *et al.* (1995) at 28 °C yielded no

hygromycin-resistant colonies. Furthermore, cocultivation in 1/10 unbuffered Hagers medium yielded a low number of hygromycin-resistant colonies, and none of these exhibited any GFP fluorescence. The pH in the diluted Hagers medium dropped from the original 5.6–3.6 during conidiospore germination and growth. The viability of *A. tumefaciens* at pH 3.6 was reduced after 44 h of incubation in pH 3.6 medium compared with that at pH 5.6 (Fig. 2), and in CF buffered to pH 3.6, no *A. tumefaciens* CFUs could be detected after 24 or 44 h of incubation (Fig. 2). In comparison, no negative effects on *A. tumefaciens* growth could be detected when CF buffered to pH 5.6 was added to the medium. To ensure optimal growth conditions for *A. tumefaciens* during the cocultivation, 1/10 Hagers medium buffered to pH 5.6 with 10 mM MES was used. Furthermore, the germination capacity of *H. annosum* conidia was moderately reduced at 28 °C compared with a temperature of 20 °C; therefore, cocultivation was carried out at c. 20 °C. After cocultivation at 20 °C in 1/10 Hagers medium buffered to pH 5.6 with 10 mM MES, about 200 hygromycin-resistant colonies were recovered. One hundred and twenty of these grew on hygromycin B-containing medium after subculturing. Seventy-two of the isolates originated from cocultivation with *A. tumefaciens* harbouring the pCD61 plasmid and 48 isolates with the pJF4 plasmid. Cocultivation with C58 alone yielded no hygromycin-resistant colonies.

Thirty-four of these 120 isolates showed GFP fluorescence in the initial screening. Eighteen of these 34 isolates were selected for further analysis based on growth rate. Seven

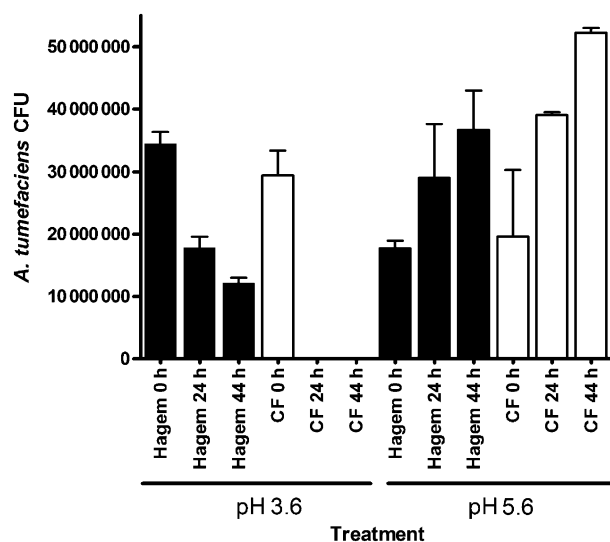


Fig. 2. *Agrobacterium tumefaciens* survival in Hagers medium and culture filtrate (CF) from germinating *Heterobasidion annosum* conidia at different pH levels. Filled bars represent the CFUs of *A. tumefaciens* incubated in 2-*N*-morpholinoethanesulphonic acid (MES)-buffered Hagers medium; open bars represent the CFUs of *A. tumefaciens* incubated in *H. annosum* CF.

Table 1. Characteristics of the transformed isolates after 3 and 18 months

Vector*	Isolate	Fluorescence (months) [†]		Radial growth (mm) [‡]	PCR analysis [§]		
		3	18		<i>gfp/egfp</i>	<i>hph</i>	<i>virD3</i>
pJF4	A	+	+	17	+	+	—
	B*	+	NA	NA	+	+	—
	C	+	+	3	+/?	+	—
	D	+	+	13	+	?	—
	E	+	+	12	+/?	+	—
	F	+	+	10	+	+	—
	G	+	+	14	+	+	—
pCD-61	H	+	+	11	+	+	—
	I	+	+	4	+	+	—
	J	+	+	15	+	+	—
	K	+	+	10	+	+	—
	L	+	+	17	+/?	+	—

*Plasmids harboured by the *Agrobacterium* strain used in the cocultivation.

[†]Fluorescence was evaluated by microscopy under ultraviolet light at 3 and 18 months posttransformation. + indicates that green fluorescent protein activity was detected in the isolate.

[‡]The radial growth of each isolate was measured along two transects (two measurements per transect) on 8-day-old fungal colonies growing on Hagers medium; the mean value is presented.

[§]Molecular analyses: PCR amplification of *gfp/egfp*, *hph* and *virD3* was carried out at approximately 3 months posttransformation, which is represented in the table. The *gfp/egfp* and *hph* analyses were verified with the remaining isolate at 18 months. + indicates the presence of an amplification product, — indicates the absence of an amplification product and +/? indicates faint products or varying results.

*Indicates isolates that were lost owing to contamination with an unknown fungus before 18 months.

NA, not analysed.

isolates originated from transformation with pJF4, while 11 originated from pCD61. Of these, 11 isolates were analysed for GFP fluorescence after 18 months (Table 1).

DNA isolation and molecular analysis

The PCR reactions were positive in all cases for all 18 selected isolates using the primers ITS1 and ITS4. Amplification of the *Vir D3* gene was unsuccessful for all isolates, indicating that the cultures were free of *A. tumefaciens*. The *hph* gene was detected in 17 isolates using the *hph*-specific primers. Amplification of the *gfp* and *egfp* genes was successful for 15 isolates, proving that these genes were present in our material.

Fluorescence microscopy

For 18 months, the isolates were exposed to subculture every 7th week on Hagers medium. At the first four of these subcultures, hygromycin B was added to the medium. The

following subcultures were grown on standard Hagem's medium to reveal whether the hygromycin resistance would remain stable over time without selective pressure. At the last subculturing, the isolates were transferred to medium containing hygromycin B, and all isolates proved to be hygromycin resistant. We observed that the GFP fluorescence became less intense and less widespread when the isolates were cultivated on standard Hagem's medium. This was independent of the construct they harboured. When the isolates were transferred back to hygromycin B-containing medium, all isolates showed increased GFP fluorescence.

The photomicrographs in Fig. 3 show GFP-expressing *H. annosum* hyphae tagged with the two different constructs (pJF4 and pCD61) at two different time intervals: 3 and 18 months after transformation, respectively. When screening for the GFP activity in the hyphae, the fluorescence appeared to be abundant in the cytosol. None of the isolates displayed global GFP fluorescence in the entire mycelium. Furthermore, 3–4 weeks after being transferred to a new subculture, the intensity and abundance of GFP fluorescence seemed to decrease in the isolates. Isolates transformed with pCD 61 showed a more uniform GFP fluorescence pattern than pJF4 isolates (data not shown).

Discussion

Our aim was to develop a transformation system for GFP-tagging *Heterobasidion annosum* under laboratory conditions in order to facilitate future microscopic hyphal–hyphal interaction studies between *H. annosum* and *P. gigantea*. Tagged isolates could also be used in host tissue interaction studies (Lee *et al.*, 2002) and gene disruption experiments.

Transformation of ascomycetes as well as basidiomycetes with hygromycin resistance has been achieved several times (Karjalainen, 1993; Asiegbu, 2000; Mikosch *et al.*, 2001; Combier *et al.*, 2003; Freitag *et al.*, 2004). However, achieving GFP fluorescence in GFP-tagged isolates of basidiomycetes has proven to be difficult. In some cases, GFP integration has been successful, but GFP activity was not detectable (Chen *et al.*, 2000; Hanif *et al.*, 2002). In other cases, e.g. *Phanerochaete chrysosporium* (Ma *et al.*, 2001) and *Schizophyllum commune* (Lugones *et al.*, 1999), GFP fluorescence has been achieved after addition of an intron to the *egfp* gene.

Temperature at the cocultivation stage is known to affect transformation efficiency. Combier *et al.* (2003) noted that a cocultivation temperature of *c.* 23 °C yielded a higher number of transformed *Hebeloma cylindrosporum* individuals compared with higher cocultivation temperatures.

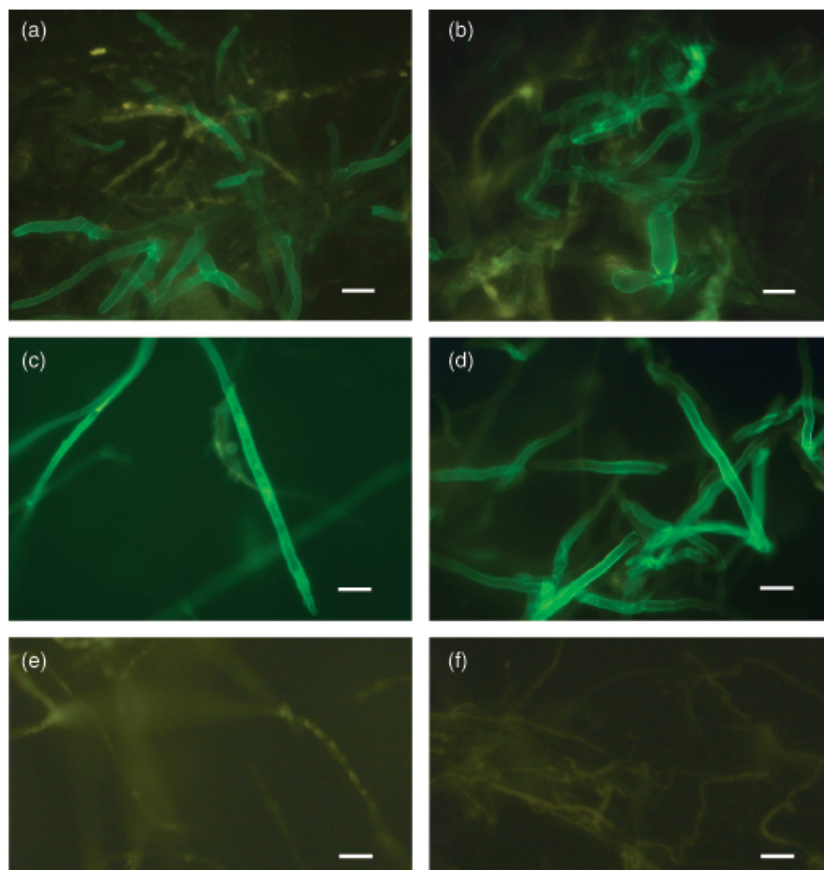


Fig. 3. Photomicrographs of green fluorescence protein in *Heterobasidion annosum* transformed with pJF4 (a and c) and pCD61 (b and d) at 3 months posttransformation (a and b) and at 18 months posttransformation (c and d). Photos (e) and (f) display untransformed *H. annosum* TC 32-1 incubated on Hagem's medium containing 50 µg mL⁻¹ hygromycin B (e) and grown on standard Hagem's medium (f). Scale bar represents 20 µm.

Similar results have been observed in *Agrobacterium*-mediated transformation of *Botrytis cinerea* (Rolland *et al.*, 2003). In the present study, some effect of cocultivation temperature on the germination rate of *H. annosum* conidia and transformation frequencies was observed. However, we found that the dominant factor in improving the transformation efficiency of *H. annosum* was to maintain the pH at 5.6. Stabilizing the pH during conidial germination enabled *A. tumefaciens* to survive and infect the germinating conidia. The growth of *A. tumefaciens* was reduced at low pH, as has been previously reported (Hammerschlag *et al.*, 1997). However, it cannot be excluded that at low pH, *A. tumefaciens* viability is further reduced by toxic secondary metabolites that are secreted into the surrounding medium by the germinating conidia. For instance, the two well-characterized secondary metabolites in *H. annosum* fommanoxin and fommanosin, produced in dual culture, have shown toxicity to bacteria (Sonnenbichler *et al.*, 1989).

All the isolates supported amplification of the *hph* gene, while at the same time they did not allow amplification of the *virD3* gene. The isolates also maintained hygromycin resistance under several consecutive subcultures on standard Hagem's medium. These data suggest that the isolates were mitotically stable. We observed GFP fluorescence in the isolates regardless of which of the two constructs they harboured, demonstrating that the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) as well as the ubiquitin promoter can drive transcription, and that both the *gfp* and the *egfp* gene can function in *H. annosum*.

Although the GFP fluorescence was stable over time, global GFP fluorescence was not detected in any of the isolates. It is not clear why the GFP fluorescence varied spatially and temporally in the material, as *gpd* and *ubiquitin* promoters are known to be highly active (van de Rhee *et al.*, 1996; Hirano *et al.*, 2000; Hanif *et al.*, 2002; Combiér *et al.*, 2003). The *gpd* promoter that we used was derived from *A. nidulans*. Using an ascomycete promoter in a basidiomycete fungus could lead to incompatibility issues, as the genetic differences between the two fungal divisions are quite substantial (Berbee & Taylor, 1993). However, van de Rhee *et al.* (1996) compared the efficiency of the *A. nidulans gpd* promoter and the *Agaricus bisporus gpd* promoter and found no significant difference in the capacity of the promoters to drive gene expression in *A. bisporus*. Furthermore, the *ubiquitin* promoter (*Ubb1*) used in pCD61 is derived from sunflower (Binet *et al.*, 1991). Hence, the evolutionary distance between the organism that the sequence originates from and *H. annosum* is even more than that in the case of the *gpd* promoter. The GFP fluorescence that we observe in our material shows that at least some *cis*-acting promoter elements of the *Ubb1* promoter are recognized in *H. annosum*.

Heterobasidion annosum conidia generally contain one to five nuclei (Korhonen & Stenlid, 1998), so it is possible that

T-DNA integrated into only one of the nuclei in the germinating conidium. The isolate would then represent a chimera with nuclei that carry the T-DNA and nuclei that do not. Our observation that the abundance of GFP fluorescent hyphae decreased after several subcultures on standard Hagem's medium indicates that this may be the case.

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