Agrobacterium-Mediated Transformation of *Ceratocystis albifundus*

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Abstract

Functional association between genomic loci and specific biological traits remains lacking in many fungi, including the African tree pathogen *Ceratocystis albifundus*. This is mainly because of the absence of suitable transformation systems for allowing genetic manipulation of this and other fungi. Here, we present an optimized protocol for *Agrobacterium tumefaciens*-mediated transformation of *C. albifundus*. Strain AGL-1 of *A. tumefaciens* and four binary T-DNA vectors (conferring hygromycin B or geneticin resistance and/or expressing the green fluorescent protein [GFP]) were used for transforming germinated conidia of three isolates of *C. albifundus*. Stable expression of these T-DNA-encoded traits was confirmed through sequential sub-culturing of fungal transformants on selective and non-selective media and by using PCR and sequence analysis. Single-copy integration of the respective T-DNAs into the genomes of these fungi was confirmed using Southern hybridization analysis. The range of experimental parameters determined and optimised included: (i) concentrations of hygromycin B and geneticin required for inhibiting growth of the wild type fungus and (ii) the dependence of transformation on acetosyringone for inducing the bacterium's virulence genes, as well as (iii) the duration of fungus-bacterium co-cultivation periods and (iv) the concentrations of fungal conidia and bacterial cells used for the latter. The system developed in this study is stable with a high-efficiency, yielding up to 400 transformants per 10^6 conidia. This is the first report of a transformation protocol for *C. albifundus* and its availability will be invaluable for functional studies in this important fungus.

Keywords: *Agrobacterium*; *Ceratocystis*; GFP; hygromycin; geneticin

1. Introduction

The genus *Ceratocystis* (phylum Ascomycota, family Ceratocystidaceae) includes many economically important pathogens of a broad spectrum of hosts globally (Wingfield *et al.*, 2013; de Beer *et al.,* 2014). In Southern Africa, the filamentous fungus *C. albifundus* De Beer, Wingfield & Morris causes wilt and canker disease of commercially propagated *Acacia mearnsii* and *Protea cynaroides*, and it is regarded as a significant threat to indigenous plant species (Wingfield *et al.,* 1996; Roux and Wingfield 1997, 2009; Lee et al., 2016). Although whole genome sequences are available for *C. albifundus* (van der Nest et al., 2014), the molecular basis for many biological properties remain poorly understood. One of the main reasons for this is the lack of a robust genetic transformation system for genetic manipulation to facilitate functional characterization of genes and processes.

Various protoplast-based transformation techniques have been used for studies on Ascomycota (Lu et al., 1994; Shi et al., 1995; Bölker et al., 1995; Thon et al., 2000). These include approaches involving electroporation (Brown et al., 1991), particle bombardment (Sunagawa and Magae, 2002) and polyethylene glycol-$CaCl_2$ mediated transformation (Honda et al., 2000). However, protoplast isolation is a difficult procedure and the quality of these propagules typically influences the rate and efficiency of transformation (Fungaro et al, 1995). Therefore, low protoplast yield and the formation of unstable transformants, along with a high probability of multi-copy integration, the formation of undesirable heterokaryons and the economic expenses associated with the process, have drastically limited the use of protoplast-based transformation in some fungi (Bundock et al., 1995; Mullins et al., 2001; Wang and Li, 2008). Also, although protoplast-based transformation systems have been developed for various model fungi (Balhadère et al., 1999; Tanaka et al., 1999; Thon et al., 2000), non-model fungi such as *Ceratocystis* and its relatives have received little to no attention in this regard (Lee et al., 2006; Tanaka et al., 1999).

Transformation using *Agrobacterium tumefaciens* is an alternative to protoplast-based transformation and provides an easy and effective avenue for generating stable transformants (Wu and O' Brien, 2008). In fact, the most significant advantage of using *A. tumefaciens*-Mediated Transformation (ATMT) for fungi lies in the flexibility regarding the type of starting material needed, where any type of fungal propagule (e.g., hyphae, reproductive spores, or blocks of agar overgrown with mycelial tissue), including protoplasts, can be used as starting

material for these experiments (Chen et al., 2000; de Groot et al., 1998). The process usually yields high transformation efficiencies, as well as high likelihood of single-copy integrations (Mullins et al., 2001; Combier et al., 2003; Bardiya and Shiu., 2007; Wu & O'Brien., 2008). Since the first published paper on ATMT of model fungi (de Groot et al., 1998), the system has been shown to be useful for many filamentous fungi, including *Magnaporthe grisea* (Rho et al., 2001) and *Fusarium oxysporum* (Mullins et al., 2001), as well as *Endoconidiophora resinifera* (Loppnau et al., 2004) and *Berkeleyomyces basicola* (Tzima et al., 2014). Like *C. albifundus*, the latter two species are part of the Ceratocystidaceae, although previously *E. resinifera* was misclassified as *Ceratocystis resinifera* and *B. basicola* as *Thielaviopsis basicola* (de Beer et al., 2014; Nel et al., 2018).

Despite the wide applicability of ATMT for fungi, its use typically requires careful optimization (Michielse et al., 2005). The aim of this study was therefore to develop a stable and high-efficiency ATMT system for *C. albifundus*. For this purpose, different *C. albifundus* strains were transformed with *A. tumefaciens* harbouring various binary vectors using the ATMT protocol originally introduced for *Aspergillus awamori* by de Groot et al. in 1998. As pointed out before (Michielse et al., 2005), several aspects associated with the co-cultivation of the two microorganisms were determined and optimized for efficient transformation. These included (i) ascertainment of whether acetosyringone is needed for induction of the bacterium's virulence, (ii) determining the optimum *A. tumefaciens* cell and *C. albifundus* conidia concentrations for efficient transformation, (iii) comparison of the transformation efficiency of different *C. albifundus* strains and (iv) establishing how the duration of co-cultivation influences transformation efficiency. The availability of an ATMT system for *C. albifundus* will allow for the functional characterization of the genes and genetic elements underlying biological properties in this important fungus and its relatives.

2. Materials and methods

2.1. Fungal and bacterial strains, plasmids and culture conditions

Three *C. albifundus* strains (CMW 13980, CMW 4068 and CMW 40625) were used in this study. These isolates were obtained from the CMW Culture Collection at the Forestry and Agricultural Biotechnology Institute at the University of Pretoria. They were maintained on malt extract agar (MEA; 2 % [w/v] Bacto™ malt extract [BD BioSciences, San Jose, CA USA], 2 % [w/v] Difco™ agar [BD BioSciences]) at 25 °C in the dark. The two bacterial strains used

were *A. tumefaciens* strain AGL-1 (Hellens et al., 2000) and *Escherichia coli* strain DH5α (Taylor et al., 1993). They were routinely grown on Luria-Bertani medium (LB; 1 % [w/v] tryptone [Sigma-Aldrich, St. Louis, MO USA], 1 % [w/v] NaCl and 0.5 % [w/v] yeast extract [Biolab, Budapest, Hungary]) containing agar (2 % [w/v] BD Difco™ agar) at 28°C.

Four binary vectors were used. Two of these (pC-HYG-GFP and pC-g-418-GFP) were provided by Dr Seogchan Kang from the Department of Plant Pathology and Environmental Microbiology at Pennsylvania State University (University Park, PA USA). The remaining two plasmids (pBHt-2 and pCAMBIA0380) were kindly provided by Dr Bridget Crampton from the Department of Plant Science at the University of Pretoria (Pretoria, South Africa).

2.2. Plasmid sequences and maps

The sequences for plasmids pC-HYG-GFP and pC-g-418-GFP were determined in this study. For this purpose, the respective plasmids were used to transform *E. coli* strain DH5α, after which transformants were grown in LB broth using a 222DS Benchtop Shaking Incubator (120 rpm at 37°C; Labnet international, Edison, NJ, USA). Cultures were grown to an optical density of 0.4 at 600 nm (OD_{600nm}), which was measured with a SmartSpec™ Plus Spectrophotometer (Bio-Rad, Hercules, CA USA). Cells were then harvested by centrifugation (4951 *rcf*) and subjected to plasmid extraction using the QIAprep Spin Miniprep Kit (Qiagen, Aarhus, Denmark).

The extracted DNAs were sequenced at the Central Analytical Facilities of Stellenbosch University (Stellenbosch, South Africa) using the Ion Torrent PGM™ platform with the 200-bp chemistry and the Ion 318™ Chip v2 (Thermo Fisher Scientific, Waltham, MA USA). Raw single reads that had been imported into the CLC Genomics Workbench 9.5.4 (Qiagen, Aarhus, Denmark) were filtered by removing poor quality reads (threshold limit of 0.05) and/or terminal residues. The quality-filtered sequence reads were assembled by allowing CLC Genomics Workbench to estimate the optimal word and bubble sizes. Sequence annotation and the making of plasmid maps were achieved using Addgene (https://www.addgene.org/analyze-sequence/) and SnapGene (www.snapgene.com), respectively.

2.3. *C. albifundus* sensitivity to Hygromycin B, Geneticin, Kanamycin and Carbenicillin

We tested whether the aminoglycoside antibiotics hygromycin B (Sigma-Aldrich, St. Louis, MO USA) and geneticin (also known as G-418; Sigma-Aldrich) could be used as selection markers for *C. albifundus*. We also confirmed the resistance of *C. albifundus* to the bacteriocides kanamycin and carbenicillin, so we could use these antibiotics in our co-cultivation media. This was done by growing the wild type isolates of *C. albifundus* on MEA supplemented with hygromycin B (at concentrations of 0, 1, 2, 5 and 10 µg/ml), geneticin (at concentrations 0, 1, 2, 5, 10 and 15 µg/ml), kanamycin (at concentrations of 0, 25, 50, 75, 100, 150 and 200 µg/ml) and carbenicillin (at concentrations of 0, 25, 50, 75 and 100 µg/ml). To determine the minimum inhibitory concentrations for each antibiotic, mycelial growth was recorded after cultivation at 25 °C for 7 d for hygromycin and geneticin and 14 d for kanamycin and carbenicillin (by taking the average of the long and short diameters of the growth surface). These experiments were done in triplicate.

2.4. Transformation of *A. tumefaciens*

We prepared competent cells for *A. tumefaciens* strain AGL-1 which is resistant to carbenicillin (Lazo et al., 1991). This was done according to the procedure described previously (McCormac et al., 1998). Briefly, it entailed the use of rapidly growing cells (in LB broth containing 100 µg/ml carbenicillin [Sigma-Aldrich, St. Louis, MO USA]) that were ice-chilled, harvested by centrifugation (2785 *rcf*) and then resuspended in ice-chilled 10% (v/v) glycerol. These competent cells were stored at -80 °C until use.

The four binary vectors were introduced separately into the *A. tumefaciens* competent cells by electroporation using a MicroPulser (Bio-Rad, Hercules, CA, USA) with a field strength of 12.5 KV/cm, the resistor setting of 200 Ω and the capacitor set at 25 µF. For this purpose, 1 µL of the plasmid (500 ng/µL) was placed on the internal side of an ice-chilled 0.2-cm-gap cuvette and mixed with 50 µL of ice-chilled competent cells of *Agrobacterium*. After electroporation, the cuvette was removed from the apparatus and 450 µL SOC medium (2% [w/v] tryptone, 0.5% [w/v] yeast extract, 20 mM glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$) was immediately applied to the cuvette. The suspension was then transferred to a clean 1.5 ml Eppendorf tube and incubated at 28°C for 1.5 h with shaking at 350 rpm. The cell suspension was poured onto LB agar medium supplemented with carbenicillin (50 µg/ml,

Sigma-Aldrich, St. Louis, MO USA) and kanamycin (100 µg/ml). After incubation at 28°C for 2-3 d, single bacterial colonies were transferred to fresh LB agar medium containing kanamycin and carbenicillin. Following another round of incubation at 28°C for 2-3 d, loopfuls of these transformed bacteria were suspended in 50% (v/v) glycerol for storage at -80 °C.

2.5. *A. tumefaciens*-mediated transformation of *C. albifundus*

The *A. tumefaciens* isolates containing the respective vectors were incubated with shaking (120 rpm and 28°C; 222DS Benchtop Shaking Incubator) in LB broth containing carbenicillin (50 µg/ml) and kanamycin (100 µg/ml), for 10-15 hours to an OD_{600nm} value of 0.2. Fifteen-ml aliquots of each cell suspension were then centrifuged (4951 *rcf*, 30 min), after which the harvested cells were resuspended in 20 ml of Induction Minimal Medium (IMM) containing carbenicillin (50 µg/ml) and kanamycin (100 µg/ml), and supplemented with either 0 or 200 µM acetosyringone (Sigma Aldrich, St. Louis, USA). IMM contained 10 mM K_2HPO_4, 10 mM KH_2PO_4, 6 mM NH_4NO_3, 2 mM NaCl, 9 µM $FeSO_4.7H_2O$, 0.09 mM $CaCl_2$, 2 mM $MgSO_4$, 20 mM MES monohydrate (morpholine-4-ethanesulfonic acid hydrate; Sigma-Aldrich, St. Louis, MO USA), 4.5 % (w/v) glucose and 25 % (v/v) glycerol and was supplemented with trace elements (0.4 mM Citric acid monohydrate [$C_6H_8O_7.H_2O$], 2 mM $ZnSO_4.7H_2O$, 0.5 mM Fe $(NH_4)_2SO_4.6H_2O$, 0.3 mM $CuSO_4.5H_2O$, 6 µM $MnSO_4$. H_2O, 16 µM H_3BO_3, 48 µM Na_2MoO_4). The respective cell suspensions were incubated at 28 °C using a 222DS Benchtop Shaking Incubator (120 rpm) and allowed to grow to five different OD_{600nm} values (i.e., 0.2, 0.3, 0.4, 0.5 and 0.6).

IMM with asexual reproductive spores (conidia) of *C. albifundus* were prepared. This was done by flooding 14-21 d old MEA cultures of each isolate with 3 ml of IMM, and then passing the liquid containing spores through cheesecloth to remove mycelia. Following quantification with a haemocytometer, aliquots of $1x10^4$, $1x10^5$, 2.5×10^5 and $1x10^6$ spores per ml of IMM were prepared and incubated at 25 °C for 15-24 h in the dark to allow germination.

For co-cultivation of the respective *Agrobacterium* transformants and fungal spores, we used IMM agar (IMM with 2 % [w/v] BD Difco™ agar) medium covered with sterile cellophane sheets (3.5 × 3.5 cm^2; Product no. Z377597, Sigma-Aldrich, St. Louis, MO USA) and containing carbenicillin (50 µg/ml), kanamycin (100 µg/ml) and acetosyringone (0 or 200 µM). This medium was inoculated with a mixture containing 500 µl of *Agrobacterium* (at OD_{600nm}

values of 0.2, 0.3, 0.4, 0.5 or 0.6) and 500 µl germinated spores of *C. albifundus* (at 10^4, 10^5, 2.5×10^5 or 10^6 spores/ml). For control purposes, equal volumes of IMM and fungal spores were mixed together and spread on the surface of IMM agar plates without antibiotics and covered with cellophane sheets. Following incubation for 48-72 h at 25 °C, the cellophane sheets were transferred and placed facing down on plates with fungal selective medium (MEA containing 50 µg/ml cefotaxime [Sigma-Aldrich, St. Louis, MO USA] to suppress bacterial growth). These plates were also supplemented with hygromycin B (10 µg/ml) or geneticin (15 µg/ml). Plates with the cellophane were incubated in the dark for at 25 °C for 10-14 d, and all these treatments were performed in triplicate. Additionally, and as suggested previously (Sullivan et al. 2002), co-cultivation of *C. albifundus* and *A. tumefaciens* under noninducing conditions (i.e., in the absence of acetosyringone; see the Results section below) served as another control for the transformation experiments.

2.6. Stability of *C. albifundus* transformants

Stability of *C. albifundus* transformants were evaluated using hygromycin B or geneticin resistance. For this purpose, the respective transformants were inoculated onto MEA without hygromycin B or geneticin for 7 d at 25 °C, after which the respective fungi were sub-cultured another five times using antibiotic-free MEA medium. To evaluate whether these sub-cultured fungi retained their antibiotic resistance traits, each transformant was then inoculated onto MEA amended with hygromycin B (10 µg/ml) or geneticin (15 µg/ml) and incubated for another 7 d. This entire procedure was repeated twice.

2.7. Analysis of the *C. albifundus* transformants

Genomic DNA was isolated from the *C. albifundus* transformants. For this purpose, the putative transformants were inoculated onto MEA supplemented with cefotaxime (50 µg/ml), and hygromycin B (10 µg/ml) or geneticin (15 µg/ml), followed by incubation at 25 °C. After 14-21 d, mycelium was harvested and DNA extracted using the CTAB method (Zhang et al, 1996). A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) was used to determine the quality and quantity of the extracted DNA.

PCR was used to confirm the presence of T-DNA in genomic DNA preparations. Primers hph-F and hph-R were used to test for the presence of the hygromycin resistance cassette, primers g-418-F and g-418-R were used to test for the presence of geneticin resistance cassette, while

primers gfp-F and gfp-R were used to test for the presence of the green fluorescent protein (GFP) encoding gene (Table 1). All 25 µl PCR mixtures contained 50 ng DNA, 2.5 mM $MgCl_2$, 150 µM of four dNTPs, 0.1µmol of each primer and 1U of *Taq* polymerase and reaction buffer (Roche Applied Science, Mannheim, Germany). PCR was carried out using an Eppendorf MasterCycler® gradient (Eppendorf, Hamburg, Germany) with initial denaturation at 94°C for 4min, followed by 30 cycles of 94°C of 45 sec, 60°C for 45 sec, 72°C for 1 min and final extension at 72°C for 15 min. PCR amplicons were visualized using 1 % (w/v) agarose gel electrophoresis, GelRed™ (Olerup SSP AB, Sweden) staining and a UV transilluminator (Sambrook and Russell 2001). Amplification products were purified with the High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim, Germany) and sequenced using the original primers and the BigDye® Terminator 3.1 cycle sequencing kit and ABI PRISIMH 3100 Genetic Analyzer from Applied Biosystems (Foster City, CA USA).

Table 1. List of primers used in this study to amplify hygromycin B (*hph*) and geneticin (*nptII*) resistance genes, as well as the gene encoding green fluorescent protein (*gfp*).

Primer	Primer sequence (5' to 3')	Annealing temperature	Amplicon size (base pairs)
gfp-F gfp-R	ACCATCTTCTTCAAGGACGAC GGGTGTTCTGCTGGTAGTG	60 ºC	271
hph-F hph-R	TAGCGCGTCTGCTGCTCCATACAAG ACCGAACTGCCCGCTGTTCTC	60 ºC	428
g-418-F g-418-R	GTGTCAACAACTAGAGTG GATGAAGAAGATGACCGAC	60 ºC	629

Confirmation of T-DNA integration and copy number of the antibiotic gene of interest was achieved using Southern blot hybridization analysis (Sambrook and Russell 2001). Genomic DNA (10-20µg/µL) of each transformant was digested with *Eco*RI (Sigma-Aldrich, St. Louis, MO USA) or *Hin*dIII (Sigma-Aldrich, St. Louis, MO USA) according to the manufacturer's recommendations. Digested DNA was then separated by 0.75% (w/v) agarose gel electrophoresis in Tris-Acetate-EDTA buffer (Sambrook and Russell 2001) and blotted onto Hybond N+ membrane (Amersham Biosciences, Little Chalfont UK) using capillary action. Five-hundred bp fragments of the hygromycin B and geneticin resistance genes were digoxigenin (DIG) end-labelled and used as probes. These fragments were obtained by PCR using the hph and g-418 primer sets (Table 1) as described above. Preparation of DIG-labelled

probes, hybridization and immunological detection was performed according to the manufacturer's protocol (Roche, Munich, Germany).

Expression of GFP in transformants of the three *C. albifundus* isolates carrying the vectors pC-HYG-GFP, pC-g-418-GFP and pBHt-2 were evaluated by fluorescence microscopy. For this purpose, actively growing hyphae from 10-day old, randomly selected transformants (grown on MEA plates containing 10 µg/ml hygromycin B or 15 µg/ml geneticin), were examined under Nikon AZ-100 epifluorescence microscope (Nikon, Tokyo, Japan) with a UV fitc (fluorescein isothiocyanate) filter and UV fluorescence at 20× lens magnification. The samples were exposed to light at 460-500 nm wavelengths with a fixed exposure of 2 s. Photos were taken with a Nikon digital Slight camera (Nikon, Tokyo, Japan) connected to the microscope, using NIS-ELEMENTS software (Nikon, Tokyo, Japan). The three different wild type strains of *C. albifundus* were used as negative controls.

The biological fitness of the *C. albifundus* transformants were assessed by comparing the growth of transformants to those of the wild types. Here twelve transformants (one for each of the three fungal isolates carrying any of the various binary vectors) and the three wild type fungi were used in a growth study. Three replicates per isolate were incubated at six different temperatures (10-35 °C, at 5 °C intervals) in the dark for 7 d. Mycelial growth was recorded as before by taking the average of the long and short diameters of the growth surface of the colony.

2.8. Statistical analysis

Each of the parameters considered in this study was compared using one-way analysis of variance (ANOVA) ($P<0.05$). Differences among means were evaluated using Tukey's tests at the 5% significance level. All statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. Plasmid sequences and maps

Of the four binary vectors used in this study, the sequences of pCAMBIA0380 and pBHt-2-gfp were known and available (GenBank accession number AF234290; Mullins et al., 2001), while those of the pC-HYG-GFP and pC-g-418-GFP were determined in this study (GenBank accession numbers MK303569 and MK303570). All four of the vectors encode the kanamycin

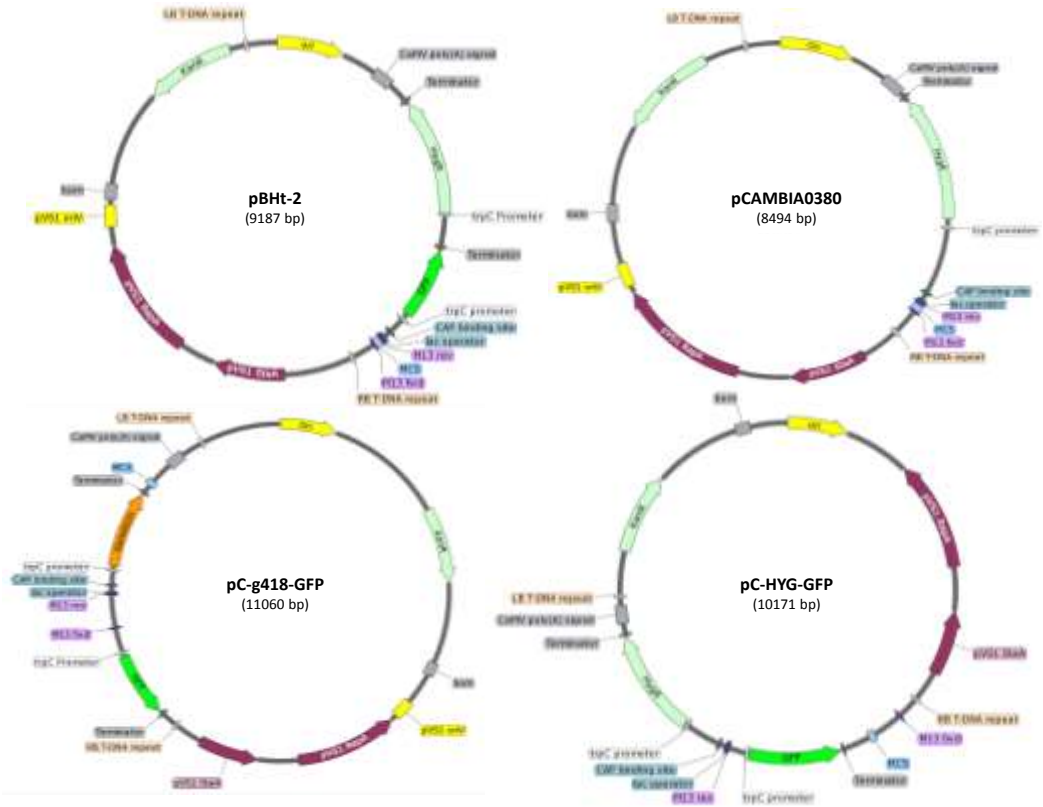

Figure 1. Schematic representation of the plasmids used in this study. The respective abbreviations are as follows: HygR = hygromycin B resistance cassettes; GFP = green fluorescent protein; Geneticin = *npt II*, the product of which confers resistance to geneticin, kanamycin and neomycin; KanR = *aph*A-3, the product of which confers resistance to kanamycin; Ori = high-copy-number ColE1/pMB1/pBR322/pUC origin of replication; PVS1 repA = replication protein from *Pseudomonas* plasmid pVS1; PVS1 staA = stability protein from *Pseudomonas* plasmid pVS1; RB T-DNA repeat = right border repeat from nopaline C58 T-DNA; LB T-DNA repeat = left border repeat from nopaline C58 T-DNA; M13 fwd and M13 Rev = common sequencing primers; MSC = multiple cloning site; lac operator = *lac* repressor encoded by *lacI*; CAP binding site = binding site for the *E. coli* catabolite activator protein; CAMV poly(A) signal = cauliflower mosaic virus polyadenylation signal; bom = basis of mobility region from pBR322.

resistance gene as selectable marker for *Agrobacterium* (Fig. 1 and Supplementary figure S1). The pCAMBIA0380 and pBHt-2 vectors further harbour the *hph* gene (encoding hygromycin B phosphotransferase) under the control of the *Aspergillus nidulans* trpC promoter (Rho et al., 2001). Plasmid pBHt-2 also harbours the *gfp* gene under control of the *Aspergillus* trpC promoter. The pC-HYG-GFP and pC-g-418-GFP plasmids carry T-DNA that contain *hph* or the neomycin phosphotransferase II gene (*nptII*) for geneticin resistance, respectively, both under the control of the *Aspergillus* trpC promoter. Both plasmids also contain the *gfp* gene under control of the *Aspergillus nidulans* gpdA promoter and trpC terminator. The pC-HYG-GFP vector lacks a multiple cloning site at one side of the *hph* gene.

3.2. *C. albifundus* sensitivity to Hygromycin B, Geneticin, Kanamycin and Carbenicillin

Among the different concentrations of hygromycin and geneticin evaluated, all three the fungal strains tested ceased to grow on MEA containing 5 µg/ml or more of hygromycin B or 10 µg/ml or more of geneticin (Fig. 2). As growth was completely inhibited at these concentrations for all three isolates, we used 10 µg/ml of hygromycin B or 15 µg/ml of geneticin in subsequent ATMT experiments to ensure that putative transformants are indeed resistant to these antibiotics (see below). In the case of kanamycin and carbenicillin, no growth inhibition was observed for any of the three fungal isolates, at all concentrations tested, thus confirming their resistance to these bacteriocidal antibiotics (Supplementary Figure S5).

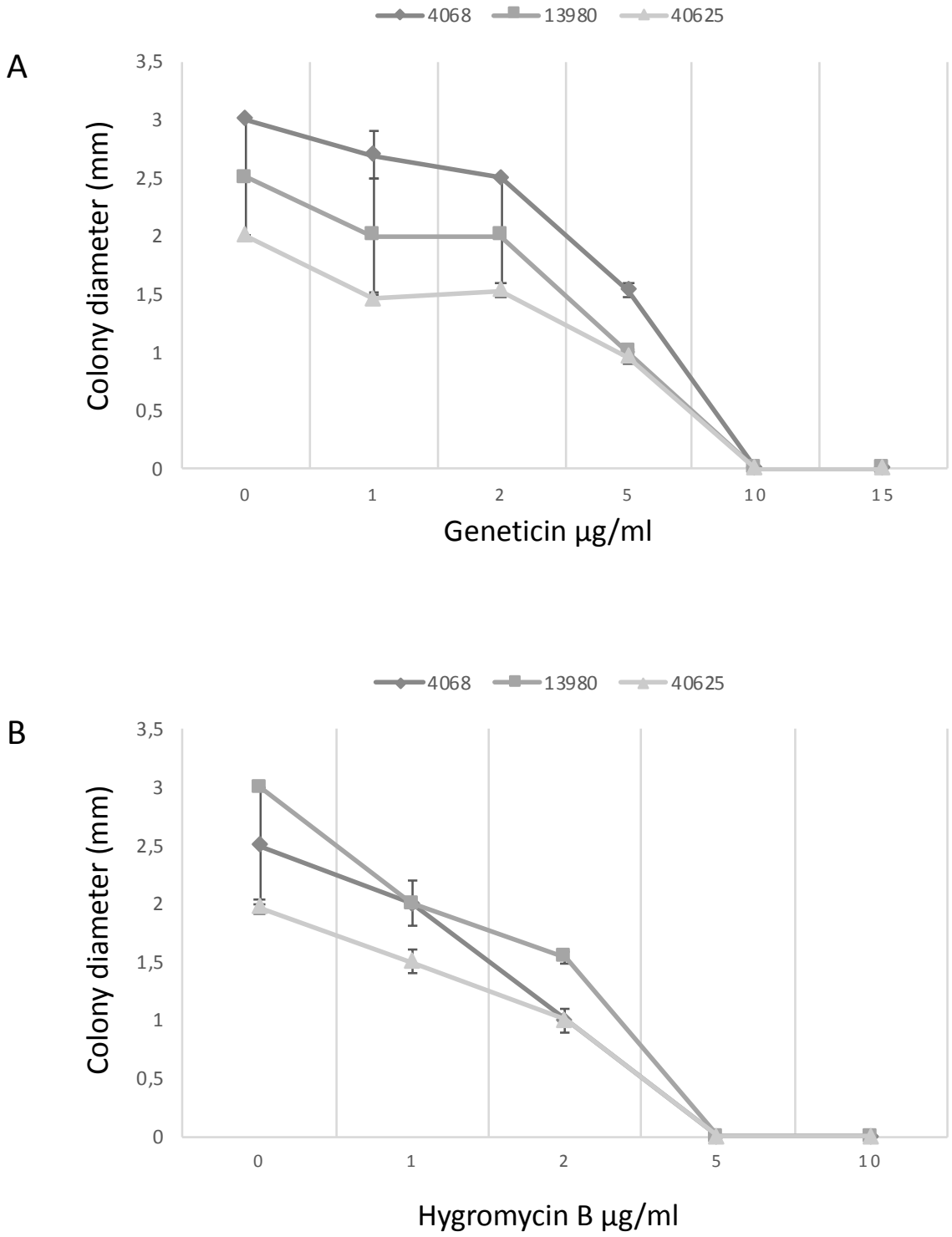

Figure 2. Colony diameter of *C. albifundus* isolates CMW 13980, CMW 4068 and CMW 40625 after 7 d at 25 °C on 2% MEA amended with different concentrations of either hygromycin B **(A)** or geneticin **(B)**.

3.3. *C. albifundus* transformation efficiency

From the number of transformants generated with a specified set of factors in three different replications, our results showed that some improved the transformation efficiency. However,

no colonies were observed on any of the control plates. One of these was the addition of acetosyringone to the co-cultivation media, and no transformants were recovered from experiments without this compound. However, co-cultivation of *C. albifundus* conidia with *A. tumefaciens* in the presence of 200 µM acetosyringone led to the formation of many hygromycin B or geneticin-resistant fungal colonies.

Our results showed that the transformation rates among the three *C. albifundus* isolates differed significantly (P value <0.05; Supplementary Fig. S2). While high transformation rates were achieved for isolates CMW 13890 and CMW 4068, those observed for isolate CMW 40625 was low (Supplementary Fig. S2). A similar trend was observed when the binary vectors were considered, where the use of vector pC-g-418-GFP with strain CMW 4068 were most efficient (P value <0.05). In terms of the concentration of the fungal conidia and bacterial cells, the use of 10^6 conidia/ml and $OD_{600\ nm}$ values of 0.3 and 0.4 for the *Agrobacterium* cells significantly (P value <0.05) improved the transformation efficiencies (Fig. 3 and Supplementary Fig. S2). Generally, a higher number of fungal conidia improved ATMT efficiency and rate, but $OD_{600\ nm}$ values > 0.4 seemed to be less efficient than the lower *Agrobacterium* cells concentrations. Finally, in terms of the co-cultivation period, highest transformation efficiency was achieved with 72 hours (Fig. 3).

The stability of *C. albifundus* transformants were confirmed by subjecting them to successive rounds of sub-culturing in the absence of any antibiotics. After five rounds of growth on MEA medium, all of the tested transformants were resistant to and grew well on MEA medium supplemented with either hygromycin B or geneticin depending on which plasmid was used.

3.4. Analysis of the *C. albifundus* transformants

Confirmation of the presence of *Agrobacterium* T-DNA in the putative transformants was achieved with PCR and sequence analyses. For detecting the presence of the *hph* gene, primers hph-F and hph-R allowed amplification and sequencing of a 427 bp product (Supplementary Fig. S3). For detecting the presence of the *gfp* gene, primers gfp-F and gfp-R allowed amplification and sequencing of a 270 bp product (Supplementary Fig. S3). PCR and sequencing with primers g-418-F and g-418-R confirmed the presence of the *nptII* gene (Supplementary Fig. S3).

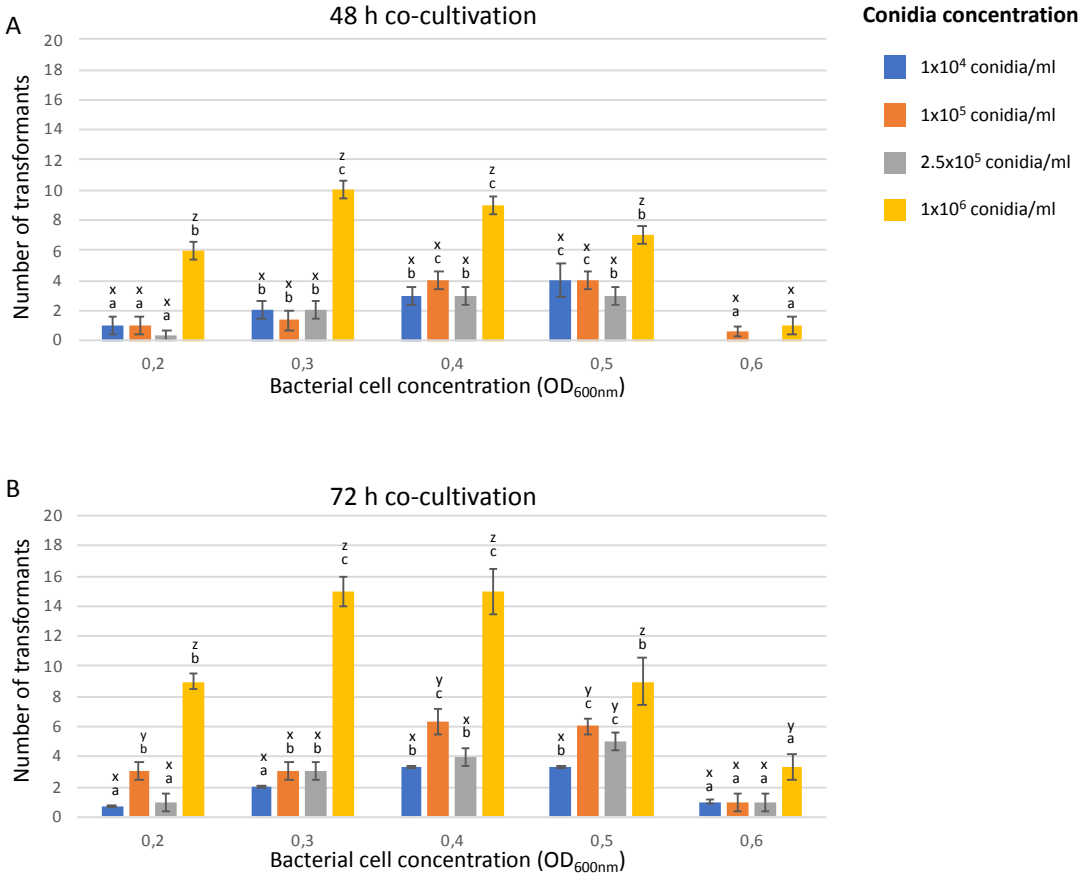

Figure 3. Transformation efficiency using different concentrations of *A. tumefaciens* cells (0.2, 0.3, 0.4, 0.5 and 0.6 OD600nm) and conidia of *Ceratocystis albifundus* (using 100 µL of the respective spore suspensions) isolate CMW 13890 carrying binary vector pC-HYG-GFP, following co-cultivation for 48h (**A**) and 72h (**B**) at 25 ˚C. Bars represent standard error, while different letters indicate significantly different means ($p < 0.05$) based on Tukey's tests. The letters a-c shows differences among bacterial cell concentrations (OD_{600nm}) relative to specific conidial concentrations, while the letters x-z show differences among conidial concentration relative to specific bacterial cell concentrations.

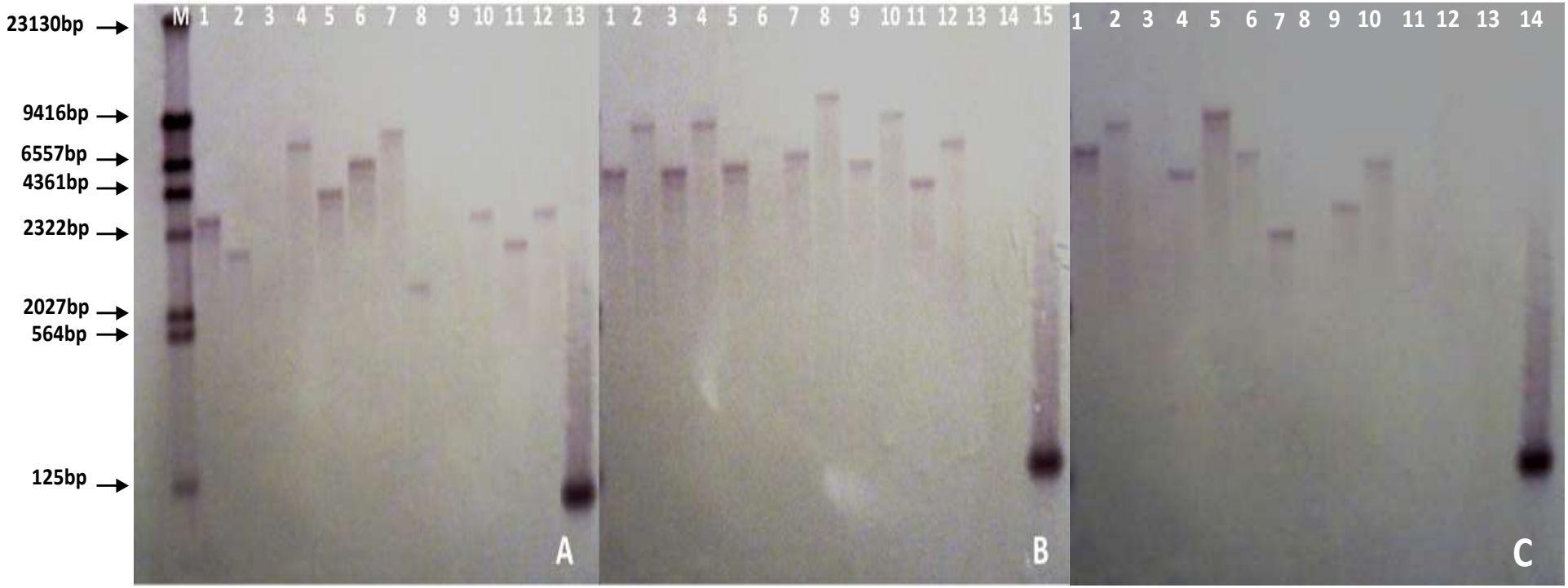

Figure 4. Southern blot analysis of genomic DNA isolated from *C. albifundus* colonies, digested with *Hind*III or *Eco*RI and hybridized with g-418 (**A**) or *hph* (**B and C**) probes. **A:** lanes 1-4: CMW 13980 transformants carrying plasmid pC-g-418-GFP; lanes 5-8: CMW 4068 transformants carrying plasmid pC-g-418-GFP; lanes 9-12: CMW 40625 transformants carrying plasmid pC-g-418-gfp; lane 13: positive control; lane M contains DNA Mol-weight Marker II, digoxigenin-labelled (Roche Applied Science, Mannheim, Germany). Lanes 1, 3, 5, 7, 9 and 11: digested with *Hind*III; Lanes 2, 4, 6, 8, 10 and 12: the same DNA but digested with *Eco*RI. **B:** Lanes 1-2: CMW13980 transformants carrying plasmid pC-HYG-GFP; lanes 3-4: CMW13980 transformants carrying plasmid pCAMBIA0380; lanes 5-6: CMW 40625 transformants carrying plasmid pC-HYG-GFP; lanes 7-8: CMW 40625 transformants carrying plasmid pCAMBIA0380; lanes 9-10: CMW 4068 transformants carrying plasmid pC-HYG-GFP; lane 11-12: CMW 4068 transformants carrying plasmid pCAMBIA0380; lane 15: positive control. lanes 1, 3, 5, 7, 9 and 11: digested with *Hind*III; Lanes 2, 4, 6, 8, 10 and 12: the same DNA but digested with *Eco*RI. **C:** Lanes 1-4: CMW 13980 carrying plasmid pBHt2; Lanes 5-8: CMW 40625 transformants carrying plasmid pBHt2; Lanes 9-10: CMW 4068 transformants carrying plasmid pBHt2; Lane 14: positive control. Lanes 1, 3, 5, 7 and 9: digested with *Hind*III; Lanes 2, 4, 6, 8 and 10: the same DNA but digested with *Eco*RI.

Confirmation of the integration of T-DNA into the genomes of the various *C. albifundus* transformants was achieved by Southern blot hybridization analysis (Fig. 4). Hybridization with a probe that targets portions of the *hph* or *nptII* genes yielded single and unique bands, which varied in size depending on the transformant examined. This suggested that T-DNAs were integrated randomly into the fungal genomes of the transformants examined, and in each case only a single copy was integrated into the genome.

For transformants carrying plasmids pC-HYG-GFP, pC-g-418-GFP and pBHt-2, we employed the emission of fluorescence to assess whether the hygromycin B or geneticin-resistant colonies expressed GFP (Fig. 5 and Supplementary Fig. S4). Images obtained with fluorescent microscopy showed that the growing hyphae of the transformants strongly expressed the green fluorescent signal characteristic of GFP. Also, these fluorescent signals were detected throughout entire hyphae. As was expected, no green fluorescence was observed in experiments with the wild type fungi.

Like the wild type isolates, the optimal growth temperature for the transformants was between 25°C and 30°C (Results not shown). However, noticeable differences were observed in fertile hyphae (conidiophores, conidiogenous cells and conidia) and in the production of the sexual morph (i.e., ascomata and ascospores). The wild type of three strains produced asexual morphs either abundantly (CMW 13980) or scarcely (CMW 40625, CMW 4068) (Fig. 6, A, B, G and M). A sexual morph was absent in the wild type of CMW 13980, CMW 40625 but present in CMW 4068 that produced some ascomata with ascospores and malformed ostiolar necks. By contrast, sexual morphs were produced in some transformants of all three strains, while a few transformants of CMW 4068 also produced ascomata with malformed ostiolar necks (Fig. 6, C and H). Conidiophores were reduced to conidiogenous cells (phialides) both in the wild types and transformants. Phialides between the wild type and the transformants were varied; some were similar to the wild types (Fig. 6 K and Q) but some produced much longer and more septated phialides (Fig. 6 J and P). Conidial morphology was varied: some similar to the wild types (cylindrical-shape) and some with inflated apex or ends (Fig. 6 F, Q and R).

With respect to growth and colony characteristics on MEA medium, no clear trends were observed (Fig. 7). For example, for most of the transformants, colony morphology and colour differed from that of their corresponding wild types. Also, among the transformants of a particular isolate, large variation was observed regarding these properties

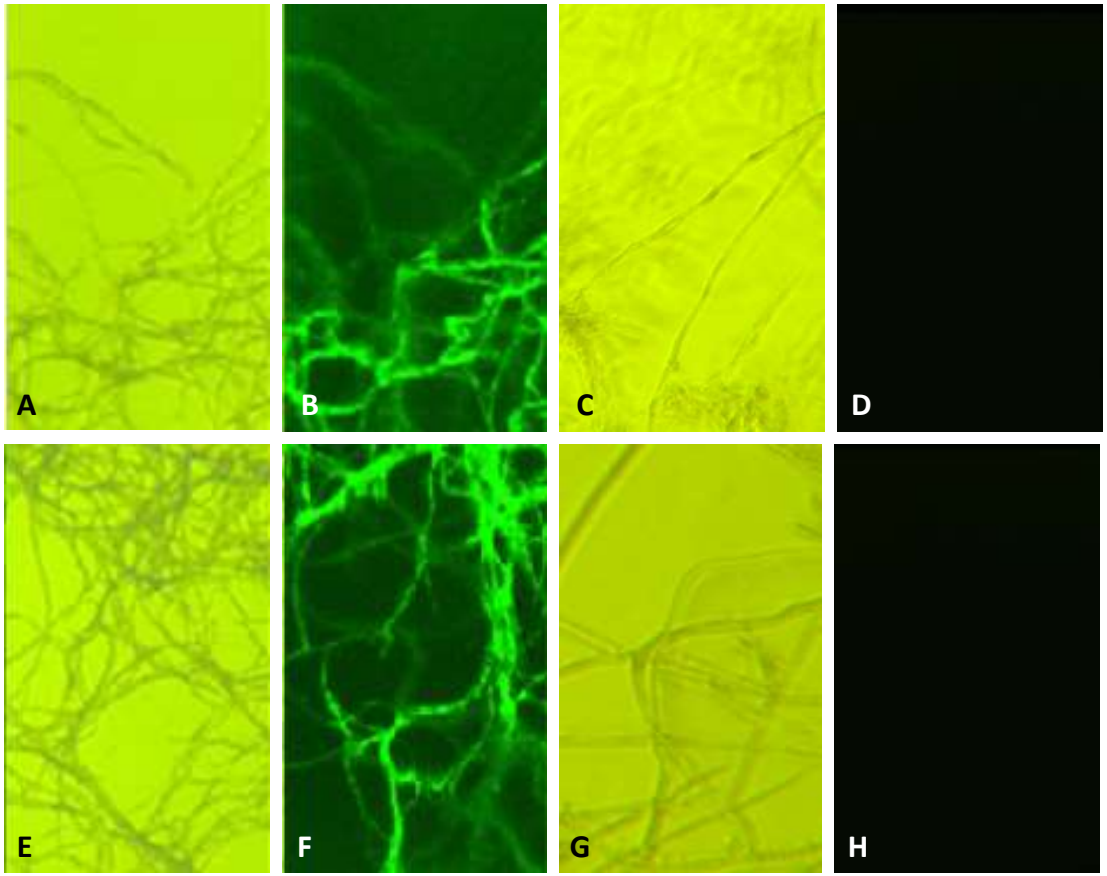

Figure 5. Micrographs of *C. albifundus* transformants of isolates CMW 13980 (**A-D**) and CMW 40625 (**E-H**) carrying plasmid pC-HYG-GFP and their corresponding wild types. A and B: Bright field image of the hyphae of a CMW 13980 transformant and the green fluorescence of the same hyphae visualized using fluorescent microscopy, respectively. **C and D:** Bright field and fluorescence micrographs, respectively of the wild type of isolate CMW 1398. **E and F:** Bright field and fluorescence micrographs, respectively, of a CMW 40625 transformant. **G and H:** Bright field and fluorescence micrographs of the wild type of isolate CMW 40625. Microscopy under UV fluorescence showed constitutive expression of GFP in the respective transformants, where the GFP signal was also distributed homogenously throughout the hyphae.

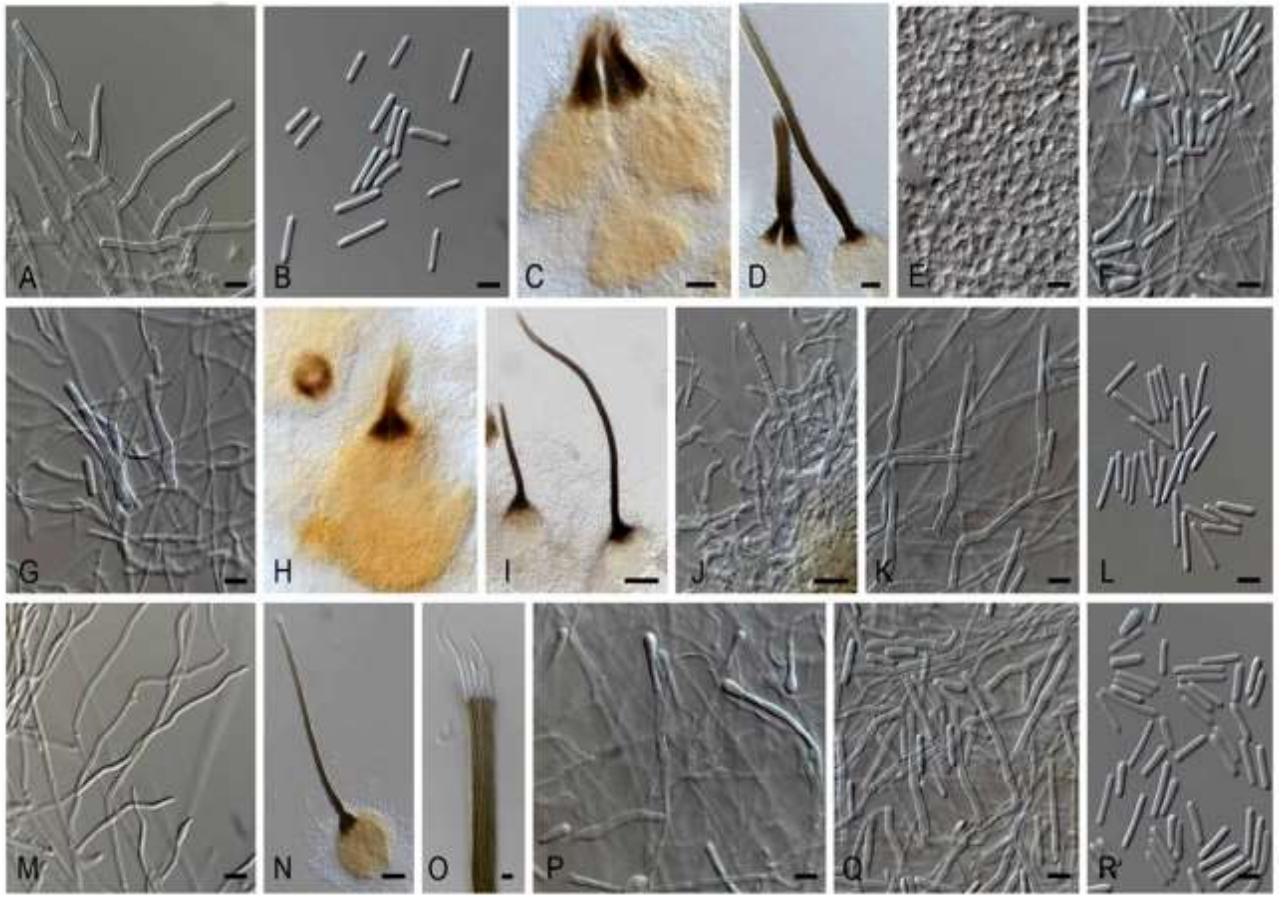

Figure 6. Microscopic features of wild type and transformant strains of Ceratocystis albifundus (A–F. CMW 13980, G–L. CMW 40625. M–R. CMW 4068). A, B, G, M. Wild type strains. C–F, H–L, N–R. Transformant strains. A, G, J, K, P, Q. Conidiogenous cells (phialides). B, F, L, R. Conidia. C, H. Ascoma with poorly developed ostiolar neck. D, I, N. Ascomata with fully developed necks. E. Ascospores. M. Vegetative hyphae. O. Ostiolar hyphae. Scale bars: A, B, E, F, G, K–R = 10 µm, J = 25 µm; C, D, H = 50 µm; I, N = 100 µm.

Figure 7. Colony variation of wild type and transformant cultures of *Ceratocystis albifundus* strains (A–E. CMW 40625, F–J. CMW 4068, K–O. CMW 13980). A, F, K. Wild type. B–E, G–J, L–O. Transformants.

4. Discussion

In this study, we developed an ATMT system for the filamentous fungus *C. albifundus*. A similar system has been previously reported for two other members of the Ceratocystidaceae (i.e., *E. resinifera* and *B. basicola*), but to the best of our knowledge, this is the first report of successful transformation for any true *Ceratocystis* species using *A. tumefaciens*. As is often

the case with fungi methodology developed for one genus does not work for other genera in the same family. With this system, single copies of the respective antibiotic resistance and GFP encoding genes, fused to the *Aspergillus* gpdA or trpC promoters, were incorporated into the genomes of multiple *C. albifundus* isolates. The system used in this study is stable with a high-efficiency, yielding up to 400 transformants per 10^6 conidia.

Co-cultivation of *A. tumefaciens* cells carrying any one of the pBHt2, pCAMBIA0380, PC-HYG-GFP or PC-g-418-GFP vectors with the conidia of three different isolates of *C. albifundus* led to the formation of hygromycin B or geneticin-resistant colonies 10-14 d after transfer to the selection medium in three independent experiments. The vectors used in this study is small in size, which makes them useful and amenable for further manipulations involving the incorporation of additional genes (Mullins et al., 2001). Furthermore, the pBHt2 vector contains nine unique restriction sites that enhances its use in cloning experiments (Mullins et al., 2001). Three of the vectors used also expressed the small and stable protein GFP, although only in the hyphae of *C. albifundus* and not in conidia. This could be a consequence of the thick cell walls that prevent entry of light and thus detection of fluorescence, which also has been reported for *Nomuraea rileyi* (Shao et al., 2015). This is different from GFP-transformed *F. oxysporum* and *Fusarium proliferatum* where fluorescence was observed in both conidia and hyphae (Islam et al. 2012; Bernardi-Wenzel et al., 2016). Nevertheless, the expression of GFP allows detection under UV light in both *in vitro* and *in vivo* experiments (Armesto et al., 2012), and it can be used as reporter for gene expression and localization in cell development studies (Lorang et al., 2001).

Hygromycin B or geneticin were used as the selectable markers for transformed *C. albifundus*. Even though the concentrations used for these antibiotics were lower than those typically used for Ascomycota (Rogers et al., 2004; Weld et al., 2006), our results showed that *C. albifundus* is highly susceptible to these agents. Tests with as little as 5 µg/ml hygromycin B or 10 µg/ml geneticin stopped its growth completely. However, transformation with the respective binary vectors rendered *C. albifundus* resistant to these antibiotics. Also, in all of our tested transformants, T-DNA remained in the fungal genome, even in the absence of antibiotics, which is contrast to the fact that ATMT-derived transformants of Ascomycota are often not stable. For example, Mora-Lugo et al. (2014) reported that only 40% of their *Aspergillus sojae* transformants were mitotically stable and for transformed *Backusella lamprospora*, Nyilasi et al. (2008) showed the loss of T-DNA in subsequent generations. Our findings therefore showed

that the *C. albifundus* ATMT system reported here yields mitotically stable transformants, which is a vital aspect of any gene insertion or disruption procedure.

The ATMT system used for *C. albifundus* is similar to those for other filamentous Ascomycota with respect to transformation efficiency and the need for acetosyringone. In terms of transformation rate, we typically obtained 200-400 transformants per 10^6 spores, which is in the same range as the reported transformation efficiencies for *Verticillium albo-atrum* (Knight et al. 2009), *Nomuraea rileyi* (Shao et al., 2015) and *Harpophora oryzae* (Lui et al., 2016). It is, however, much lower than those reported for the rice blast fungus *M. grisea* (300 to > 1,000 transformants per 10^6 spores) (Mullins et al., 2001; Rho et al., 2001). In terms of acetosyringone, our results showed that this compound is essential for ATMT of *C. albifundus*, as is often the case for fungal transformation (de Groot et al., 1998; Chen et al., 2000; Mullins et al., 2001). ATMT systems requiring acetosyringone include those for *Harpophora oryzae*, *Lecanicillium lecanii* and *Aspergillus awamori* (de Groot et al., 1998, Michielse et al., 2005; Liu et al., 2016; Martınez-Cruz et al., 2017). This compound stimulates expression of the *vir* genes, the products of which are responsible for the integration of the T-DNA into the genome of the fungus, thereby increasing the efficiency of transformation (Michielse et al. 2005).

Among the factors influencing transformation efficiency and the rate at which T-DNA was inserted into the *C. albifundus* genome, isolate identity appeared to be important. The number of transformants obtained for the three isolates examined differed significantly across the various parameters evaluated. Such intraspecific variation in transformation efficiency has been reported previously in various fungi, including *Rosellinia necatrix*, *Penicillium digitatum* and *Aspergillus awamori* (Michielse et al., 2004; Wang and Li, 2008; Kano et al., 2011). Originally, de Groot et al. (1998) ascribed such within-species patterns of transformation efficiency to the biological differences among individuals of the same species, but future work using ATMT and other gene replacement/disruption systems are required to reveal the precise molecular traits underlying this property.

Our study showed that *A. tumefaciens* cell and *C. albifundus* conidia concentrations, and co-cultivation times strongly influence transformation efficiency. We found that *Agrobacterium* suspensions with OD_{600} values of 0.4 and 0.5 and a conidial concentration of 10^6 spores/ml yielded the best transformation efficiencies. It was also demonstrated in other fungi that increasing the amount of *A. tumefaciens* cells and conidia improved transformation (Mullins et

al., 2001; Rho et al., 2001; Michielse et al. 2005; Wang and Li, 2008; Liu et al., 2016; Martınez-Cruz et al., 2016; Lu et al., 2017). Additionally, as was observed in other fungi, especially in the case of slower growing species, longer co-cultivation periods increased the number of transformants (Michielse et al., 2005; Mullins et al., 2001; Rho et al., 2001; Lu et al., 2017). In our study, the numbers of *C. albifundus* transformants increased in all three isolates when the co-cultivation period was increased to 72 h. The choice of *C. albifundus* recipient strains, concentration of fungal and bacterial cells, and the duration of the co-cultivation period used are therefore crucial for optimal transformation efficiencies.

Southern analysis of all the examined transformants showed single-copy integration of the T-DNA into the *C. albifundus* genome. Such high rates of single-copy integration events are a common feature of ATMT (Islam et al., 2012). For example, high rates of single-copy insertion of T-DNA have been shown for various species of *Colletotrichum*, including *C. trifolii* (Takahara et al., 2004), *C. lagenarium* (Tsuji et al., 2003), *C. acutatum* and *C. falcatum* (Maruthachalam et al., 2008). This feature is essential for an efficient ATMT system, as it allows association of a particular phenotype with a specific locus in the genome of the fungus (Michielse et al., 2005). Furthermore, the Southern hybridization patterns observed for our transformants suggested that the T-DNA integration happened at different locations of the genome. This is similar to what has been reported for fungi such as *Aspergillus carbonarius* and *Fusarium circinatum* (Covert et al., 2001; Morioka et al., 2006). As expected, our ATMT system thus allowed efficient generation of transformants carrying random and single-copy insertions of the manipulated T-DNA (Michielse et al., 2005).

The conidia and sexual features of the *C. albifundus* transformants differed from those of the wild types. Changes in morphological traits of transformed fungi due to random integration of T-DNA into the genome are a common phenomenon (Lu et al., 2017). For example, phenotypic alterations such as faster growing hyphae, swollen colony surface and production of more pigment, have been also observed in transformants of *Aspergillus terreus* (Wang et al., 2014). Such changes morphology, growth rate and conidial germination in the mutant's strains may thus offer ideal opportunities for studying the molecular basis of these biological features in *C. albifundus* and other fungi.

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Supplementary material

Supplementary Figure S1. Restriction map of the plasmids used in this study (**A**: pBHt-2; **B**: pCAMBIA0380; **C**: pC-g-418-GFP; **D**: pC-HYG-GFP). HygR= hygromycin B resistance cassettes, ZsGreen 1= *gfp* gene, g-418= geneticin resistance cassette and KanR= kanamycin resistance cassette.

Supplementary Figure S2. Effect of different parameters (number of conidia and concentration of bacterial cells) during co-cultivation at 25 °C with *A. tumefaciens* used for ATMT on *Ceratocystis albifundus* strains CMW 13980 **(A)** CMW 4068 **(B)** and CMW40625 **(C)** for the 4 vectors included in this study (pC-HYG-GFP, pC-g-418-GFP, pCAMBIA0380 and pBHt2). Bars represent standard error, where different letters indicate significantly different means ($p < 0.05$) based on Tukey's tests. The letters a-c show differences among bacterial cell concentrations (optical density at 600nm) relative to specific conidial concentrations, while the letters x-z, show differences among conidial concentration relative to specific bacterial cell concentrations.

Supplementary Figure S3. **A, B:** Agarose gel of PCR products obtained with hph specific primers from the *C. albifundus* transformants carrying plasmids pC-HYG-GFP (A), pBHt2 (A) and pCAMBIA0380 (B). **A:** Randomly selected hygromycin-resistant transformants obtained with the binary vectors pC-HYG-GFP (lanes 2-3: CMW 13980; lanes 5-6: CMW 4068 and lanes 9-10: CMW 40625) and pBHt-2 (lanes 4: CMW 13980; 7-8: CMW 4068; 11-12: CMW 40625) were used as PCR templates. Lane 13: plasmid DNA of pBHt-2 used as positive control; lane 14: negative control; lane M: 100bp DNA ladder (Invitrogen, USA). **B:** gDNA of randomly selected hygromycin resistant transformants carrying the binary vector pCAMBIA0380 (lanes 2–3: CMW 13980; 4-5: CMW 4068; 6-7: CMW 40625) were used as PCR templates. Lane 8: plasmid DNA of pCAMBIA0380 used as positive control; lane M: 100bp DNA ladder (Invitrogen, USA). **C, D:** Agarose gel of PCR products obtained with gfp specific primers from the *C. albifundus* transformants carrying plasmids pC-HYG-GFP (A), pBHt-2 (A) and pC-g-418-GFP (B). **C:** gDNA of randomly selected gfp-expressed transformants obtained with the binary vectors pC-HYG-GFP (lanes 1-2: CMW 13980; lanes 4-5: CMW 4068 and lanes 8-9: CMW 40625) and pBHt-2 (lanes 3: CMW 13980; 6-7: CMW 4068; 10-11: CMW 40625) were used as PCR templates. Lane 12: plasmid DNA of pBHt-2 used as positive control; lane 13: negative control; lane M: 100bp DNA ladder (Invitrogen,

USA). **D:** gDNA of randomly selected hygromycin resistant transformants carrying the binary vector pC-g-418-GFP (lanes 2–3: CMW 13980; 4-5: CMW 4068; 6-7: CMW 40625) were used as PCR templates. Lane 8: plasmid DNA of pC-g-418-GFP used as positive control; lane 9: negative control; lane M: 100bp DNA ladder (Invitrogen, USA). **E.** Agarose gel of PCR products obtained with g-418 specific primers from the *C. albifundus* transformants. gDNA of randomly selected geneticin resistant transformants obtained with the binary vectors pC-g-418-GFP (lanes 1–3: 13980; 4-7: CMW 4068; 8-11: CMW 40625) were used as PCR templates. The positive control, plasmid DNA of pC-g-418-GFP (lane 12). The negative control, gDNA of *C. albifundus* wild type (lane 13). Lane M contains 100bp DNA ladder (Invitrogen, USA).

Supplementary Figure S4. Fluorescence micrographs of *C. albifundus* transformants showing constitutive expression of GFP. A-H. CMW 13980, I-P. CMW4068 and Q-X. CMW 40625. **A, C, E:** Bright field image of the CMW 13980 hyphae carrying plasmids pC-HYG-GFP, pC-g-418-GFP and pBHt2, respectively; **B, D, F:** Green fluorescence of the same hyphae. **I, K, M:** Bright field image of the CMW 4068 hyphae carrying plasmids pC-HYG-GFP, pC-g-418-GFP and pBHt2, respectively; J, L, N: Green fluorescence of the same hyphae. **Q, S, U:** Bright field image of the CMW 40625 hyphae carrying plasmids pC-HYG-GFP, pC-g-418-GFP and pBHt2, respectively. **R, T, V:** Green fluorescence of the same hyphae **G, O, W:** Bright field image of the wild type control of strains CMW 13980, CMW 4068 and CMW 40625, respectively, **H, P and X:** Same hyphae under the fluorescent microscope.

Supplementary Figure S5. Colony diameter of *C. albifundus* isolates CMW 13980, CMW 4068 and CMW 40625 after 21 d at 25 °C on 2% MEA amended with different concentrations of either kanamycin **(A)** or carbenicillin **(B)**.