Transferring an *Agrobacterium*-mediated transformation protocol across eight genera in the Ceratocystidaceae

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Abstract

Establishing a transformation system is an essential first step for functional studies. Numerous transformation systems have been developed and optimised for filamentous ascomycetes, including *Agrobacterium*-mediated transformation. Recently, such a system was developed for the African tree pathogen *Ceratocystis albifundus*. This fungus is part of the family Ceratocystidaceae, which includes many tree and plant pathogens of economic importance. Despite the many advantages associated with *Agrobacterium*-mediated transformation, establishing and optimising this system within a species is extremely arduous due to the many species-specific parameters that must be optimised. This study aimed to apply the existing *C. albifundus Agrobacterium*-mediated transformation protocol to eight diverse Ceratocystidaceae species from eight different genera. Minor adjustments were made to the already established protocol to increase transformant yield before it was used to successfully produce transformants for six of the eight targeted species without the need for any further species-specific optimizations. Although the number of transformants obtained was less than for *C. albifundus*, this study proves that protocols for *Agrobacterium*-mediated transformation might be more transferable than previously thought. It also provides a useful benchmark for future transformation studies in the Ceratocystidaceae and will make species-specific optimisation easier.

Keywords

Non-model fungi; genetic modification; tree pathogens

Introduction

Functional studies are crucial to our understanding of fungal biology and have become more important as the number of publicly available genome sequences has increased. The diversity of fungal species for which genome sequences are available (http://1000.fungalgenomes.org) provides a comprehensive source of candidate genes needing functional characterisation. Understanding gene function, however, is often based on the extrapolation of knowledge from homologues identified and functionally studied in non-related species, mostly model organisms. The assumption that the function of a gene is conserved among both closely related and diverse species is mostly correct, but examples exist where this is not the case (Kwon et al., 2011). The problem worsens for species-specific genes for which there is no point of reference, such as novel mating-type genes (Turgeon & Yoder, 2000; Wilken et al., 2017). In these cases, functional studies in non-model systems are needed, as genetic manipulation of target genes can advance our understanding of gene function for both species-specific genes, as well as known homologues. However, before targeted genome editing can be achieved, a transformation system must first be established in the organism of interest.

Agrobacterium-mediated transformation has been used to genetically alter fungi for more than two decades, and still remains one of the most popular transformation techniques (de Groot et al., 1998). *Agrobacterium tumefaciens* is a bacterium that can transmit a segment of its tumour-inducing (Ti) plasmid into a target cell. The transferred piece is aptly named the transfer DNA (T-DNA), and randomly integrates into the genome when there is a lack of sequences homologous to the recipient DNA (Hooykaas et al., 2018). These are usually single-copy integrations (Blaise et al., 2007; Michielse et al., 2005). *Agrobacterium*-mediated transformation is also useful for targeted integrations where the T-DNA is targeted to a specific genomic location by incorporating guide sequences into the T-DNA. This form of integration has proven very successful as homologous recombination is generally more likely to occur than non-homologous recombination in *Agrobacterium* transformations (Amey et al., 2003; Bundock et al., 1999; Michielse et al., 2005). *Agrobacterium*-transformation has been successfully applied to a range of starting material including conidia and fresh mycelia, a distinct advantage over other transformation techniques that

rely on the production of enzymatically digested protoplasts (de Groot et al., 1998; Zhang et al., 2003).

While *Agrobacterium*-mediated transformation is a highly efficient method, there are many parameters of the protocol that should be optimised for individual species (Michielse et al., 2005). This makes it a difficult and time-consuming process to establish and optimise an *Agrobacterium* protocol in species where none exists (Michielse et al., 2005; Poyedinok & Blume, 2018), as is often the case in non-model species. These parameters include the type and quantity of fungal material used, the amount of bacteria used, the ratio of fungal cells to *A. tumefaciens* cells in the co-incubation suspension, the need for and concentration of acetosyringone, as well as co-incubation conditions such as time, temperature, pH and choice of membrane used (Michielse et al., 2005). For example, a co-incubation period of 48 hours in *Penicillium parvum* resulted in an approximate four-fold increase in transformant yield as opposed to a 36 hour period, and 24 hours did not yield any transformants (Long et al., 2018). In *Kabatiella zeae*, a ratio of 3:1 of conidia to bacteria (conidia suspension of 10^6 conidia per ml and *A. tumefaciens* with an OD_{600} of 0.6) was optimal, resulting in more than 142 transformants per 10^6 conidia, while ratios of 2:1 and 4:1 yielded less than 50 transformants (Sun et al., 2018). With so many factors influencing transformation success, attempting these optimisations for many different species would be an arduous task.

Recently, an *Agrobacterium*-mediated transformation protocol was established for *Ceratocystis albifundus*, a filamentous ascomycete in the family Ceratocystidaceae (Sayari et al., 2019a). This species is an important pathogen on plantation of *Acacia mearnsii* and cultivated *Protea cynaroides* plants in South Africa (de Beer et al., 2014). A genome sequence is available for this species, as well as for numerous other members of the family (van der Nest et al., 2014a, b; Wingfield et al., 2015, 2018). These genomes have been used for studies related to host specificity, pathogen aggressiveness, the degradation of plant-derived sucrose, sexual reproductive strategies and recombination, as well as secondary metabolite biosynthesis gene clusters (Fourie et al., 2019; Nel et al., 2018; Sayari et al., 2018; Sayari et al., 2019b; Simpson et al., 2018; van der Nest et al., 2015, 2019; Wilken et al., 2014; Wilken et al., 2017; Wilson et al.,

2018; Witthuhn et al., 2000). However, almost all of these were limited to *in silico* analyses as transformation systems are only available for five Ceratocystidaceae species; three of which are *Agrobacterium* protocols (including the recent *C. albifundus* protocol) and the remaining two are PEG/$CaCl_2$-protoplast transformation systems (Al-jaaidi, 2007; Loppnau et al., 2004; Niu et al. 2019; Sayari et al., 2019a; Tzima et al., 2014; Wilson et al., 2020). Considering the importance of this family in plant health, as well as the amount of interest generated through *in silico* analyses, there is clearly a need to establish more transformation systems that will drive functional studies for these fungi.

The aim of this study was to transform eight Ceratocystidaceae species using the *Agrobacterium*-mediated transformation system established in *C. albifundus* (Sayari et al., 2019a). The existing protocol was first minorly adjusted and applied to *C. albifundus*, before being used on the closely related species *Ceratocystis manginecans*, as well as a representative member of the genera *Ambrosiella*, *Berkeleyomyces*, *Chalaropsis*, *Davidsoniella*, *Endoconidiophora*, *Huntiella* and *Thielaviopsis*. In this way, the transferability of the *Agrobacterium*-mediated transformation protocol of *C. albifundus* to other species within the same family was evaluated. The results of this study will address questions related to the transferability of *Agrobacterium*-mediated transformation systems across genera, while providing a starting point for future optimizations for this transformation system in the Ceratocystidaceae.

Materials and methods

Strains and plasmid

The AGL-1 strain of *A. tumefaciens*, that contains a chromosomal carbenicillin resistance gene and makes use of a bivalent vector system, was used for this study (Hellens et al., 2000). This bacterium contained the Ti plasmid pC-HYG-GFP (Sayari et al., 2019a). In addition to a kanamycin resistance gene within the backbone of this plasmid, the T-DNA region contains two selectable markers to allow for effective selection of transformed fungal hosts. These markers include a hygromycin resistance gene (*hyg*^{R}) and a gene encoding green fluorescent protein (*GFP*). *Agrobacterium tumefaciens* was maintained on standard Luria-Bertani agar plates (10 g/l tryptone,

Sigma-Aldrich, Johannesburg, South Africa; 10 g/l NaCl, 5 g/l yeast extract, 1.5% agar, Biolab, Germiston, South Africa) supplemented with 50 µg/ml carbenicillin and 100 µg/ml kanamycin (Sigma-Aldrich, Johannesburg, South Africa) incubated at 28°C.

The nine fungal isolates used in this study were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) based at the University of Pretoria (Table 1). These included *Ceratocystis albifundus* isolate CMW 4068 that was included in the initial study that established the *Agrobacterium*-mediated transformation protocol (Sayari et al., 2019a). *Ceratocystis manginecans* was chosen as a closely related relative, while one representative species was chosen from seven other Ceratocystidaceae genera. For most of the species, genome sequences are available for the specific isolates used in this study (Table 1). Although genome sequences are not available for the isolates of *Huntiella moniliformis* and *Berkeleyomyces basicola* used here, representative assemblies are publicly available (Table 1). A reference assembly for the genome of *Chalaropsis ovoidea* is expected to be made publicly available in the near future (P.M. Wilken, personal communication). All fungal isolates were maintained at room temperature (~25°C) on 2% MEA (2% malt extract, 2% agar, Biolab, Germiston, South Africa), supplemented with 100 mg/l thiamine and 150 mg/l streptomycin (Sigma-Aldrich, Johannesburg, South Africa), through the sterile transfer of a mycelial block or spore drop mass. To produce liquid cultures, five mycelial blocks of between 5 - 10 mm were transferred into 200 ml of 1% malt extract and incubated for one week in the dark at 25°C with 140 rpm shaking.

Hygromycin sensitivity

To determine if a single, selective dosage of hygromycin could be used to screen for transformants across all the targeted species, the wild-type isolate of each species was grown in triplicate on media supplemented with either 25 µg/ml or 50 µg/ml of the antibiotic (Sigma, Johannesburg, South Africa). A 5 mm mycelial plug of each species was plated on 2% MEA supplemented with hygromycin in 65 mm plates and incubated at room temperature. Growth was measured as an average across two perpendicular diameters for each plate after 14 days of incubation.

Table 1: Isolates used in the *Agrobacterium*-mediated transformations

Species	**Culture collection number(s)[1]**	**Country**	**Host**	**Collected/ isolated by**	**Genome sequence**	**Genome assembly accession number**
Ambrosiella cleistominuta	CMW 50464; CBS 141682; C 3843	USA	*Anisandrus maiche*	C.G. Mayers 2015	Wilken et al., 2020	ASM1713954v1
Berkeleyomyces basicola	CMW 25440; CBS 142829	Indonesia	*Styrax benzoin*	M.J. Wingfield & M. van Wyk 2007	Nel et al., 2018[2]	ASM367143v1
Ceratocystis albifundus	CMW 4068; CBS 128992	South Africa	*Acacia mearnsii*	J. Roux 1997	van der Nest et al., 2019	ASM274225v2
C. manginecans	CMW 17570; CBS 138185	Oman	*Prosopis cineraria*	A. Al Adawi 2005	van der Nest et al., 2014b	CMang1.0
Chalaropsis ovoidea	CMW 22733; CBS 354.76; C 1375	Netherlands	Firewood	W. Gams 1976	P.M. Wilken, personal communication	-
Davidsoniella virescens	CMW 17339; CBS 17339	USA	*Acer saccharum*	D. Houston 1987	Wingfield et al., 2015	ASM151380v1
Endoconidiophora polonica	CMW 20930; CBS 100205	Norway	*Picea abies*	H. Solheim 1990	Wingfield et al., 2016	ASM185676v1
Huntiella moniliformis	CMW 36919; CBS 144008	Cameroon	*Theobroma cacao*	M. Mbenoun 2009	van der Nest et al., 2014b[2]	CMo_1.0
Thielaviopsis cerberus		Cameroon	*Elaeis guineensis*	M. Mbenoun &	Krämer et al. 2021	ASM1685922v1

	CMW 36653; CBS 130763	J. Roux 2010

[1] C: Culture collection of T.C. Harrington, Iowa State University, USA; CBS: Culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CMW: Culture collection of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[2] A genome assembly is not available for the specific isolates tested here, however, representative genome sequences are publicly available.

Transformation

The *Agrobacterium*-mediated transformation protocol established in *C. albifundus* (Sayari et al., 2019a) was used as a basis for all the transformations attempted in this study. Minor adjustments were made to the established protocol in an effort to improve transformant yield and increase throughput rate, and the effect of these were tested in *C. albifundus* (Table 1). The first adjustment was the use of liquid cultures to isolate conidia instead of cultures grown on solid agar. Following filtration of the liquid cultures through Miracloth (Merck, New Jersey, USA), conidia were pelleted by centrifugation at 5 000 *g* for 30 min at 4°C. The second adjustment was the addition of a one-hour incubation step in liquid at 25°C (with shaking at 140 rpm) before plating the conidial and *A. tumefaciens* suspensions. Lastly, transformants were selected using a hygromycin concentration of 50 µg/ml instead of 10 µg/ml, as determined by the hygromycin assay (see results). The altered protocol was then applied to the remaining eight Ceratocystidaceae species (Table 1), without any further alterations.

Two controls were included during every transformation reaction. To ascertain the viability of the conidia following transformation, a sample of the conidia / *A. tumefaciens* mixture from the co-incubation step was placed onto MEA containing cefotaxime (100 µg/ml, Aspen Pharmacare, Durban, South Africa), but lacking the selective hygromycin antibiotic. This would kill the bacteria, but allow both transformed and untransformed conidia to grow. For a second control, conidia that had not been mixed with *A. tumefaciens*, but that were further subjected to all other transformation steps, were plated on selective media containing cefotaxime and hygromycin. Growth of these conidia would screen for false positives that could emerge on selective media even when not transformed.

The plates were checked regularly for the emergence of colonies for up to 30 days. Where possible, transformation efficiency was calculated as the average number of transformants produced from 10^6 conidia (Sayari et al., 2019a). As colonies emerged, a maximum of five colonies were transferred to 2% MEA plates with 50 µg/ml hygromycin by collecting mycelia with a sterile needle. Homokaryotic transformants were obtained by first transferring mycelia of each transformant onto 2% water agar and incubating these overnight. These were then used to collect

hyphal tips onto hygromycin-supplemented MEA using a stereomicroscope. This excluded the possibility of heterokaryotic transformants that may contain multinucleated cells with some nuclei lacking T-DNA integrations.

Analysis of transformants

DNA was isolated from 1 to 2 week-old cultures of both the wild-type isolates and homokaryotic transformants using an adapted version of the Damm et al. (2008) CTAB extraction protocol. Modifications included the use of glass beads (212 – 300 µm; Sigma-Aldrich, Johannesburg, South Africa) to macerate the fungal mycelia by vortexing until homogenised in the CTAB buffer (0.2 M Tris, 1.4 M NaCl, 20 mM EDTA, 0.2 g/l CTAB). The tube was then incubated at 100°C for 3 min and immediately placed on ice for a further 10 min before adding the chloroform: isoamyl alcohol (24:1) mixture. Cold ammonium acetate (to a final concentration of 2.5 M) and 1.5 volumes of isopropanol were added to the supernatant collected after centrifugation (Damm et al., 2008), and the samples were incubated overnight at -20°C. This was followed by ethanol precipitation and resuspension of the DNA as per the Damm et al. (2008) protocol.

The hygromycin resistance (*hyg*^{R}) and green fluorescent protein (*GFP*) genes were targeted in PCR reactions as markers for T-DNA presence. Primers hph-F and hph-R (Sayari et al., 2019a) were used to amplify 428 bp of the *hyg*^{R} gene, while primers ZSG-F and ZSG-R were used to amplify 594 bp of the *GFP* open reading frame (Suppl. Table 1). PCR reactions were conducted in 25 µl volumes that consisted of 1 U KAPA *Taq* DNA Polymerase (KAPA Biosystems, Boston, MA, USA), 1 X KAPA *Taq* Buffer A, 0.2 mM dNTP mix, 0.4 µM of each primer and 10 – 50 ng of template DNA. The Ti plasmid and DNA from the wild-type isolates served as a positive and negative control, respectively. The initial denaturation step was at 96°C for 5 min followed by 25 cycles of denaturation at 96°C, annealing at 60°C and elongation at 72°C, with each step lasting 30 sec. This was followed by a final elongation step at 72°C for 7 min. PCR products and GeneRuler 100 bp DNA ladder (ThermoScientific, Waltham, USA) were mixed with GelRed (Biotium, Hayward, USA) and analysed by electrophoresis through a 1% agarose gel (Lonza, Basel, Switzerland) using a 1 X sodium boric acid (SB) buffer (Sigma-Aldrich, Johannesburg, South Africa; Brody & Kern, 2004).

The transformants were evaluated for expression of GFP using fluorescent microscopy. Slides were prepared from mycelia collected from one-week-old cultures and mounted in sterile water. Both the wild-type isolates and the transformants were evaluated using an Axioskop microscope (Zeiss, Oberkochen, Germany), with the filter set 00 (excitation 530 – 585 nm and emission 615 nm) and a fluorescent illuminator with a HBO50 mercury lamp. Pictures were taken using a magnification of 20 x with an AxioCam ICc 5 camera (Zeiss, Oberkochen, Germany) using the AxioVison program (v4.9.1) with exposure set to 4 s. For comparisons, brightfield images were also taken.

Stability of T-DNA integration

To determine the stability of the T-DNA integrations into the genomes of the transformed isolates, the retention of hygromycin resistance over multiple sub-culturing rounds were evaluated. A block of mycelia from each homokaryotic transformant was placed onto MEA plates without the hygromycin antibiotic. These plates were incubated at room temperature for a week, before being used for a new round of inoculations. This assay was repeated three more times, so that each isolate was sub-cultured on antibiotic free media for a total of five consecutive sub-plating rounds. A block of mycelia was transferred from the last non-antibiotic media culture onto MEA containing 50 µg/ml hygromycin, before being visually inspected for mycelial growth.

Results

Hygromycin sensitivity

The hygromycin assay confirmed that the wild-type isolates of all species used in this study were sensitive to this antibiotic. When grown on 2% MEA media lacking hygromycin, the culture grew to a size of between 41 mm and 65 mm (covering the entire plate) during 14 days of incubation (Suppl. Table 2). Only *D. virescens* and *T. cerberus* showed growth on media supplemented with 25 µg/ml hygromycin, although the growth rate of both species was noticeably reduced (65 mm to 14.8 mm and 65 mm to 43 mm respectively). At a 50 µg/ml hygromycin concentration, none of the species showed any growth, and this concentration was considered sufficient for the selection of transformants.

Transformant yield

Small modifications that were made to the existing *Agrobacterium*-mediated transformation protocol improved the transformant yield in *C. albifundus*. The modified protocol yielded an average of 237 colonies on each of four replicate plates for an efficiency of 2 370 transformants per 10^6 conidia. The fifth plate lacked any fungal growth even after one month of incubation.

The modified protocol was used to successfully transform six additional Ceratocystidaceae species. Colonies became visible on the selective media after 5 to 20 days for *C. manginecans*, *Ch. ovoidea*, *D. virescens*, *H. moniliformis* and *T. cerberus*. *D. virescens* and *H. moniliformis* producing at least five colonies each, while *Ch.* ovoidea and *T. cerberus* showed only two and three colonies, respectively. Two colonies were obtained from *B. basicola* after 30 days of incubation that had grown underneath the cellophane sheet and were only visible after the sheet was removed from the plate, making the exact time of emergence unknown. All hyphal tip isolations of the colonies from the different species onto fresh hygromycin-MEA produced full-sized cultures. While transformation was successful in these six species, the transformant yield was less than in *C. albifundus*. In many cases, this could be attributed to noticeable variation at different stages of the protocol. After the overnight incubation of the conidia, some isolates had germinated to the point of having visible mycelia in the spore suspension. Many of these also had colonies emerging on the induction agar plates before the cellophane sheets were transferred to selective media. In some cases, after transfer to the selective media more than five transformed colonies were present, but these emerged at the edges of the plates and many overlapped, making it difficult to determine the exact number of colonies present. For *A. cleistominuta* and *E. polonica*, no growth was observed on the selective media after the 30-day incubation period, despite isolates on hygromycin-free MEA growing normally during this time.

Analysis of transformants

PCR amplification of the *hyg*^{R} and *GFP* gene served as moderately good indicators of successful transformation. As expected, no amplification of either the *hyg*^{R} gene or *GFP* gene was possible from the wild-type isolates. Both genes were successfully amplified from most of the transformants, producing amplicons of the expected size (Suppl. Table 1). However, in one of the *C. manginecans*

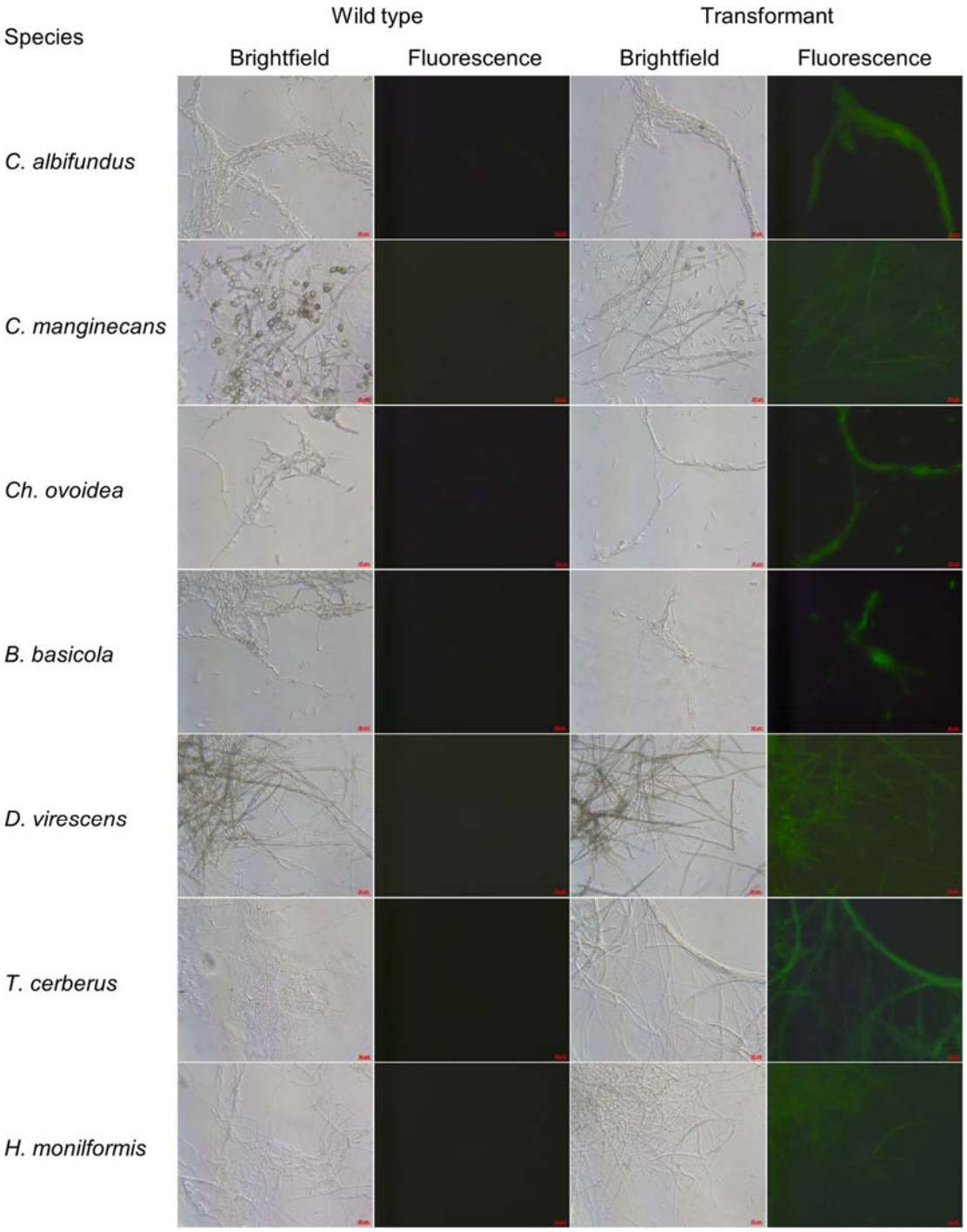

Figure 1. Microscopy images of fungal isolates indicating fluorescence in mutant individuals. Brightfield and fluorescence micrographs are presented for comparisons and to visualise the GFP emission in transformants. For each species, the wild type and a single, representative transformant is shown. Scale bars 50 µm.

transformant no amplicon corresponding to the *GFP* gene was produced, although the *hyg*^{R} gene fragment was successfully amplified.

GFP expression was observed in at least one transformed homokaryon from each of the different Ceratocystidaceae species tested (Figure 1). Auto-fluorescence was detected in the wild-type *C. manginecans* and *D. virescens* isolates, although neither exhibited the bright green emissions seen in the transformed cultures. GFP fluorescence was detected in both mycelia and conidia of most isolates tested. For *H. moniliformis*, fluorescence could only be detected in the mycelia from four homokaryons. Additionally, one of the transformants from *B. basicola*, *C. manginecans*, *D. virescens* and *H. moniliformis* did not show detectable GFP. The *C. manginecans* transformant that lacked detectable GFP was the same one from which the *GFP* gene could not be amplified.

Stability of T-DNA integration

Most transformants retained the T-DNA inserts through multiple rounds of sub-culturing. These were able to grow on hygromycin-supplemented media following five rounds of growth of MEA lacking the antibiotic. The ability to maintain resistance in the absence of antibiotic selection indicates that the T-DNA was stably integrated into the host genome. For all three *T. cerberus* transformants, no growth was observed on the hygromycin-supplemented media, even after one month of incubation, showing that the T-DNA was likely lost due to unstable T-DNA integration.

Discussion

Establishing an *Agrobacterium*-mediated transformation system can be extremely arduous largely due to the numerous parameters of the protocol that need optimization. A comparison between *Agrobacterium*-mediated protocols for six *Aspergillus* species show that variations in the number of conidia and *A. tumefaciens* cells used, co-cultivation temperatures and incubation periods as well as the types of membranes used for co-cultivation all influence transformation success and efficiency (Kunitake et al., 2011; Meyer et al., 2003; Michielse et al., 2004; Nguyen et al., 2016; Sugui et al., 2005; Wang et al., 2014). This indicates that, even among closely related species, optimisations at different stages of the transformation process are needed. Studies such as these creates the valid expectation that *Agrobacterium*-mediated transformation protocols are not easily

transferred between even closely related species. The findings presented here challenge this preconception, as a single protocol was successfully applied to seven Ceratocystidaceae species from six different genera, without the need for any species-specific optimisation. This indicates that *Agrobacterium*-mediated transformation might be more transferable than generally expected and provides a basis for further optimization of this protocol in these Ceratocystidaceae species.

Six of the eight Ceratocystidaceae species targeted in this study were successfully transformed, producing multiple transgenic cultures that are resistant to hygromycin and express detectable levels of GFP. Although the protocol used was specifically developed for *C. albifundus* (Sayari et al., 2019a), minor adjustments were made that likely contributed to the transferability reported here. For example, the introduction of a one-hour liquid co-incubation step likely enhanced interactions between *A. tumefaciens* and the fungal cells, resulting in a higher transformation rate. The inclusion of such a co-incubation step in *Kabatiella zeae* increased the number of transformants obtained (Sun et al., 2018). Changes (such as conidia isolation from liquid cultures as opposed to isolating them from cultures grown on agar plates, and the use of a single selective hygromycin concentration) were made to streamline the protocol and optimize it for multiple transformations in a single run. This would be useful when transforming multiple isolates or species, or when performing various knock-ins/knockouts in tandem.

The lack of species-specific optimization did result in some shortcomings during the transformation attempts. The number of transformants produced from the six Ceratocystidaceae species targeted was noticeably less than that obtained for *C. albifundus* in either this study, or that of Sayari et al. (2019a). An exact transformation efficiency for these Ceratocystidaceae species could not be determined due to the position of the emerging colonies, making it impossible to discern single colonies. The lower colony number and unexpected location of emerging colonies may be linked to the use of cellophane membranes, as Tzima et al. (2014) reported that transformation was only successful in *Berkeleyomyces basicola* when a Hybond N+ membrane was used. These two membranes influence different various aspects of transformation, including the movement of the acetosyringone as well as interactions between the bacteria and fungus (Tzima et al., 2014). The presence of visible mycelia in the conidial suspensions after overnight incubation was likely a result

of variability in the growth rates of the different species. Membrane choice and incubation time are both variables that can be optimised in future to increase transformation efficiency, as the current study clearly shows the importance of these factors.

A comparison of the transformation protocol used here with those previously established in *B. basicola* and *E. resinifera* allows for easy identification of additional areas for optimization (Table 2; Loppnau et al., 2004; Tzima et al., 2014). Increasing the concentration of conidia and/or bacteria could raise transformation efficiency as this could improve the interactions between the two (Michielse et al., 2005). However, care should be taken as increasing the bacteria too much could lead to less transformations as the fungus struggles to compete for nutrients and the bacteria become difficult to kill during subsequent selection, while too many fungal cells can result in false positive colonies emerging. The co-incubation period on induction minimal media (IMM) agar could also be adjusted to the growth rates of each species, allowing the conidia to be at an optimum point of germination that makes the fungal cells more susceptible to *Agrobacterium* infection (Michielse et al., 2005; Tzima et al., 2014), and supplementing the induction and selection media with V8 juice (a commonly used supplement that is a blend of 8 vegetable juices) can increase their germination. Although such optimizations were not attempted here, these could not only increase transformant yield, but might also prove crucial to inducing transformation in *A. cleistominuta* and *E. polonica*, the two species that could not be transformed using the current protocol.

The demonstrated transferability of this *Agrobacterium*-mediated transformation protocol, together with the suggested modifications to improve transformant yield, makes this study a benchmark for genetic modification in the Ceratocystidaceae. It provides a useful starting point in using this transformation system as a powerful tool for future mutagenic studies, either through random mutations as displayed here, or for targeted mutagenesis, a strategy that is invaluable when trying to discern the functions of specific genes. The transferability of this *Agrobacterium*-mediated transformation protocol amongst the family Ceratocystidaceae displayed here, as well as the ever-growing availability of genome sequences for this group, now provides the opportunity to functionally study these economically important fungi. Although it will be important to first improve

Table 2: Comparison of the parameters used in the *Agrobacterium*-mediated transformation protocol established in *Ceratocystis albifundus*, *Berkeleyomyces basicola* and *Endoconidiophora resinifera*

	***C. albifundus*[1]**	***B. basicola*[2]**	***E. resinifera*[3]**
Concentration of conidia suspension	1 x 10^6	5 x 10^6	1 x 10^8
Co-cultivation media	IMM[4]	IMM[4] + V8 juice	IMM[4]
Concentration of acetosyringone	200 µM	600 µM	200 µM
Ratio (bacteria : conidia)	1:1	1:1	10:1
Membrane	Cellophane	Hybond-N+[5]	Cellophane
Co-incubation period	3 days	2 days	5 days

[1] The protocol used in this study and adapted from Sayari *et al.* (2019a).

[2] From Tzima *et al.* (2014).

[3] From Loppnau *et al.* (2004).

[4] Induction minimal media.

[5] Hybond N+ was found to yield the most transformants, although nytran- and nitrocellulose membranes were also tested.

the transformation efficiency of this protocol within these species, especially when using targeted mutation, the application of the current protocol to various species yielded GFP-emitting transformants. The ability to detect GFP emissions in these few transformants means the infection pathway of these fungi within their hosts can be tracked (Maor et al., 1998; Moročko-Bičevska & Fatehi, 2011; Tzima et al., 2014; Vu et al., 2018), an important aspect considering that most of the Ceratocystidaceae family are well-known plant pathogens.

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