Development of a genetic transformation system in the family Ceratocystidaceae with specific reference to *Ceratocystis albifundus*

by

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Declaration

I, Frances Alice Lane, declare that the dissertation, which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own and has not previously been submitted by me for the degree at this or any other tertiary institution.

Signature: 

Date: 11 December 2019

Ethics statement

The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that he/she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

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Preface

The Ceratocystidaceae is a family of filamentous ascomycetes that includes many important tree and plant pathogens with a global distribution, as well as some cosmopolitan saprobes. Due to their importance to the agricultural and forestry industries, members of the Ceratocystidaceae have been extensively studied, mostly utilizing the large number of genome sequences that are publicly available. The vast majority of these studies were in silico, and the lack of functional work can largely be attributed to a deficiency in transformation systems within these species. An established transformation system allows one to study many aspects of the biology of a fungus including, but not limited to, pathogenicity, host specificity and sexual reproduction. Despite the existence of many transformation systems for filamentous ascomycetes, establishing them de novo within a species is time consuming and often proves challenging, with unique optimizations required for even closely relates species. As such, the focus of this dissertation is on establishing transformation systems within the family Ceratocystidaceae, as well as exploring the transferability of this technology amongst different members.

The first chapter of this dissertation is a review of the different transformation systems and genome editing tools that have been used in filamentous ascomycetes. Advantages and disadvantages associated with these technologies are also discussed. Chapter two looks at the transferability of an *Agrobacterium*-mediated transformation system that was previously developed in *Ceratocystis albifundus*. In this chapter, the transformation protocol was first modified to improve transformation efficiency in this fungus before being applied to eight other members of the Ceratocystidaceae across different genera. The transformants obtained were assessed for transformation stability and expression of the introduced green fluorescent protein. The final chapter outlines the progress made towards establishing a PEG/$CaCl_2$-protoplast transformation system in *C. albifundus*. In this chapter, a working protocol to produce *C. albifundus* protoplasts is described, and suggestions are put forward to transform these specialised cells using PEG/$CaCl_2$ transformation.

The data presented in this dissertation was produced at the Forestry and Agricultural Biotechnology Institute (FABI) and the Department of Biochemistry, Genetics and Microbiology at the University of Pretoria. This research was performed under the guidance of Prof Brenda D. Wingfield and Dr P. Markus Wilken. All fungal isolates were obtained from the CMW culture collection at FABI, while *Agrobacterium tumefaciens*, and the plasmids therein, were provided by Dr Mohammad Sayari at FABI.

This dissertation is written in the form of three standalone articles, of which the first is a review paper, and the others are independent research articles. As a result, there is some overlap of information and references that could not be avoided. All articles are written in the style required for submission to the journal Genetics.

Chapter 1

Literature Review:

The arsenal of transformation systems in filamentous ascomycetes

Introduction

In its infancy, functional studies in filamentous fungi began with random mutagenesis (Beadle and Tatum 1941). In this way, gene functions were characterised through random mutagenesis by treating the fungi with radiation or chemical mutagens and identifying a phenotypic change seen in the mutant (Casselton and Zolan 2002; Scazzocchio 2014). In later years, methods of mutation became more sophisticated, starting with the first fungal transformation of *Neurospora crassa* in 1973 (Mishra *et al.* 1973). The study took an existing mutant with random mutations and transformed it with DNA extracted from a wild-type strain. This led to the functional restoration of previously disrupted genes as the wild-type strain's DNA was incorporated into the mutant's genome. This experiment was the first crucial steppingstone that led to numerous possibilities in genetic modification of *N. crassa* as well as many other fungi.

Over the last half a century, numerous methods to induce changes in the genetic makeup of fungi have emerged. In the genetic modification battle, there are two major obstacles, namely transformation and genome editing. Transformation systems are methods that allow one to introduce DNA into the fungal cell. Transformation systems have proven difficult to establish in many fungi and form the basis for all GMO studies (Poyedinok and Blume 2018). The intricacies involved in generating a desired genomic change requires techniques collectively referred to as genomic editing tools. As a rule, transformation systems lead to random integration of transforming DNA if the DNA sequence has no regions of homology with the host's genome. The efficiency of generating random mutant libraries can be improved using genome editing tools specialised for this purpose. Furthermore, some genome editing tools allow for targeted mutation, meaning specific genes or DNA sequences can be altered. In the case of both transformation systems and genome editing tools, many different methods are currently being used in fungi. Each method has its own advantages and disadvantages which should be considered before developing a transformation and gene editing system in a fungus for which this technology has not previously been developed.

In an exciting time where countless fungal genomes are available (http://1000.fungalgenomes.org), we have access to a myriad of genes for which functions are undetermined. As a result, there has been an increasing number of transformational studies in fungi that have previously been neglected. In this way, transformation systems have been employed to either better understand the biology of these fungi, or to modify them for pharmaceutical or industrial uses. Transformation

systems are well established in model species (Roche *et al.* 2014; Cairns *et al.* 2018), but these often do not include fungi that are important as forestry and agricultural pathogens. Moreover, the findings from functional studies in one fungal species does not necessarily apply to another, even in species that are closely related (Kwon *et al.* 2011). This indicates that the extrapolation of findings from model species could be grossly inaccurate if applied to other less studied fungi. As such, there is a need to establish transformation systems tailored to individual species.

Considering the rising need to functionally study non-model fungi, this review discusses numerous transformation systems currently available for filamentous ascomycetes. The mechanisms of how these systems work is explained along with any drawbacks or limitations. Furthermore, some of the genome editing tools that allow researchers to make a desired genomic change in filamentous ascomycetes are briefly reviewed.

Transformation systems

Filamentous fungi do not readily take up exogenous DNA into their cells from the surrounding environments as is commonly seen in bacteria (Griffith 1928; Avery *et al.* 1944; Shockley and Tatum 1962). The cell walls of fungi are complex, often being composed of chitin, glucans and glycoproteins, making the cells impermeable to foreign DNA molecules (Bowman and Free 2006). There is also much physiological variation among the filamentous ascomycetes, making some species recalcitrant to transformation methods developed and successfully used in this group. As such, the cell wall is a major obstacle to fungal transformations, requiring a tailored transformation system to induce the uptake of the exogenous DNA in each species (Poyedinok and Blume 2018). Fortunately, numerous transformation systems have been developed using different methods that may be applied to fungi (Table 1), and each system comes with its own advantages and disadvantages (Table 2). These methods can be broadly categorised into three types of transformation treatments, namely chemical, physical and biological treatments.

Chemical transformation systems

Fungal cells are often treated with different chemicals to mediate the transformation process (Li *et al.* 2017; Poyedinok and Blume 2018). As such, these types of methods are referred to as chemical transformation systems in this review, namely PEG/$CaCl_2$-protoplast transformation and lithium acetate transformation. The chemicals cause disruption or digestion of the cell wall, allowing the passage of DNA into the cell. These chemically treated cells are the equivalent of competent cells used in bacterial transformation procedures. In addition to making the fungal cells more conducive to transformation, other chemicals such as polyethylene glycol (PEG) play a major role in facilitating the uptake of the DNA molecules by the cells (Timberlake and Marshall 1989).

Protoplast-mediated transformation is arguably the best-known system that relies on the chemical manipulation of the cell wall. Cell walls of young hyphae or germinating conidia are partially

digested with lysogenic enzymes to generate protoplasts. As with competent bacterial cells, protoplasts need to be induced to take up transforming DNA. Therefore, protoplasts are used as the starting material for numerous transformation systems as their permeable cell walls are more receptive to external DNA compared with intact fungal cells. One such transformation system is the PEG/$CaCl_2$-protoplast transformation system that depends on protoplasts and is often referred to as the protoplast-mediated transformation system (Case *et al.* 1979).

The PEG/$CaCl_2$-protoplast method was the first transformation system used in filamentous ascomycetes (Case *et al.* 1979). During this procedure, the protoplasts are combined with transforming DNA, polyethylene glycol and calcium chloride in an osmotically stable environment. The presence of calcium ions are important as they cause cohesion between the DNA and cell membrane and are thought to take part in the formation of pores and channels in the cell membrane which assists in the uptake of DNA (Olmedo-Monfil *et al.* 2004; Asif *et al.* 2017). In turn, PEG increases the permeability of the protoplasts by interacting with the lipids in the cell membrane. This allows for the fusion of protoplasts, where DNA molecules which are trapped between the protoplasts, are forced to enter the cells (Fincham 1989).

The PEG/$CaCl_2$-protoplast transformation system remains the most commonly used method for transforming ascomycetes and has been used in numerous species (Li *et al.* 2017). This is attributed to a relatively simple and reproducible procedure that is quick to perform once the protoplasts are made. Another advantage is that either plasmid or linear exogenous DNA can be used in the transformation procedure (Weyda *et al.* 2017). While plasmids are simpler to use in random mutagenic studies, the use of linear DNA allows the use of PCR generated DNA and does not necessitate tedious cloning steps often needed when targeting specific genes (Yu *et al.* 2004). Furthermore, the use of linear DNA allows a bipartite gene targeting substrate approach to be used to increase gene-targeting efficiency (Nielsen *et al.* 2008). This approach relies on transformation with two fragments of DNA, both with homologous sequence to the middle of the marker gene used, that will only result in expression of the selective marker when the two DNA fragments have integrated in the same locus.

Protoplast-mediated transformation systems such as the PEG/$CaCl_2$ method are dependent on the use of high-quality protoplasts, an inherent drawback of these systems. These specialised cells are difficult to produce, are extremely fragile and require care during handling. Variation in cell wall composition requires optimizing the digestion process with regards to the types and concentration of enzymes used, as well as digestion times (Li *et al.* 2017). Protoplast yield and viability is also influenced by the preparation of the enzymes, leading to quality variations among different batches (Michielse *et al.* 2005b). Once made, the protoplasts need to be maintained in an osmotically stable environment during the transformation process. After the protoplasts have been transformed, they must undergo a recovery process to allow the cells to regain normal cell wall stability. As PEG causes the fusion of protoplasts, and therefore results in multinucleated cells,

heterokaryotic transformants are often produced (Lorito *et al.* 1993), needing tedious sub-culturing steps to isolate pure transformants.

Another chemical strategy to transform filamentous fungi is the use of high concentrations of lithium acetate that make the cells more receptive to the uptake of external DNA. This protocol relies on lithium ions to increase the permeability of the intact cells, allowing PEG to facilitate the uptake of either linear or circular DNA. Compared to the PEG/$CaCl_2$-protoplast transformation system, the use of lithium acetate produces more consistent results through a simpler and faster method (Dhawale *et al.* 1984; Marek *et al.* 1989). However, despite showing success in some filamentous ascomycetes (Dhawale *et al.* 1984; Dickman 1988; Marek *et al.* 1989; Soliday *et al.* 1989), this procedure has not been widely used. This lack of popularity may be due to the method not showing success in some species, or the low yield of transformants obtained, with many unstable genomic integrations (Tsai *et al.* 1992; Malonek and Meinhardt 2001).

Physical transformation systems

Transformation of fungi has also been achieved by using physical forces to introduce the DNA molecules into the cells (Rivera *et al.* 2014). These forces may interrupt the cell wall and cell membrane, as is the case with electroporation, allowing the DNA molecules to pass through these natural barriers. Alternatively, the DNA molecules may be projected into the cell, as with hydraulic shock wave treatment, or the DNA is transmitted into the cell on microparticles that are “shot” into the cells with a gene gun.

Electroporation is a method in which an electrical charge is used to disrupt the cell membrane, inducing reversible permeability. The high voltage pulses modify the polarity of the cell membrane and components thereof, resulting in the formation of temporary pores through which DNA molecules can enter the cell (Rivera *et al.* 2014). After the electric pulse, the cell membrane recovers, trapping the exogenous DNA inside the cell. Some species have proven recalcitrant to the electroporation of intact cells (Ward *et al.* 1989; Goldman *et al.* 1990), although this has been addressed by using protoplasts, as the partially degraded cell walls are more receptive to the electrical charge. In the few cases where intact cells have been successfully transformed through electroporation, germinating conidia were used as starting material (Ozeki *et al.* 1994; Jin *et al.* 2008; Timofeev *et al.* 2019). It is not known why the intact cell successes are limited to the use of conidia, however, the overall cell wall composition of these fungi might make them more receptive to electroporation. The use of intact cells as opposed to protoplasts makes the process faster and cheaper as initial digestion of the cell wall is not needed. Electroporation is the most popular physical method of transformation because of its relative simplicity, rapid nature and efficiency, which can be further enhanced by using PEG (Goldman *et al.* 1990). However, the exposure of cells to high voltage is extremely toxic, and still results in high cell mortality despite employing laborious regeneration protocols (Jiang *et al.* 2013a).

Biolistics is a highly efficient but less popular physical transformation method. This technique uses a gene gun to bombard the cells with gold, tungsten or platinum micro particles coated with the target DNA (Rivera *et al.* 2014). Once within the cells, the DNA dissolves off the micro particles and can then integrate into the genome. This technique involves a very quick procedure that can use intact cells, such as conidia or mycelia. Particle bombardment yields similar to lower numbers of transformants when compared to the PEG/$CaCl_2$-protoplast technique and, in most cases, these bombardment integrations tend to also be more stable (Lorito *et al.* 1993; Herzog *et al.* 1996). The cost associated with the sophisticated equipment, as well as the expertise required for using the equipment, are major drawbacks of this technique. This coupled with multicopy DNA integrations has severely limited the wide-spread use of this technique.

Hydraulic shock wave-mediated transformation is a recently developed system used to introduce exogenous DNA into a cell (Jagadeesh *et al.* 2004). The shock waves cause microbubbles of air to form in the cell suspension that produce a microjet of fluid when they collapse (Ohl and Ikink 2003). These microjets produce temporary pores in the cell membrane, causing momentary permeability that allows exogenous DNA to enter the cell (Lauer *et al.* 1997). This technique is quick and uses a low complexity protocol that gives reproducible results (Rivera *et al.* 2014). The efficiency of the system is much higher than PEG/$CaCl_2$-protoplast transformation, electroporation and *Agrobacterium*-mediated transformation methods in some species of filamentous ascomycetes (Magaña-Ortíz *et al.* 2013). However, shock wave-mediated transformation has had limited use in most filamentous fungi, likely due to the need for expensive equipment. Furthermore, large amounts of transforming DNA are required as many of the DNA molecules are damaged by the shock wave (Campos-Guillén *et al.* 2012).

Biological transformation systems

The final category of transformation methods are biological transformation systems. These biological systems include *Agrobacterium*-mediated transformation and liposome-mediated transformation as they make use of organic vectors to introduce DNA into the host cells. In the case of the *Agrobacterium*-mediated transformation system, the bacteria, *Agrobacterium tumefaciens*, transmits the DNA into the host cell under conducive conditions (Gelvin 2003; Hooykaas *et al.* 2018). Although liposomes are synthesised, they are vesicles made up of lipid bilayers that bear a resemblance to the cell membrane of the host cell, and act as vectors for the transforming DNA molecules.

Agrobacterium-mediated transformation is a popular biological transformation system used in filamentous ascomycetes. It relies on *Agrobacterium tumefaciens*, a gram-negative bacterium that naturally causes gall tumours in dicotyledonous plants and some gymnosperm species (De Cleene and De Ley 1976; Stafford 2000). The bacteria carry a tumour-inducing (Ti) plasmid that contains a portion of DNA known as transferred DNA (T-DNA; Hooykaas *et al.* 2018). The T-DNA is

transmitted into the host cell where it randomly integrates into the plant genome and the oncogenes present on the T-DNA cause rapid cell growth, thus tumours are formed. The transformational ability of *A. tumefaciens* was first harnessed for controlled mutational studies in plants (Gelvin 2003), before *Agrobacterium*-mediated transformation systems were established for yeasts and filamentous fungi (Bundock *et al.* 1995; de Groot *et al.* 1998).

Agrobacterium tumefaciens is adept at transferring T-DNA into a host cell and integrating the molecule into the host genome. This process is orchestrated by virulence proteins that make up the transfer machinery (Gelvin 2003). In natural strains of *A. tumefaciens*, the genes encoding the virulence proteins (*vir* genes) are located on the Ti plasmid outside the T-DNA region and are not transmitted into the host cell (Michielse *et al.* 2005b). Strains used for experimental transformations most often use a binary vector system in which the *vir* genes and the T-DNA are located on separate plasmids (Figure 1; Hoekema *et al.* 1983). This allows for easier manipulation of the T-DNA region into which constructed cassettes can be inserted and will then be transferred into the host cell and integrated into the genomic DNA. The virulence proteins facilitate the production of T-DNA copies by producing single-stranded nicks at the T-DNA borders, inducing synthesis of a new T-DNA strand and resulting in the displacement and release of a single-stranded T-DNA copy from the Ti plasmid (Michielse *et al.* 2005b; Hooykaas *et al.* 2018). The virulence proteins also enable the transport of the T-DNA into the host cell by coating the copies with single-stranded DNA binding proteins, and by forming the pilus structure and membrane pores needed for transfer (Hooykaas *et al.* 2018). A phenolic chemical, acetosyringone, is often required to trigger the expression of the *vir* genes, and so induces the transformation process. As such, the addition of acetosyringone is often required in transformation protocols for fungi (Malonek and Meinhardt 2001; Mullins and Kang 2001).

Agrobacterium-mediated transformation has many advantages over other transformation systems (Michielse *et al.* 2005b; Hooykaas *et al.* 2018). Most significant of these is the flexibility of starting material that can be used for *Agrobacterium*-mediated transformation, including both protoplasts and intact cells such as mycelia and conidia (de Groot *et al.* 1998; Zhang *et al.* 2003). Additionally, this transformation method yields a high percentage of single-copy T-DNA integrations (Blaise *et al.* 2007), likely due to the single-stranded nature of the T-DNA and the fact that it is coated with virulence proteins (Michielse *et al.* 2005b). The layer of proteins coating the DNA not only assists in transport through the membrane pore, but also serve as protection against host nucleases. Additionally, the coating proteins play a role in delivery of the T-DNA to the host nucleus and are thought to assist with integration of the T-DNA into the host chromosomes (Citovsky *et al.* 1989; Michielse *et al.* 2004). *Agrobacterium*-mediated transformation has been an effective transformation system in some species that have been recalcitrant to alternative methods (Michielse *et al.* 2005b). However, this protocol requires numerous optimisations to work efficiently

(Michielse *et al.* 2005b), including the concentration of acetosyringone used, the ratios of host cells to bacteria, as well as the time and temperature needed for co-cultivation.

Liposome-mediated transformation makes use of synthetic lipid vesicles containing the exogenous DNA to transform fungal cells (He *et al.* 2017). Liposomes containing transforming DNA fuse with the host cell membrane and the contents of the liposome is released into the host cytoplasm. This technique is limited by the presence of cell walls, although the transformation of intact cells has been successful in a few species (Chai *et al.* 2013). The efficiency of liposome-mediated transformation has been reported as similar to that of other transformation systems (Chai *et al.* 2013), while offering advantages such as the ability of the liposomes to encapsulate large DNA molecules which can be produced in large numbers, and a low immunogenicity (Joshi and Müller 2009). Surprisingly, liposome-mediated transformation is mainly used in non-ascomycete filamentous fungi, such as basidiomycetes, with only two reports of liposome-mediated delivery in ascomycetes, one for DNA in *Neurospora crassa* and a case for protein delivery into *Tuber borchii* (Radford *et al.* 1981; Poma *et al.* 2005). The lack of further investigation into this transformation system could be due to numerous drawbacks associated with this approach. Success has mainly been seen when using protoplasts which adds laborious additional steps, comes with its own set of challenges and limits yield (Fincham 1989; Jiang *et al.* 2013a). Additionally, the liposomes must also be produced, which is complex, expensive and lacking in reproducibility (Mach 2004; Joshi and Müller 2009).

Genome editing tools

Random mutagenesis

Once an effective delivery vehicle has been established through a transformation system, the delivered nucleic material must integrate into the fungal genome. The simplest of these outcomes, random integration of transforming DNA into host chromosomes, has been invaluable to many functional studies. Forward genetics relies on mutations that are randomly induced in an organism of interest to create mutagenic libraries (Moresco *et al.* 2013). Transformants with identifiable phenotypic changes can then be further studied, through methods such as DNA sequencing, to identify and characterise the genes that were disrupted by the random integration. Numerous genome editing tools have been developed that are efficient at creating these mutagenic libraries in filamentous ascomycetes.

Some of the genome editing tools used to induce random mutations rely on the non-homologous DNA end joining machinery of the host cell. Restriction enzyme mediated integration (REMI) is usually combined with the PEG/$CaCl_2$-protoplast transformation system to introduce a restriction enzyme and transforming DNA that has been digested by the same restriction enzyme into a target cell (Figure 2; Kahmann and Basse 1999). The restriction enzyme makes double-stranded breaks in the fungal chromosomes, leaving sticky ends compatible with the transforming DNA. As a result,

the transforming DNA is randomly integrated into the host's genome at the restriction sites. REMI is useful as it yields a high number of stable transformants, while the integrated DNA and flanking regions can easily be recovered through plasmid rescue (Jin *et al.* 2005). Unfortunately, REMI is biased towards insertion at highly transcribed regions, and some insertions may lack the marker gene (Sweigard *et al.* 1998).

The *Agrobacterium*-mediated transformation system has also been extensively used to create random mutant libraries in various ascomycetes (Jiang *et al.* 2013a). T-DNA lacking homology to its host will randomly integrate into the host's chromosomes through non-homologous recombination (Hooykaas *et al.* 2018). The pattern of integration does not show the integration bias seen in other systems and retains a high transformation efficiency (Michielse *et al.* 2005b). As previously discussed, transformation with T-DNA results in single-copy integrations making it a useful tool in reverse genetics where a phenotype can easily be attributed to a single mutation.

Tools for targeted transformation

Targeted mutagenesis relies on homologous recombination through homology between the flanking regions of the transforming DNA and the targeted region in the host's genome (Mullins and Kang 2001). As non-homologous recombination occurs at a higher frequency than homologous recombination in filamentous ascomycetes, long stretches of homologous sequences are needed to ensure targeted recombination (Meyer *et al.* 2007; Meyer 2008). *Agrobacterium*-mediated transformation can be used to target specific genes through homologous recombination. Interestingly, T-DNA-based transformation appears to preferentially integrate on target through homologous recombination (Michielse *et al.* 2005a). This contrasts with other methods, such as PEG/$CaCl_2$-protoplast transformation and electroporation, in which non-homologous recombination is prevalent, despite the use of homologous sequences, and commonly results in ectopic mutations. It is thought that the single-stranded nature of the T-DNA, as well as the coating of virulence proteins, may play a role in homologous recombination by the host cell's repair machinery (Michielse *et al.* 2005b).

Transposon-arrayed gene knockouts (TAGKO) make use of the natural "jumping" ability of transposons (Hamer *et al.* 2001). This method lends itself to producing cosmid libraries with random mutations which can then be used for targeted mutations in a fungus (Casselton and Zolan 2002). To create the cosmid library with random mutations, transposons are allowed to randomly integrate into a cosmid library of the fungal genome of interest (Hamer *et al.* 2001). Specific cosmids that contain a transposon-disrupted target gene can then be isolated. The isolated cosmid is then used to transform the fungus, where homologous recombination would be preferentially employed due to the high sequence similarity between the cosmid and the chromosomal target region. TAGKO has been used in numerous filamentous ascomycetes (Jiang *et al.* 2013a), and is

a valuable targeting tool because it does not require a highly efficient transformation system (Weld *et al.* 2006).

The Clustered, Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system is an exciting new genome-editing technology that has become a popular tool for targeted gene mutagenesis in fungi (Idnurm and Meyer 2018). This system makes use of a single guide RNA (sgRNA) and Cas9 DNA endonuclease to target the region of interest (Jinek *et al.* 2012). The sgRNA pairs with the target region, guiding the Cas9 nuclease to create a double-stranded break in that region. The repair of this break results in a targeted mutation, as the homologous recombination machinery uses exogenous DNA that contains regions flanking the target gene (Nødvig *et al.* 2015). CRISPR/Cas9 technology is relatively easy to use, has a high efficiency of homologous recombination, and can be used to mutate more than one target at a time (Jiang *et al.* 2013b; Deng *et al.* 2017). However, the sgRNA and Cas9 protein must first be integrated into the host's chromosomes, and off-target mutations still provide a challenge (Wang *et al.* 2017). Transformation efficiency is also dependent on the accuracy of the host's repair system and can show a delay in the emergence of the mutant phenotype (Weyda *et al.* 2017).

Conclusions and future prospects

While there are recent and continuous advances in the realm of genome editing tools, no significant developments in transformation methods have occurred over the last twenty years (Li *et al.* 2017). At first, this appears strange as the first and most difficult hurdle to genetic manipulation in filamentous fungi, or any organism for that matter, is the introduction of the transforming DNA into the host cell. Moreover, the efficiency of the transformation system naturally influences the efficiency of the genome editing tool. It is also apparent that, while the choice in genome editing tool can mostly be based on the desired outcome, transformation systems appear to be more dependent on the physiology of the species and the availability of specialised equipment. Although there should be excitement about the prospects that new, easy and reportedly efficient genome editing technologies, such as CRISPR/Cas-9, provide (Wang *et al.* 2017; Schuster and Kahmann 2019), it is clear that attempting genomic manipulation in non-model species lacking a transformation system may not be as simple as studies on models or other fungi promise.

Despite the many difficulties, reports of transformation systems being established in non-model filamentous ascomycetes are becoming more common (Díaz-Trujillo *et al.* 2018; Long *et al.* 2018; Liu *et al.* 2019; Sayari *et al.* 2019; Timofeev *et al.* 2019). These reports further illustrate the importance of having an efficient transformation system in place before attempting targeted genomic manipulations. This also speaks to a trend where studying non-model species is becoming more important. As mentioned earlier, this can at least in part be attributed to the huge drive towards genome sequencing of numerous and diverse fungal species (Wilken *et al.* 2019). Perhaps another attributing factor lies in an emerging trend of favouring outcomes-based science.

Currently, a vast amount of research is focussed either on important pathogens in efforts to control them (Kahmann and Basse 1999; Münch *et al.* 2011; Latinović *et al.* 2019), or on investigating species with novel characteristics that can be exploited for pharmaceutical or industrial uses (Hoffmeister and Keller 2007; Meyer *et al.* 2011). This focus on outcomes-based science has broadened the scope of filamentous ascomycetes that are now intimately studied, but all of this relies on the development of appropriate genetic tools. Arguably, the most important of these is the establishment of transformation systems, as this must be in place before any kind of genome editing can be done. One can reasonably expect that there will be difficulties in doing so, but it is possible that these obstacles may lead to the development of innovative methods of transformation that have been lacking in recent years.

References

Armaleo, D., G. N. Ye, T. M. Klein, K. B. Shark, J. C. Sanford *et al.*, 1990 Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi. Curr. Genet. 17: 97–103.

Asif, A., H. Mohsin, R. Tanvir, and Y. Rehman, 2017 Revisiting the mechanisms involved in calcium chloride induced bacterial transformation. Front. Microbiol. 8: 2169.

Avery, O. T., C. M. MacLeod, and M. McCarty, 1944 Studies on the chemical nature of the substances inducing transformation of Pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III. J. Exp. Med. 79: 137–158.

Ball, A. M., M. K. Sawczyc, A. M. Ashby, D. S. Ingram, and K. Johnstone, 1991 Transformation of the fungus *Pyrenopeziza brassicae*, cause of light leaf spot of brassicas, and complementation of mutants using a genomic library. Exp. Mycol. 15: 243–254.

Beadle, G. W., and E. L. Tatum, 1941 Genetic control of biochemical reactions in *Neurospora*. Proc. Natl. Acad. Sci. U. S. A. 27: 499–506.

Blaise, F., E. Rémy, M. Meyer, L. Zhou, J. P. Narcy *et al.*, 2007 A critical assessment of *Agrobacterium tumefaciens*-mediated transformation as a tool for pathogenicity gene discovery in the phytopathogenic fungus *Leptosphaeria maculans*. Fungal Genet. Biol. 44: 123–138.

Bowman, S. M., and S. J. Free, 2006 The structure and synthesis of the fungal cell wall. BioEssays 28: 799–808.

Bundock, P., A. den Dulk-Ras, A. Beijersbergen, and P. J. Hooykaas, 1995 Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J. 14: 3206–3214.

Cairns, T. C., C. Nai, and V. Meyer, 2018 How a fungus shapes biotechnology: 100 years of *Aspergillus niger* research. Fungal Biol. Biotechnol. 5: 13.

Campos-Guillén, J., F. Fernández, X. Pastrana, and A. M. Loske, 2012 Relationship between plasmid size and shock wave-mediated bacterial transformation. Ultrasound Med. Biol. 38: 1078–1084.

Case, M. E., M. Schweizer, S. R. Kushner, and N. H. Giles, 1979 Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc. Natl. Acad. Sci. 76: 5259–5263.

Casselton, L., and M. Zolan, 2002 The art and design of genetic screens: filamentous fungi. Nat. Rev. Genet. 3: 683–697.

Chai, R., G. Zhang, Q. Sun, M. Zhang, S. Zhao *et al.*, 2013 Liposome-mediated mycelial transformation of filamentous fungi. Fungal Biol. 117: 577–583.

Chakraborty, B. N., and M. Kapoor, 1990 Transformation of filamentous fungi by electroporation. Nucleic Acids Res. 18: 6737.

Chen, L., Q. Wang, H. Chen, G. Sun, H. Liu *et al.*, 2016 *Agrobacterium tumefaciens*-mediated

transformation of *Botryosphaeria dothidea*. World J. Microbiol. Biotechnol. 32: 106.

Citovsky, V., M. L. Wong, and P. Zambryski, 1989 Cooperative interaction of *Agrobacterium* VirE2 protein with single-stranded DNA: implications for the T-DNA transfer process. Proc. Natl. Acad. Sci. U. S. A. 86: 1193–1197.

Cullen, D., V. Yang, T. Jeffries, J. Bolduc, and J. H. Andrews, 1991 Genetic transformation of *Aureobasidium pullulans*. J. Biotechnol. 21: 283–288.

De Cleene, M., and J. De Ley, 1976 The host range of crown gall. Bot. Rev. 42: 389–466.

de Groot, M. J. A., P. Bundock, P. J. J. Hooykaas, and A. G. M. Beijersbergen, 1998 *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat. Biotechnol. 16: 839–842.

Deng, H., R. Gao, X. Liao, and Y. Cai, 2017 CRISPR system in filamentous fungi: current achievements and future directions. Gene 627: 212–221.

Dhawale, S. S., J. V. Paietta, and G. A. Marzluf, 1984 A new, rapid and efficient transformation procedure for *Neurospora*. Curr. Genet. 8: 77–79.

Díaz-Trujillo, C., A. K. Kobayashi, M. Souza, P. Chong, H. J. G. Meijer *et al.*, 2018 Targeted and random genetic modification of the black Sigatoka pathogen *Pseudocercospora fijiensis* by *Agrobacterium tumefaciens*-mediated transformation. J. Microbiol. Methods 148: 127–137.

Dickman, M. B., 1988 Whole cell transformation of the alfalfa fungal pathogen *Colletotrichum trifolii*. Curr. Genet. 14: 241–246.

Fincham, J. R., 1989 Transformation in fungi. Microbiol. Rev. 53: 148–170.

Fungaro, M. H. P., E. Rech, G. S. Muhlen, M. H. Vainstein, R. C. Pascon *et al.*, 1995 Transformation of *Aspergillus nidulans* by microprojectile bombardment on intact conidia. FEMS Microbiol. Lett. 125: 293–298.

Gelvin, S. B., 2003 *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. Microbiol. Mol. Biol. Rev. 67: 16–37.

Gentile, G., 2011 Using polyethylene glycol-mediated transformation when identifying pathogenicity genes in *Thielaviopsis basicola*, University of New England, Armidale, Australia, PhD thesis.

Goldman, G. H., M. Van Montagu, and A. Herrera-Estrella, 1990 Transformation of *Trichoderma harzianum* by high-voltage electric pulse. Curr. Genet. 17: 169–174.

Griffith, F., 1928 The significance of pneumococcal types. J. Hyg. 27: 113–159.

Hamer, L., K. Adachi, M. V. Montenegro-Chamorro, M. M. Tanzer, S. K. Mahanty *et al.*, 2001 Gene discovery and gene function assignment in filamentous fungi. Proc. Natl. Acad. Sci. 98: 5110–5115.

He, L., J. Feng, S. Lu, Z. Chen, C. Chen *et al.*, 2017 Genetic transformation of fungi. Int. J. Dev. Biol. 61: 375–381.

Herzog, R. W., H. Daniell, N. K. Singh, and P. A. Lemke, 1996 A comparative study on the transformation of *Aspergillus nidulans* by microprojectile bombardment of conidia and a more

conventional procedure using protoplasts treated with polyethyleneglycol. Appl. Microbiol. Biotechnol. 45: 333–337.

Hoekema, A., P. R. Hirsch, P. J. J. Hooykaas, and R. A. Schilperoort, 1983 A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179–180.

Hoffmeister, D., and N. P. Keller, 2007 Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat. Prod. Rep. 24: 393–416.

Hooykaas, P. J. J., G. P. H. van Heusden, X. Niu, M. R. Roushan, J. Soltani *et al.*, 2018 *Agrobacterium*-mediated transformation of yeast and fungi, pp. 349–374 in Agrobacterium *Biology. Current Topics in Microbiology and Immunology, vol 418*, edited by S. B. Gelvin, Springer, Cham.

Idnurm, A., and V. Meyer, 2018 The CRISPR revolution in fungal biology and biotechnology, and beyond. Fungal Biol. Biotechnol. 5.

Jagadeesh, G., K. N. Nataraja, and M. Udayakumar, 2004 Shock waves can enhance bacterial transformation with plasmid DNA. Curr. Sci. 87: 734–735.

Jiang, D., W. Zhu, Y. Wang, C. Sun, K. Q. Zhang *et al.*, 2013a Molecular tools for functional genomics in filamentous fungi: recent advances and new strategies. Biotechnol. Adv. 31: 1562–1574.

Jiang, W., D. Bikard, D. Cox, F. Zhang, and L. A. Marraffini, 2013b RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31: 233–239.

Jin, X., M. Ming-He, H. Xiao-Wei, and Z. Ke-Qin, 2005 Improvement on genetic transformation in the nematode-trapping fungus *Arthrobotrys oligospora* and its quantification on dung samples. Mycopathologia 159: 533–538.

Jin, K., Y. Zhang, Z. Luo, Y. Xiao, Y. Fan *et al.*, 2008 An improved method for *Beauveria bassiana* transformation using phosphinothricin acetlytransferase and green fluorescent protein fusion gene as a selectable and visible marker. Biotechnol. Lett. 30: 1379–1383.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna *et al.*, 2012 A programmable dual-RNA – guided endonuclease in adaptive bacterial immunity. Science 337: 816–821.

Joshi, M. D., and R. H. Müller, 2009 Lipid nanoparticles for parenteral delivery of actives. Eur. J. Pharm. Biopharm. 71: 161–172.

Kahmann, R., and C. Basse, 1999 REMI (Restriction Enzyme Mediated Integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants. Eur. J. Plant Pathol. 105: 221–229.

Kars, I., M. McCalman, L. Wagemakers, and J. A. L. van Kan, 2005 Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of strain B05.10. Mol. Plant Pathol. 6: 641–652.

Kwon, M. J., M. Arentshorst, E. D. Roos, C. A. M. J. J. van den Hondel, V. Meyer *et al.*, 2011 Functional characterization of Rho GTPases in *Aspergillus niger* uncovers conserved and

diverged roles of Rho proteins within filamentous fungi. Mol. Microbiol. 79: 1151–1167.

Latinović, N., M. S. Sabovljević, M. Vujičić, J. Latinović, and A. Sabovljević, 2019 Bryophyte extracts suppress growth of the plant pathogenic fungus *Botrytis cinerea*. Bot. Serbica 43: 9–12.

Lauer, U., E. Bürgelt, Z. Squire, K. Messmer, P. H. Hofschneider *et al.*, 1997 Shock wave permeabilization as a new gene transfer method. Gene Ther. 4: 710–715.

Lee, T., Y. K. Han, K. H. Kim, S. H. Yun, and Y. W. Lee, 2002 *Tri13* and *Tri7* determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. Appl. Environ. Microbiol. 68: 2148–2154.

Li, D., Y. Tang, J. Lin, and W. Cai, 2017 Methods for genetic transformation of filamentous fungi. Microb. Cell Fact. 16: 1–13.

Liu, L., Y. R. Cao, C. C. Zhang, H. F. Fan, Z. Y. Guo *et al.*, 2019 An efficient gene disruption system for the nematophagous fungus *Purpureocillium lavendulum*. Fungal Biol. 123: 274–282.

Long, L., Q. Lin, Y. Shi, J. Wang, and S. Ding, 2018 Highly efficient transformation of a (hemi-) cellulases-producing fungus *Eupenicillium parvum* 4–14 by *Agrobacterium tumefaciens*. J. Microbiol. Methods 146: 40–45.

Loppnau, P., P. Tanguay, and C. Breuil, 2004 Isolation and disruption of the melanin pathway polyketide synthase gene of the softwood deep stain fungus *Ceratocystis resinifera*. Fungal Genet. Biol. 41: 33–41.

Lorito, M., C. K. Hayes, A. Di Pietro, and G. E. Harman, 1993 Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. Curr. Genet. 24: 349–356.

Mach, R. L., 2004 Transformation and gene manipulation in filamentous fungi: an overview., pp. 109–120 in *Handbook of Fungal Biotechnology*, edited by D. K. Arora, Marcel Dekker, New York.

Magaña-Ortíz, D., N. Coconi-Linares, E. Ortiz-Vazquez, F. Fernández, A. M. Loske *et al.*, 2013 A novel and highly efficient method for genetic transformation of fungi employing shock waves. Fungal Genet. Biol. 56: 9–16.

Malonek, S., and F. Meinhardt, 2001 *Agrobacterium tumefaciens*-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii*. Curr. Genet. 40: 152–155.

Marek, E. T., C. L. Schardl, and D. A. Smith, 1989 Molecular transformation of *Fusarium solani* with an antibiotic resistance marker having no fungal DNA homology. Curr. Genet. 15: 421–428.

Meyer, V., M. Arentshorst, A. El-Ghezal, A. C. Drews, R. Kooistra *et al.*, 2007 Highly efficient gene targeting in the *Aspergillus niger kusA* mutant. J. Biotechnol. 128: 770–775.

Meyer, V., 2008 Genetic engineering of filamentous fungi - progress, obstacles and future trends. Biotechnol. Adv. 26: 177–185.

Meyer, V., B. Wu, and A. F. J. Ram, 2011 *Aspergillus* as a multi-purpose cell factory: current status and perspectives. Biotechnol. Lett. 33: 469–476.

Michielse, C. B., A. F. J. Ram, P. J. J. Hooykaas, and C. A. M. J. . van den Hondel, 2004 Role of bacterial virulence proteins in *Agrobacterium*-mediated transformation of *Aspergillus awamori*. Fungal Genet. Biol. 41: 571–578.

Michielse, C. B., M. Arentshorst, A. F. J. Ram, and C. A. M. J. J. van den Hondel, 2005a *Agrobacterium*-mediated transformation leads to improved gene replacement efficiency in *Aspergillus awamori*. Fungal Genet. Biol. 42: 9–19.

Michielse, C. B., P. J. J. Hooykaas, C. A. M. J. J. van den Hondel, and A. F. J. Ram, 2005b *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr. Genet. 48: 1–17.

Mishra, N. C., G. Szabo, and E. L. Tatum, 1973 Nucleic acid-induced genetic changes in *Neurospora*, pp. 259–268 in *The Role of RNA in Reproduction and Development*, edited by M. C. Niu, and S. J. Segal, North-Holland Publ. Co., Amsterdam.

Moresco, E. M. Y., X. Li, and B. Beutler, 2013 Going forward with genetics: recent technological advances and forward genetics in mice. Am. J. Pathol. 182: 1462–1473.

Mullins, E. D., and S. Kang, 2001 Transformation: a tool for studying fungal pathogens of plants. Cell. Mol. Life Sci. 58: 2043–2052.

Münch, S., N. Ludwig, D. S. Floss, J. A. Sugui, A. M. Koszucka *et al.*, 2011 Identification of virulence genes in the corn pathogen *Colletotrichum graminicola* by *Agrobacterium tumefaciens*-mediated transformation. Mol. Plant Pathol. 12: 43–55.

Nielsen, J. B., M. L. Nielsen, and U. H. Mortensen, 2008 Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*. Fungal Genet. Biol. 45: 165–170.

Nødvig, C. S., J. B. Nielsen, M. E. Kogle, and U. H. Mortensen, 2015 A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLoS One 10: e0133085.

Ohl, C. D., and R. Ikink, 2003 Shock-wave-induced jetting of micron-size bubbles. Phys. Rev. Lett. 90: 214502.

Olmedo-Monfil, V., C. Cortés-Penagos, and A. Herrera-Estrella, 2004 Three decades of fungal transformation., pp. 297–313 in *Recombinant Gene Expression. Methods in Molecular Biology*, edited by P. Balbás, and A. Lorence, Humana Press,Totowa.

Ozeki, K., F. Kyoya, K. Hizume, A. Kanda, M. Hamachi *et al.*, 1994 Transformation of intact *Aspergillus niger* by electroporation. Biosci. Biotechnol. Biochem. 58: 2224–2227.

Poma, A., S. Colafarina, T. Limongi, and G. Pacioni, 2005 *Tuber borchii* mycelial protoplasts isolation, characterization and functional delivery of liposome content, a new step towards truffles biotechnology. FEMS Microbiol. Lett. 253: 331–337.

Poyedinok, N. L., and Y. B. Blume, 2018 Advances, problems, and prospects of genetic transformation of fungi. Cytol. Genet. 52: 139–154.

Radford, A., S. Pope, A. Sazci, M. J. Fraser, and J. H. Parish, 1981 Liposome-mediated genetic transformation of *Neurospora crassa*. Mol. Gen. Genet. 184: 567–569.

Richey, M. G., E. T. Marek, C. L. Schardl, and D. A. Smith, 1989 Transformation of filamentous fungi with plasmid DNA by electroporation. Phytopathology 79: 844–847.

Rivera, A. L., D. Magaña-Ortíz, M. Gómez-Lim, F. Fernández, and A. M. Loske, 2014 Physical methods for genetic transformation of fungi and yeast. Phys. Life Rev. 11: 184–203.

Roche, C. M., J. J. Loros, K. McCluskey, and N. L. Glass, 2014 *Neurospora crassa*: looking back and looking forward at a model microbe. Am. J. Bot. 101: 2022–2035.

Rollins, J. A., 2003 The *Sclerotinia sclerotiorum pac1* gene is required for sclerotial development and virulence. Mol. Plant-Microbe Interact. 16: 785–795.

Sayari, M., M. A. van der Nest, E. T. Steenkamp, O. O. Adegeye, S. Marincowitz *et al.*, 2019 *Agrobacterium*-mediated transformation of *Ceratocystis albifundus*. Microbiol. Res. 226: 55–64.

Scazzocchio, C., 2014 Fungal biology in the post-genomic era. Fungal Biol. Biotechnol. 1: 7.

Schuster, M., and R. Kahmann, 2019 CRISPR-Cas9 genome editing approaches in filamentous fungi and oomycetes. Fungal Genet. Biol. 130: 43–53.

Shockley, T. E., and E. L. Tatum, 1962 A search for genetic transformation in *Neurospora crassa*. Biochim. Biophys. Acta 61: 567–572.

Sivan, A., T. E. Stasz, M. Hemmat, C. K. Hayes, and G. E. Harman, 1992 Transformation of *Trichoderma* spp. with plasmids conferring hygromycin B resistance. Mycologia 84: 687–694.

Soliday, C. L., M. B. Dickman, and P. E. Kolattukudy, 1989 Structure of the cutinase gene and detection of promoter activity in the 5'-flanking region by fungal transformation. J. Bacteriol. 171: 1942–1951.

Stafford, H. A., 2000 Crown gall disease and *Agrobacterium tumefaciens*: a study of the history, present knowledge, missing information, and impact on molecular genetics. Bot. Rev. 66: 99–118.

Sun, J., R. Xu, S. Xiao, Y. Lu, Q. Zhang *et al.*, 2018 *Agrobacterium tumefaciens*-mediated transformation as an efficient tool for insertional mutagenesis of *Kabatiella zeae*. J. Microbiol. Methods 149: 96–100.

Sweigard, J. A., A. M. Carroll, L. Farrall, F. G. Chumley, and B. Valent, 1998 *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. Mol. Plant-Microbe Interact. 11: 404–412.

Tilburn, J., C. Scazzocchio, G. G. Taylor, J. H. Zabicky-Zissman, R. A. Lockington *et al.*, 1983 Transformation by integration in *Aspergillus nidulans*. Gene 26: 205–221.

Timberlake, W. E., and M. A. Marshall, 1989 Genetic engineering of filamentous fungi. Science 244: 1313–1317.

Timofeev, S., A. Tsarev, I. Senderskiy, E. Rogozhin, G. Mitina *et al.*, 2019 Efficient transformation of the entomopathogenic fungus *Lecanicillium muscarium* by electroporation of germinated

conidia. Mycoscience 60: 197–200.

Tsai, H. F., M. R. Siegel, and C. L. Schardl, 1992 Transformation of *Acremonium coenophialum*, a protective fungal symbiont of the grass *Festuca arundinacea*. Curr. Genet. 22: 399–406.

Tzima, A. K., E. J. Paplomatas, C. Schoina, E. Domazakis, S. Kang *et al.*, 2014 Successful *Agrobacterium* mediated transformation of *Thielaviopsis basicola* by optimizing multiple conditions. Fungal Biol. 118: 675–682.

Wang, S., H. Chen, X. Tang, H. Zhang, W. Chen *et al.*, 2017 Molecular tools for gene manipulation in filamentous fungi. Appl. Microbiol. Biotechnol. 101: 8063–8075.

Ward, M., K. H. Kodama, and L. J. Wilson, 1989 Transformation of *Aspergillus awamori* and *A. niger* by electroporation. Exp. Mycol. 13: 289–293.

Weld, R. J., K. M. Plummer, M. A. Carpenter, and H. J. Ridgway, 2006 Approaches to functional genomics in filamentous fungi. Cell Res. 16: 31–44.

Weyda, I., L. Yang, J. Vang, B. K. Ahring, M. Lübeck *et al.*, 2017 A comparison of *Agrobacterium*-mediated transformation and protoplast-mediated transformation with CRISPR-Cas9 and bipartite gene targeting substrates, as effective gene targeting tools for *Aspergillus carbonarius*. J. Microbiol. Methods 135: 26–34.

Wilken, S. E., C. L. Swift, I. A. Podolsky, T. S. Lankiewicz, S. Seppälä *et al.*, 2019 Linking 'omics' to function unlocks the biotech potential of non-model fungi. Curr. Opin. Syst. Biol. 14: 9–17.

Yu, J. H., Z. Hamari, K. H. Han, J. A. Seo, Y. Reyes-Domínguez *et al.*, 2004 Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. 41: 973–981.

Zhang, A., P. Lu, A. M. Dahl-Roshak, P. S. Paress, S. Kennedy *et al.*, 2003 Efficient disruption of a polyketide synthase gene (*pks1*) required for melanin synthesis through *Agrobacterium*-mediated transformation of *Glarea lozoyensis*. Mol. Genet. Genomics 268: 645–655.

Zhang, T., P. Ren, V. Chaturvedi, and S. Chaturvedi, 2015 Development of an *Agrobacterium*-mediated transformation system for the cold-adapted fungi *Pseudogymnoascus destructans* and *P. pannorum*. Fungal Genet. Biol. 81: 73–81.

Tables and figures

Table 1: Examples of transformation systems developed for a diverse range of species from four classes of the Pezizomycotina

Species	Transformation system	Cell type used	Reference
	Eurotiomycetes		
Aspergillaceae			
Aspergillus awamori	*Agrobacterium*-mediated transformation	Protoplasts and conidia	de Groot *et al.* (1998)
	Electroporation	Protoplasts	Ward *et al.* (1989)
A. carbonarius	*Agrobacterium*-mediated transformation	Conidia	Weyda *et al.* (2017)
	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Weyda *et al.* (2017)
A. nidulans	Biolistic transformation	Conidia	Fungaro *et al.* (1995)
	Electroporation	Protoplasts	Richey *et al.* (1989)
	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Tilburn *et al.* (1983)
A. niger	*Agrobacterium*-mediated transformation	Conidia	de Groot *et al.* (1998)
	Electroporation	Germinated conidia	Ozeki *et al.* (1994)
	Shock wave-mediated transformation	Conidia	Magaña-Ortíz *et al.* (2013)
	Dothideomycetes		
Botryosphaeriaceae			
Botryosphaeria dothidea	*Agrobacterium*-mediated transformation	Protoplasts	Chen *et al.* (2016)
Saccotheciaceae			
Aureobasidium pullulans	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Cullen *et al.* (1991)
Kabatiella zeae	*Agrobacterium*-mediated transformation	Conidia	Sun *et al.* (2018)
	Leotiomycetes		
Ploettnerulaceae			
Pyrenopeziza brassicae	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Ball *et al.* (1991)
Pseudeurotiaceae			
Pseudogymnoascus destructans	*Agrobacterium*-mediated transformation	Conidia	Zhang *et al.* (2015)

Sclerotiniaceae			
Botrytis cinerea	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Kars *et al.* (2005)
Sclerotinia sclerotum	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Rollins (2003)
Sordariomycetes			
Ceratocystidaceae			
Berkeleyomyces basicola	*Agrobacterium*-mediated transformation	Germinated conidia	Tzima *et al.* (2014)
	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Gentile (2011)
Ceratocystis albifundus	*Agrobacterium*-mediated transformation	Germinated conidia	Sayari *et al.* (2019)
Endoconidiophora resinifera	*Agrobacterium*-mediated transformation	Germinated conidia	Loppnau *et al.* (2004)
Clavicipitaceae			
Epichloe coenophiala	Electroporation	Protoplasts	Tsai *et al.* (1992)
Cordycipitaceae			
Akanthomyces muscarius	Electroporation	Germinated conidia	Timofeev *et al.* (2019)
Beauveria bassiana	Electroporation	Germinated conidia	Jin *et al.* (2008)
Glomerellaceae			
Colletotrichum gloeosporoides	*Agrobacterium*-mediated transformation	Conidia	de Groot *et al.* (1998)
C. trifolii	Lithium acetate transformation	Mycelium	Dickman (1988)
C. capsici	Lithium acetate transformation	Mycelium	Soliday *et al.* (1989)
	PEG/$CaCl_2$-protoplast transformation	Mycelium	Soliday *et al.* (1989)
Hypocreaceae			
Trichoderma hazianum	Biolistic transformation	Germinated conidia	Lorito *et al.* (1993)
	Electroporation	Protoplasts	Goldman *et al.* (1990)
	PEG/$CaCl_2$-protoplast transformation	Mycelium	Sivan *et al.* (1992)
T. reesei	*Agrobacterium*-mediated transformation	Conidia	de Groot *et al.* (1998)
	Shock wave-mediated transformation	Conidia	Magaña-Ortíz *et al.* (2013)

Nectriaceae			
Fusarium graminearum	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Lee *et al.* (2002)
F. oxysporum	Shock wave-mediated transformation	Conidia	Magaña-Ortíz *et al.* (2013)
F. solani	Electroporation	Protoplasts	Richey *et al.* (1989)
	Lithium acetate transformation	Germinated conidia	Marek *et al.* (1989)
	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Marek *et al.* (1989)
F. venenatum	*Agrobacterium*-mediated transformation	Conidia	de Groot *et al.* (1998)
Gibberella zeae	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Lee *et al.* (2002)
Sordariaceae			
Neurospora crassa	*Agrobacterium*-mediated transformation	Conidia	de Groot *et al.* (1998)
	Biolistic transformation	Conidia	Armaleo *et al.* (1990)
	Electroporation	Germinated conidia	Chakraborty and Kapoor (1990)
	Liposome-mediated transformation	Protoplasts	Radford *et al.* (1981)
	Lithium acetate transformation	Germinated conidia	Dhawale *et al.* (1984)
	PEG/$CaCl_2$-protoplast transformation	Protoplasts	Case *et al.* (1979)

Table 2: The advantages and disadvantages of transformation systems in filamentous ascomycetes

Transformation system	Advantages	Disadvantages
PEG/$CaCl_2$-protoplast transformation	Simple Reproducible Uses linear or plasmid DNA	Requires protoplasts Heterokaryotic transformants
Lithium acetate transformation	Simple Reproducible Uses linear or plasmid DNA Uses intact cells	Low yield Unstable DNA integration Recalcitrant species
Electroporation	Simple Efficient	Protoplasts required in most species High cell mortality Specialised equipment
Biolistic transformation	Uses intact cells Stable DNA integrations Fast	Expensive specialised equipment Multicopy DNA integrations
Shock wave-mediated transformation	Simple Reproducible High efficiency	Expensive specialised equipment Requires high quantities of DNA DNA damage common
Agrobacterium-mediated transformation	Uses intact cells or protoplasts High efficiency Single-copy integrations	Many conditions to be optimised
Liposome-mediated transformation	Large DNA molecules can be used Efficient Low immunogenicity	Protoplasts required in most species High toxicity Liposomes are difficult to produce Unstable DNA integrations

Figure 1: The process by which *Agrobacterium tumefaciens*, with a binary vector system, transmits the T-DNA into a host cell. The helper plasmid contains the *vir* genes while the small Ti plasmid contains the T-DNA region. Environmental acetosyringone acts as an inducer of the *vir* genes, which mediate the transformation process of *A. tumefaciens*. Copies of the T-DNA are produced and transmitted into the host cell through pores and a pilus connecting the bacteria to the host cell. The T-DNA copy is transported across the membranes, into the host cell and then further into the host nucleus where it integrates into the chromosomal DNA.

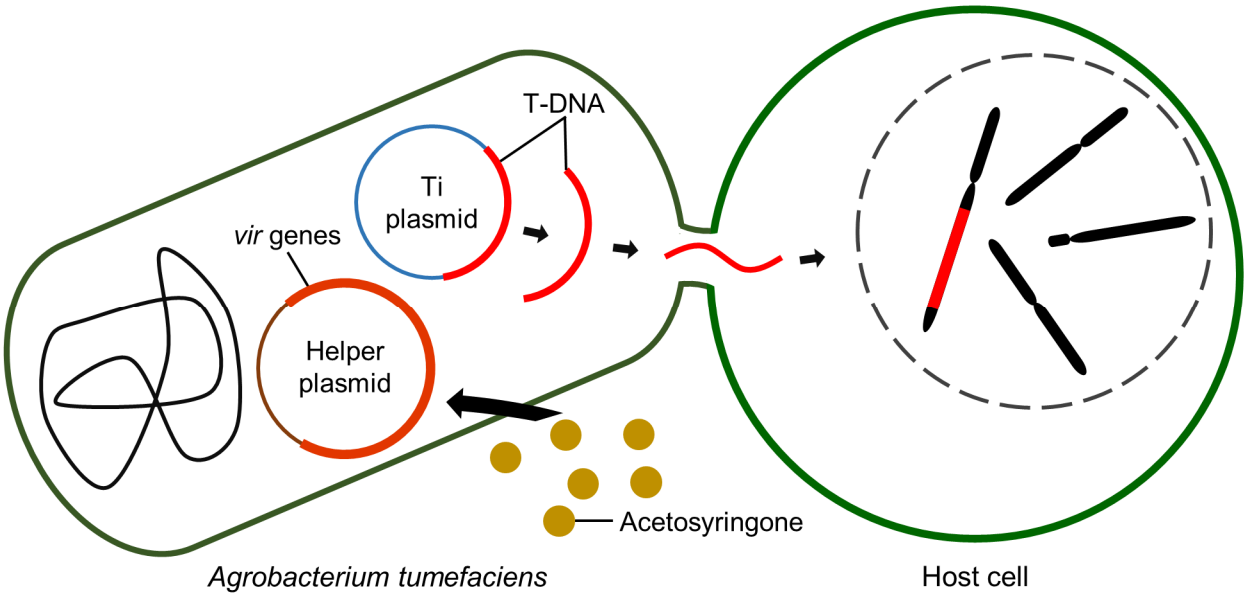
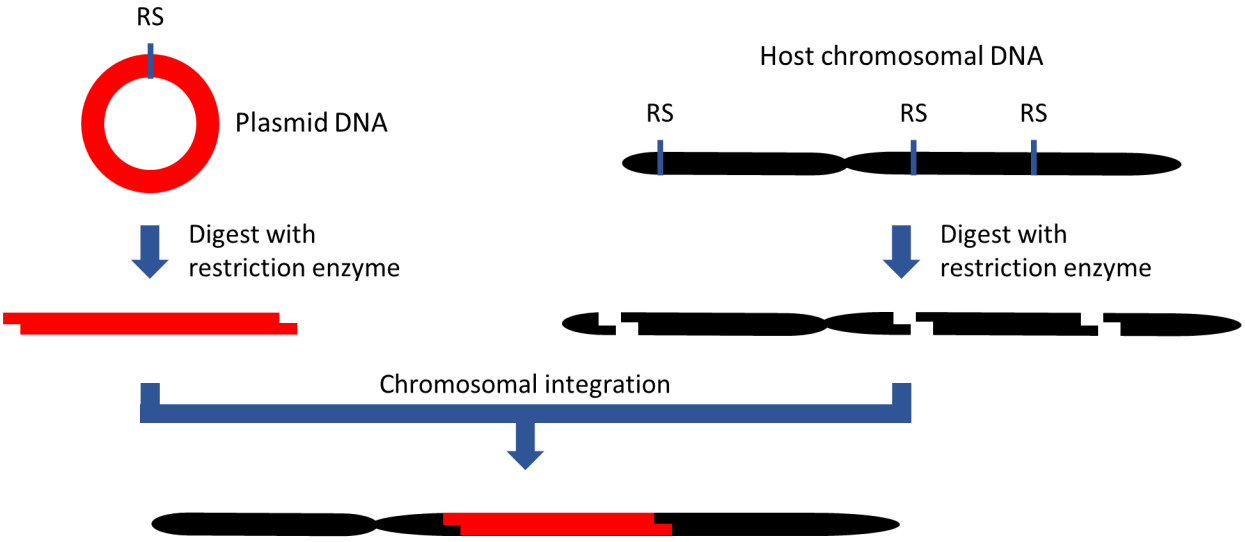


Figure 2: The process of REMI transformation. Plasmid DNA is digested with a restriction enzyme to create a linear molecule with sticky ends. The genomic DNA of the host is also digested with the same restriction enzymes, resulting in numerous double-stranded nicks across the genome with sticky ends complementary to the linearized plasmid molecule, mediating integration of the plasmid DNA into the chromosome. Adapted from Kahmann and Basse 1999.



Chapter 2

Agrobacterium-mediated transformation across the Ceratocystidaceae

Abstract

Establishing a transformation system is essential for the functional study of genes for any fungus. As such, numerous transformation systems have been developed and optimised for filamentous ascomycetes. One such transformation system is *Agrobacterium*-mediated transformation which has recently been employed in the African tree pathogen *Ceratocystis albifundus*. This fungus is a part of the family Ceratocystidaceae, which encompasses a wide range of important tree and plant pathogens, that have yet to be functionally studied. Establishing and optimising an *Agrobacterium*-mediated transformation system for many of these species would be extremely arduous as the protocol has many species-specific parameters that must be optimised. For this reason, this study aimed to determine how easily and successfully the *Agrobacterium*-mediated transformation protocol for *C. albifundus* could be applied to eight species, each representing different genera within the family. Apart from improving the transformation efficiency for *C. albifundus*, the protocol was successful in producing transformants for six of the eight different species targeted. These six species were *C. manginecans, Berkeleyomyces basicola, Chalaropsis ovoidea, Davidsoniella virescens, Huntiella moniliformis* and *Thielaviopsis cerberus* while transformation was unsuccessful in *Ambrosiella cleistominuta* and *Endoconidiophora polonica*. This study thus produced seven species with detectable green fluorescent protein expression that could in future be used for functional studies, such as tracking the infection process. Although the transformant yield for the six species was less than for *C. albifundus*, this study still provides a useful benchmark for transformation studies in the Ceratocystidaceae, and would make species-specific optimisation simpler.

Introduction

Functional studies are crucial to our understanding of fungal biology, and the ever-growing number of available genome sequences has fuelled this further. There is a rich diversity of fungal species for which genome sequences are available (http://1000.fungalgenomes.org), providing a rich source of candidate genes needing functional characterisation. These genes in turn are used for taxonomy, understanding pathogenicity and identifying genomic clusters involved in the production of secondary metabolites, amongst others (Butler *et al.* 2009; Choi and Kim 2017; Gilchrist *et al.* 2018; van der Nest *et al.* 2019). Understanding gene function, however, is often based on the extrapolation of knowledge from homologues identified and functionally studied in other 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 mating-type genes (Turgeon and Yoder 2000; Wilken *et al.* 2017). In these cases, functional studies in non-model systems can prove invaluable, 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.

Transformation systems provide a means to introduce exogenous DNA into the host cell. The greatest barrier to transformation in fungi is their thick and complex cell walls, as they make the cells impermeable to transforming DNA (Poyedinok and Blume 2018). Currently, there are numerous transformation systems that have shown success to varying degrees in different filamentous ascomycetes. The first such method, which also remains one of the most commonly used, is the PEG/$CaCl_2$-protoplast transformation system (Case *et al.* 1979; Li *et al.* 2017). This method requires the use of protoplasts, which are young hyphae or germinating conidia that are partially digested with lysis enzymes to the point where the cell wall is degraded. Thereafter, the protoplasts are treated with polyethylene glycol (PEG) and calcium chloride which further increase the permeability of the cells and mediate the uptake of transforming DNA. Although relatively efficient, PEG/$CaCl_2$-protoplast transformation has proven unsuccessful in some species as they are recalcitrant to the formation of protoplasts (Michielse *et al.* 2005b). A common alternative to overcome this is the use of the *Agrobacterium*-mediated transformation system.

Agrobacterium tumefaciens is a gram-negative bacterium that naturally causes cankers in plants (Stafford 2000). This is achieved by introducing oncogenic genes into the host cell. The segment of DNA containing these genes integrates into the plant genome and is expressed (Hooykaas *et al.* 2018). The transforming DNA segment is known as T-DNA and is located on the tumour-inducing (Ti) plasmid between two repeat borders. The transformational ability of *A. tumefaciens* was first exploited in plants, and only later established in fungi (Bundock *et al.* 1995; de Groot *et al.* 1998). There are many parameters of the *Agrobacterium*-mediated transformation protocol that must be

optimised for individual species (Michielse *et al.* 2005b), making it a time-consuming system to establish and optimise in species lacking an established protocol. However, this system has been successfully applied to numerous species of filamentous ascomycetes, including more recently the important African tree pathogen *Ceratocystis albifundus* (Sayari *et al.* 2019a).

Ceratocystis albifundus is a filamentous ascomycete in the family Ceratocystidaceae, an assemblage of fungi known as important plant and tree pathogens causing huge economic losses to the agricultural and forestry industry (de Beer *et al.* 2014). For example, *C. manginecans* causes disease on mango and *Acacia mangium* trees while *Berkeleyomyces basicola* infects the roots of numerous plants, including cotton, cowpea and tobacco (Johnson 1916; van Wyk *et al.* 2007; Tarigan *et al.* 2011). Genome sequences are available for numerous members of this family, including both pathogenic and saprophytic species (van der Nest *et al.* 2014a, b; Wingfield *et al.* 2015, 2018). These genome sequences have underpinned studies involved in aspects such as host specificity, pathogen aggressiveness, the degradation of plant-derived sucrose, sexual reproductive strategies and recombination, as well as secondary metabolite biosynthesis gene clusters (Witthuhn *et al.* 2000; Wilken *et al.* 2014; van der Nest *et al.* 2015; Wilken *et al.* 2018; Nel *et al.* 2018; Sayari *et al.* 2018; Simpson *et al.* 2018; Wilson *et al.* 2018; Fourie *et al.* 2019; van der Nest *et al.* 2019; Sayari *et al.* 2019b). However, almost all of these were limited to in silico analyses as transformation systems are not well established in these species.

Currently, transformation systems are available for only five species within the family Ceratocystidaceae. These species include *Ceratocystis albifundus*, *Berkeleyomyces basicola*, *Endoconidiophora resinifera, Huntiella omanensis* and a *Thielaviopsis* species (reported as *C. paradoxa*; Wilson *et al.* 2019; Loppnau *et al.* 2004; Al-Jaaidi 2007; Tzima *et al.* 2014; Niu *et al.* 2019; Sayari *et al.* 2019a). Similar to *C. albifundus*, *B. basicola* and *E. resinifera* have been transformed using *Agrobacterium tumefaciens*. PEG/$CaCl_2$-protoplast transformation systems are available for *E. resinifera*, as well as *H. omanensis* and a species of *Thielaviopsis*. Considering the importance of this family in plant health, as well as the amount of interest shown to in silico analyses, it is surprising that so few species have an established transformation system, which in turn results in very few functional studies reported for these fungi (Wilson *et al.* 2019; Loppnau *et al.* 2004; Tanguay *et al.* 2006).

The aim of this study was to attempt transformations in eight Ceratocystidaceae species using the *Agrobacterium*-mediated transformation system established in *C. albifundus* (Sayari *et al.* 2019a). This protocol was first optimised to increase the number of *C. albifundus* transformants obtained. The optimised protocol was then applied to the closely related species *Ceratocystis manginecans*, as well as a representative member of the genera *Berkeleyomyces*, *Chalaropsis*, *Endoconidiophora*, *Davidsoniella*, *Thielaviopsis*, *Huntiella* and *Ambrosiella*. In this way, we could determine the transferability of the *Agrobacterium*-mediated transformation system of *C. albifundus* to other related species within the same family. The results of this study will inform efforts to

establish a protocol for genetic modification of species in the Ceratocystidaceae. An established transformation protocol will be the first step towards efforts for elucidating gene functions in these species that have, until now, not been possible in this group.

Materials and methods

Strains and plasmid

The AGL-1 strain of *Agrobacterium 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*). *A. 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, which was one of the isolates used 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. Selection of isolates for all species were guided by the availability of genome sequences, with the exception of *Chalaropsis ovoidea* and *Thielaviopsis cerberus* for which genome sequences are currently being generated. 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 where transferred into 200 ml of 1% malt extract and incubated for one week at 25°C with 140 rpm shaking.

Hygromycin sensitivity

In order to determine the selective dosage of hygromycin needed to screen for transformants, the wild-type isolates of each fungus was grown on media supplemented with different hygromycin concentrations (Sigma, Johannesburg, South Africa). All plates were 2% MEA supplemented with either 0, 25 or 50 µg/ml, with three replicates for each concentration. A single mycelial plug was

placed in the centre of each plate and incubated at room temperature for 14 days after which growth was measured as an average across two perpendicular diameters.

Transformation

As a basis for the transformations, the *Agrobacterium*-mediated transformation protocol established in *C. albifundus* by Sayari *et al.* (2019a) was used. Minor adjustments were made to increase the transformation efficiency in *C. albifundus*, before testing the protocol on the eight other Ceratocystidaceae species. 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 through centrifugation at 5 000 *g* for 30 min at 4°C. The second adjustment was the addition of a one-hour incubation period at 25°C with 140 rpm shaking in liquid after the conidia- and *A. tumefaciens* suspensions were mixed, and before plating this on the induction minimal media agar. Lastly, transformants were selected using a hygromycin concentration of 50 µg/ml instead of 10 µg/ml, allowing for selection of transformants for all the species tested here.

Two controls were included for each attempted transformation. The first was to make sure the conidia remained viable following transformation. For this, a sample of the conidia that had been co-incubated with *A. tumefaciens* was placed onto MEA with cefotaxime (100 µg/ml, Aspen Pharmacare, Durban, South Africa), but lacking the selective hygromycin antibiotic. The second control was included to test if false positives were emerging on the selective media. For this, conidia that had not been mixed with *A. tumefaciens*, but that were subjected to all the transformation steps, were plated on selective media containing cefotaxime and hygromycin.

The plates were checked regularly for the emergence of colonies for up to a month. The efficiency of transformation was calculated by determining the average number of transformants that would be produced from the 10^6 conidia used per transformation (Sayari *et al.* 2019a). As colonies emerged, a maximum of five separate colonies were transferred to 2% MEA plates with 50 µg/ml hygromycin by scraping some mycelia with a sterile needle. To obtain homokaryotic transformants, some mycelia from these plates was transferred onto 2% water agar and incubated overnight for hyphal tip isolations onto hygromycin-supplemented MEA.

Analysis of transformants

DNA was isolated from 1 to 2 week-old cultures of the wild-type isolates and transformants using an adapted version of the Damm *et al.* (2008) CTAB extraction protocol. The 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, 0.2g/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). Cold ammonium acetate (to a 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 at -20°C overnight. This was followed by ethanol precipitation and DNA resuspension as per the Damm et al. (2008) protocol.

The hygromycin resistance and *GFP* genes were targeted in PCR reactions to assess for the presence of the T-DNA in the transformants. For the *hyg*^{R} gene, the hph-F and hph-R primers (Sayari *et al.* 2019a) were used while primers ZSG-F and ZSG-R were used to amplify the *GFP* gene (primers from Santana *et al.* unpublished; Suppl. Table 1). The KAPA *Taq* PCR kit (KAPA Biosystems, Boston, MA, USA) was used for all PCR reactions, and consisted of 1 U KAPA *Taq* DNA Polymerase, 1 X KAPA *Taq* Buffer A, 0.2 mM dNTP mix, 0.4 µM of each primer and 10 – 50 ng of template DNA in 25 µl volumes. The wild-type isolates and Ti plasmid DNA served as controls. 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. All PCR products and a GeneRuler 100 bp DNA ladder, ready-to-use (ThermoScientific, Waltham, USA) were mixed with GelRed (Biotium, Hayward, USA) and analysed via electrophoresis through a 1% agarose gel (Lonza, Basel, Switzerland) using a 1 X Borax buffer (Sigma-Aldrich, Johannesburg, South Africa).

The expression of GFP was identified using fluorescent microscopy. Slides were prepared from one-week-old cultures where mycelia were collected and mounted in sterile water. Both the wild-type isolates and the transformants were evaluated using an Axioskop microscope (Zeiss, Oberkochen, Germany), with a filter set 48 79 00 and a fluorescent laminator 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. Pictures were also taken of the samples under normal light conditions for comparison.

Stability of T-DNA integration

Stability of the T-DNA integrations into the genomes of the transformed isolates were evaluated based on hygromycin resistance. A block of mycelia from each transformant was placed onto MEA lacking hygromycin and incubated at room temperature for a week. Another block of mycelia was taken from these cultures and placed on another MEA plate lacking antibiotics and incubated as before. This was repeated three more times so that each isolate was sub-cultured on antibiotic free media for a total of five consecutive rounds. A block of mycelia was transferred from the last non-antibiotic media culture onto MEA with 50 µg/ml hygromycin and given another week to grow before being visually inspected for growth.

Results

Hygromycin sensitivity

All isolates grown on 2% MEA without hygromycin covered between half and the whole plate after 14 days of incubation (Table 2). *D. virescens* and *T. cerberus* were the only isolates to grow on media supplemented with 25 µg/ml hygromycin, although the growth rate was noticeably reduced in *D. virescens*. None of the isolates showed any growth on MEA with 50 µg/ml hygromycin, and this concentration was subsequently used for selection of transformants in this study.

Transformation

Using the modified *Agrobacterium*-mediated transformation protocol on *C. albifundus*, colonies were visible approximately a month after the cellophane sheets were transferred to MEA. Of the five replicate plates, four yielded an average of 237 colonies per plate, while one plate had no growth. Using the average across the four plates, efficiency was calculated as 2 370 transformants per 10^6 conidia.

Using this modified protocol on the other Ceratocystidaceae species proved less successful than for *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. Colonies became visible on the selective media after 5 to 20 days for *C. manginecans*, *Ch. ovoidea*, *D. virescens*, *H. moniliformis* and *T. cerberus*, while for *A. cleistominuta* and *E. polonica*, no growth was observed on the selective media after the 30-day incubation period. In all cases, isolates on the control plates lacking hygromycin grew normally during this time. *D. virescens* and *H. moniliformis* produced at least five colonies each, while *Ch.* ovoidea and *T. cerberus* showed only two and three colonies respectively. In some cases, it appeared as if more than five 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. However, it was clear that, for all these species, less colonies were present compared to *C. albifundus*. 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.

Analysis of transformants

PCR results of the hygromycin resistance gene and *GFP* gene indicated that both selective marker genes were absent in the wild-type isolates, which was as expected. These genes were successfully amplified from most of the transformants, with product sizes corresponding to that of

the positive control (Ti plasmid DNA). A single *C. manginecans* transformant produced an amplicon for the hygromycin resistance gene, although the *GFP* gene failed to amplify.

GFP expression was identified in at least one transformant from each of the different Ceratocystidaceae species tested (Figure 1). Although some of the wild-type isolates displayed detectable auto-fluorescence, none of them exhibited the bright green emissions seen in their transformed mutant counterparts. In the transformants expressing GFP, fluorescence could be seen in both the mycelia and conidia of all the species, with the exception of *H. moniliformis* where green fluorescence could not be detected in the conidia. A single mutant, each from *B. basicola*, *C. manginecans*, *D. virescens* and *H. moniliformis*, did not show detectable GFP expression. Unsurprisingly, the *C. manginecans* transformant was the one from which the *GFP* gene could not be amplified.

Stability of T-DNA integration

All transformants successfully grew on the hygromycin-supplemented media in the final round of sub-culturing, with the exception of the *T. cerberus* transformants. The ability to maintain hygromycin resistance in the absence of antibiotic selection suggests that the T-DNA is stably integrated into the host genome. In the case of the three *T. cerberus* transformants, no growth was observed even after one month of incubation, showing that the T-DNA was likely lost, an indication of unstable T-DNA integration or transfer.

Discussion

The purpose of this study was to determine the ease with which an established *Agrobacterium*-mediated transformation system could be applied to related species without any optimization. In spite of the many advantages of the *Agrobacterium*-mediated transformation system that has led to its wide-spread use, it remains difficult and time consuming to establish such a system for species in which this has not been done before (Michielse *et al.* 2005b; Poyedinok and Blume 2018). Numerous parameters need to be optimised for each species in which the protocol is being developed. These include factors such as, the type and quantity of fungal material used, the amount of bacteria used, the ratio of fungal cells to *A. tumefaciens* in the co-incubation suspension, the need for and concentration of acetosyringone, as well as co-incubation conditions such as time, temperature and choice of membrane used (Michielse *et al.* 2005b). Attempting these optimisations for many different species would be extremely arduous, and therefore we attempted to evaluate whether an existing protocol for *C. albifundus* could be used in other related species, bypassing some of the tedious optimising steps normally required.

The minor adjustments that were made to the existing *Agrobacterium*-mediated transformation protocol in *C. albifundus* resulted in increased transformation efficiency. These adjustments were also likely key to the successful use of this protocol in the other species, while also simplifying its

use on multiple isolates in a single round of transformation. For example, isolating conidia from liquid cultures reduced the time needed for executing the protocol. In the original protocol, cultures on agar media were flooded with induction minimal media and scraped with a hockey stick to release the conidia (Sayari *et al.* 2019a). Often, multiple plates were required to obtain enough conidia for a single transformation, making it arduous to isolate large numbers of conidia for numerous isolates. When compared to the protocol of Sayari *et al.* (2019a), the introduction of a one-hour liquid co-incubation step increased the number of transformed colonies almost six-fold. It is likely that the liquid media enhanced interactions between *A. tumefaciens* and the fungal cells when compared to only interacting on a solid medium. The inclusion of such a liquid co-incubation period in *Kabatiella zeae* also increased the number of transformants obtained and was shown to be optimal when one hour was used (Sun *et al.* 2018). Finally, all selective media used a higher concentration of hygromycin, making it useful for selection across multiple species. This circumvented the need to make selective media with hygromycin concentrations specific to each species.

Six of the eight targeted Ceratocystidaceae species were successfully transformed using the modified *Agrobacterium*-mediated transformation protocol. Some differences were noted during transformation of these isolates that were not observed in *C. albifundus* transformations. Generally, the number of transformants obtained was noticeably less than in *C. albifundus*, with these colonies also emerging on the edges of the selection plates in some species. This may be due to the cellophane membrane, as previous transformations of *Berkeleyomyces basicola* was only successful when a Hybond N+ membrane was used (Tzima *et al.* 2014). Another notable difference was the visible mycelia in the conidial suspensions that were incubated overnight. This could be attributed to differences in the growth rates between the different species, and these should be adjusted accordingly for each. In doing so, transformation efficiency will also likely be improved as the growth rate of the host is thought to influence the susceptibility of the conidial cells to transformation by *A. tumefaciens* (Michielse *et al.* 2005b).

No transformants were produced from two of the Ceratocystidaceae species tested here, although this can likely be attributed to technical challenges. When comparing the phylogenetic relationship of the two species to those successfully transformed (de Beer *et al.* 2014), there is no clear pattern, making it unlikely that, in this case, the transferability of the *Agrobacterium*-mediated transformation protocol is influenced by the relatedness of these species. Additionally, an *Agrobacterium*-mediated transformation system has been successfully established in *E. resinifera* (Loppnau *et al.* 2004), a close relative of *E. polonica* which failed to produce any transformed colonies in the present study. In order to transform *A. cleistominuta* and *E. polonica*, and possibly increase the efficiency of transformation in the other species, some parameters of the protocol could be modified. When comparing the protocol used in the current study to those in other Ceratocystidaceae species (Table 3; Loppnau *et al.* 2004; Tzima *et al.* 2014), one can easily

identify areas for potential optimization. Adjustments like increasing the concentration of conidia and/or bacteria, as well as supplementing the induction and selection media with V8 juice to improve germination of the conidia, will likely lead to further optimizations of these transformations (Michielse *et al.* 2005b; Tzima *et al.* 2014).

There were a few unexpected findings during the course of this study. Firstly, the *GFP* gene could not be amplified in one of the *C. manginecans* transformants, although a hygromycin resistance gene was present, confirming transformation. One possibility for this is the partial integration or complete loss of the T-DNA region carrying the GFP coding sequence during transformation. Such a loss is unusual as the *GFP* gene is positioned near the right T-DNA border in the pC-HYG-GFP plasmid, and not the left T-DNA border which is more susceptible to T-DNA truncation (Michielse *et al.* 2004; Chang *et al.* 2005). Although the *GFP* gene could be amplified from all other transformants, one individual, each from *B. basicola*, *D. virescens* and *H. moniliformis*, did not show fluorescence. In a previous study, no fluorescence was detected in either mycelia or conidia following transformations of *H.* omanensis protoplasts (Dr A. Wilson, personal communication). One possible explanation lies in the nature of these transformations. The lack of homologous sequences to target the T-DNA to a known position in the host genome means that these transformations resulted in random T-DNA integrations. This could have positioned the T-DNA into a region where expression is suppressed. Alternatively, it is also possible that the location of integration may have disrupted genes involved in the physiology of the fungus, distorting the fluorescent signal. Lastly, the lack of stability in the T-DNA integration exhibited in the *T. cerberus* transformants is unusual for *Agrobacterium*-mediated transformations. This form of transformation is renowned for introducing stable T-DNA integration, whereas other methods, such as the PEG/$CaCl_2$-protoplast transformation, frequently result in unstable integrations (Li *et al.* 2017; Poyedinok and Blume 2018).

Agrobacterium-mediated transformation provides a powerful tool for mutagenic studies. In addition to generating random mutations in the transformants, this technology is ideal for targeted mutagenesis, a strategy that is extremely valuable when trying to discern the function of a specific gene. The natural pathway of transformation by *A. tumefaciens* is thought to increase the targeting capabilities of this system when compared to others (Michielse *et al.* 2005a; Hooykaas *et al.* 2018). Considering 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, the opportunity to functionally study these economically important fungi is within reach. However, it will be essential to first improve the transformation efficiency of this protocol within these species. The parameters used here, and the modifications suggested provide a useful starting point, with the current study acting as a benchmark.

The ability to detect GFP emission in all of the species transformed here is very promising as these transformants could be used for additional functional studies. One example would be studies

looking at infection processes. The random transformants from this study could be used to visually track the infection pathway of these fungi within their hosts (Maor *et al.* 1998; Moročko-Bičevska and Fatehi 2011; Tzima *et al.* 2014; Vu *et al.* 2018), an important aspect considering most of the Ceratocystidaceae family are pathogenic. Additionally, through targeted mutagenesis, genes involved in pathogenicity, or other functions such as in sexual reproduction, can be fused with the *GFP* gene, creating a chimeric protein that will function normally while also emitting a fluorescent signal. This allows for the gene's expression and cellular location of the proteins to be visually tracked in real-time (Hong *et al.* 2010; Zheng *et al.* 2013; Yu *et al.* 2017). These types of studies can now be investigated in different species from the Ceratocystidaceae and will likely result in a better understanding of the biological processes of these important pathogens.

References

Al-Jaaidi, S. S., 2007 Transformation of *Thielaviopsis basicola* to study host-pathogen interactions, pp. 73–122 in University of New England, Armidale, Australia, PhD thesis.

Bundock, P., A. den Dulk-Ras, A. Beijersbergen, and P. J. Hooykaas, 1995 Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J. 14: 3206–3214.

Butler, G., M. D. Rasmussen, M. F. Lin, M. A. S. Santos, S. Sakthikumar *et al.*, 2009 Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 459: 657–662.

Case, M. E., M. Schweizer, S. R. Kushner, and N. H. Giles, 1979 Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc. Natl. Acad. Sci. 76: 5259–5263.

Chang, H. K., S. Y. Park, Y. H. Lee, and S. Kang, 2005 A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum*. Fungal Genet. Biol. 42: 483–492.

Choi, J. J., and S. H. Kim, 2017 A genome Tree of Life for the Fungi kingdom. Proc. Natl. Acad. Sci. U. S. A. 114: 9391–9396.

Damm, U., L. Mostert, P. W. Crous, and P. H. Fourie, 2008 Novel *Phaeoacremonium* species associated with necrotic wood of *Prunus* trees. Persoonia Mol. Phylogeny Evol. Fungi 20: 87–102.

de Beer, Z. W., T. A. Duong, I. Barnes, B. D. Wingfield, and M. J. Wingfield, 2014 Redefining *Ceratocystis* and allied genera. Stud. Mycol. 79: 187–219.

de Groot, M. J. A., P. Bundock, P. J. J. Hooykaas, and A. G. M. Beijersbergen, 1998 *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat. Biotechnol. 16: 839–842.

Fourie, A., M. A. van der Nest, L. de Vos, M. J. Wingfield, B. D. Wingfield *et al.*, 2019 QTL mapping of mycelial growth and aggressiveness to distinct hosts in *Ceratocystis* pathogens. Fungal Genet. Biol.: 103242.

Gilchrist, C. L. M., H. Li, and Y. H. Chooi, 2018 Panning for gold in mould: can we increase the odds for fungal genome mining? Org. Biomol. Chem. 16: 1620–1626.

Hellens, R., P. Mullineaux, and H. Klee, 2000 A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci. 5: 446–451.

Hong, S. Y., J. So, J. Lee, K. Min, H. Son *et al.*, 2010 Functional analyses of two syntaxin-like SNARE genes, *GzSYN1* and *GzSYN2*, in the ascomycete *Gibberella zeae*. Fungal Genet. Biol. 47: 364–372.

Hooykaas, P. J. J., G. P. H. van Heusden, X. Niu, M. R. Roushan, J. Soltani *et al.*, 2018 *Agrobacterium*-mediated transformation of yeast and fungi, pp. 349–374 in Agrobacterium *Biology. Current Topics in Microbiology and Immunology, vol 418*, edited by S. B. Gelvin, Springer, Cham.

Johnson, B. J., 1916 Host plants of *Thielavia basicola*. J. Agric. Res. VII: 289.

Kwon, M. J., M. Arentshorst, E. D. Roos, C. A. M. J. J. van den Hondel, V. Meyer *et al.*, 2011 Functional characterization of Rho GTPases in *Aspergillus niger* uncovers conserved and diverged roles of Rho proteins within filamentous fungi. Mol. Microbiol. 79: 1151–1167.

Li, D., Y. Tang, J. Lin, and W. Cai, 2017 Methods for genetic transformation of filamentous fungi. Microb. Cell Fact. 16: 1–13.

Loppnau, P., P. Tanguay, and C. Breuil, 2004 Isolation and disruption of the melanin pathway polyketide synthase gene of the softwood deep stain fungus *Ceratocystis resinifera*. Fungal Genet. Biol. 41: 33–41.

Maor, R., M. Puyesky, B. A. Horwitz, and A. A. Sharon, 1998 Use of green fluorescent protein (GFP) for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. Mycol. Res. 102: 491–496.

Michielse, C. B., A. F. J. Ram, P. J. J. Hooykaas, and C. A. M. J. J. van den Hondel, 2004 *Agrobacterium*-mediated transformation of *Aspergillus awamori* in the absence of full-length VirD2, VirC2, or VirE2 leads to insertion of aberrant T-DNA structures. J. Bacteriol. 186: 2038–2045.

Michielse, C. B., M. Arentshorst, A. F. J. Ram, and C. A. M. J. J. van den Hondel, 2005a *Agrobacterium*-mediated transformation leads to improved gene replacement efficiency in *Aspergillus awamori*. Fungal Genet. Biol. 42: 9–19.

Michielse, C. B., P. J. J. Hooykaas, C. A. M. J. J. van den Hondel, and A. F. J. Ram, 2005b *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr. Genet. 48: 1–17.

Moročko-Bičevska, I., and J. Fatehi, 2011 Infection and colonization of strawberry by *Gnomonia fragariae* strain expressing green fluorescent protein. Eur. J. Plant Pathol. 129: 567–577.

Nel, W. J., T. A. Duong, M. J. Wingfield, B. D. Wingfield, A. Hammerbacher *et al.*, 2018 Heterothallism revealed in the root rot fungi *Berkeleyomyces basicola* and *B. rouxiae*. Fungal Biol. 122: 1031–1040.

Niu, X., M. Pei, C. Liang, Y. Lv, X. Wu *et al.*, 2019 Genetic transformation and green fluorescent protein labeling in *Ceratocystis paradoxa* from coconut. Int. J. Mol. Sci. 20: 2387.

Poyedinok, N. L., and Y. B. Blume, 2018 Advances, problems, and prospects of genetic transformation of fungi. Cytol. Genet. 52: 139–154.

Sayari, M., E. T. Steenkamp, M. A. van der Nest, and B. D. Wingfield, 2018 Diversity and evolution of polyketide biosynthesis gene clusters in the Ceratocystidaceae. Fungal Biol. 122: 856–866.

Sayari, M., M. A. van der Nest, E. T. Steenkamp, O. O. Adegeye, S. Marincowitz *et al.*, 2019a *Agrobacterium*-mediated transformation of *Ceratocystis albifundus*. Microbiol. Res. 226: 55–64.

Sayari, M., M. A. van der Nest, E. T. Steenkamp, N. C. Soal, P. M. Wilken *et al.*, 2019b Distribution and evolution of nonribosomal peptide synthetase gene clusters in the Ceratocystidaceae.

Genes 10: 328.

Simpson, M. C., M. P. A. Coetzee, M. A. van der Nest, M. J. Wingfield, and B. D. Wingfield, 2018 Ceratocystidaceae exhibit high levels of recombination at the mating-type (*MAT*) locus. Fungal Biol. 122: 1184–1191.

Stafford, H. A., 2000 Crown gall disease and *Agrobacterium tumefaciens*: a study of the history, present knowledge, missing information, and impact on molecular genetics. Bot. Rev. 66: 99–118.

Sun, J., R. Xu, S. Xiao, Y. Lu, Q. Zhang *et al.*, 2018 *Agrobacterium tumefaciens*-mediated transformation as an efficient tool for insertional mutagenesis of *Kabatiella zeae*. J. Microbiol. Methods 149: 96–100.

Tanguay, P., P. Loppnau, C. Morin, L. Bernier, and C. Breuil, 2006 A spontaneous albino mutant of *Ceratocystis resinifera* results from a point mutation in the polyketide synthase gene, *PKS1*. Can. J. Microbiol. 52: 501–507.

Tarigan, M., J. Roux, M. van Wyk, B. Tjahjono, and M. J. Wingfield, 2011 A new wilt and die-back disease of *Acacia mangium* associated with *Ceratocystis manginecans* and *C. acaciivora sp. nov.* in Indonesia. South African J. Bot. 77: 292–304.

Turgeon, B. G., and O. C. Yoder, 2000 Proposed nomenclature for mating type genes of filamentous ascomycetes. Fungal Genet. Biol. 31: 1–5.

Tzima, A. K., E. J. Paplomatas, C. Schoina, E. Domazakis, S. Kang *et al.*, 2014 Successful *Agrobacterium* mediated transformation of *Thielaviopsis basicola* by optimizing multiple conditions. Fungal Biol. 118: 675–682.

van der Nest, M. A., L. A. Beirn, J. A. Crouch, J. E. Demers, Z. W. de Beer *et al.*, 2014a Draft genomes of *Amanita jacksonii*, *Ceratocystis albifundus*, *Fusarium circinatum*, *Huntiella omanensis*, *Leptographium procerum*, *Rutstroemia sydowiana*, and *Sclerotinia echinophila*. IMA Fungus 5: 473–486.

van der Nest, M. A., W. Bihon, L. De Vos, K. Naidoo, D. Roodt *et al.*, 2014b Draft genome sequences of *Diplodia sapinea*, *Ceratocystis manginecans*, and *Ceratocystis moniliformis*. IMA Fungus 5: 135–140.

van der Nest, M. A., E. T. Steenkamp, A. R. McTaggart, C. Trollip, T. Godlonton *et al.*, 2015 Saprophytic and pathogenic fungi in the Ceratocystidaceae differ in their ability to metabolize plant-derived sucrose. BMC Evol. Biol. 15: 273.

van der Nest, M. A., E. T. Steenkamp, D. Roodt, N. C. Soal, M. Palmer *et al.*, 2019 Genomic analysis of the aggressive tree pathogen *Ceratocystis albifundus*. Fungal Biol. 123: 351–363.

van Wyk, M., A. O. Al Adawi, I. A. Khan, M. L. Deadman, A. A. Al Jahwari *et al.*, 2007 *Ceratocystis manginecans* sp. nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. Fungal Divers. 27 I: 213–230.

Vu, T. X., T. T. Ngo, L. T. D. Mai, T. T. Bui, D. H. Le *et al.*, 2018 A highly efficient *Agrobacterium tumefaciens*-mediated transformation system for the postharvest pathogen *Penicillium*

digitatum using DsRed and GFP to visualize citrus host colonization. J. Microbiol. Methods 144: 134–144.

Wilken, P. M., E. T. Steenkamp, M. J. Wingfield, Z. W. de Beer, and B. D. Wingfield, 2014 DNA loss at the *Ceratocystis fimbriata* mating locus results in self-sterility. PLoS One 9: e92180.

Wilken, P. M., E. T. Steenkamp, M. J. Wingfield, Z. W. de Beer, and B. D. Wingfield, 2017 Which *MAT* gene? Pezizomycotina (Ascomycota) mating-type gene nomenclature reconsidered. Fungal Biol. Rev. 31: 199–211.

Wilken, P. M., E. T. Steenkamp, M. A. van der Nest, M. J. Wingfield, Z. W. de Beer *et al.*, 2018 Unexpected placement of the *MAT1-1-2* gene in the *MAT1-2* idiomorph of *Thielaviopsis*. Fungal Genet. Biol. 133: 32–41.

Wilson, A. M., M. A. van der Nest, P. M. Wilken, M. J. Wingfield, and B. D. Wingfield, 2018 Pheromone expression reveals putative mechanism of unisexuality in a saprobic ascomycete fungus. PLoS One 13: e0192517.

Wilson, A. M., P. M. Wilken, M. A. van der Nest, M. J. Wingfield, and B. D. Wingfield, 2019 The novel *H. omanensis* mating gene, *MAT1-2-7*, is essential for ascomatal maturation. Fungal Genet. Biol. Submitted.

Wingfield, B. D., I. Barnes, Z. W. de Beer, L. De Vos, T. A. Duong *et al.*, 2015 Draft genome sequences of *Ceratocystis eucalypticola*, *Chrysoporthe cubensis*, *C. deuterocubensis*, *Davidsoniella virescens*, *Fusarium temperatum*, *Graphilbum fragrans*, *Penicillium nordicum*, and *Thielaviopsis musarum*. IMA Fungus 6: 493–506.

Wingfield, B. D., G. F. Bills, Y. Dong, W. Huang, W. J. Nel *et al.*, 2018 Draft genome sequence of *Annulohypoxylon stygium*, *Aspergillus mulundensis*, *Berkeleyomyces basicola* (syn. *Thielaviopsis basicola*), *Ceratocystis smalleyi*, two *Cercospora beticola* strains, *Coleophoma cylindrospora*, *Fusarium fracticaudum*, *Phialophora* cf. *hyalina*, and *Morchella septimelata*. IMA Fungus 9: 199–223.

Witthuhn, R. C., T. C. Harrington, B. D. Wingfield, J. P. Steimel, and M. J. Wingfield, 2000 Deletion of the *MAT*-2 mating-type gene during uni-directional mating-type switching in *Ceratocystis*. Curr. Genet. 38: 48–52.

Yu, Y., J. Amich, C. Will, C. E. Eagle, P. S. Dyer *et al.*, 2017 The novel *Aspergillus fumigatus MAT1-2-4* mating-type gene is required for mating and cleistothecia formation. Fungal Genet. Biol. 108: 1–12.

Zheng, Q., R. Hou, J. Zhang, J. Ma *et al.*, 2013 The *MAT* locus genes play different roles in sexual reproduction and pathogenesis in *Fusarium graminearum*. PLoS One 10: e66980.

Tables and figures

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

Species	Culture collection number(s)[1]	Country	Host	Collected/ isolated by
Ambrosiella cleistominuta	CMW 50464; CBS 141682; C 3843	USA	*Anisandrus maiche*	C.G. Mayers 2015
Berkeleyomyces basicola	CMW 25440; CBS 142829	Indonesia	*Styrax benzoin*	M.J. Wingfield & M. van Wyk 2007
Ceratocystis albifundus	CMW 4068; CBS 128992	South Africa	*Acacia mearnsii*	J. Roux 1997
C. manginecans	CMW 17570; CBS 138185	Oman	*Prosopis cineraria*	A. Al Adawi 2005
Chalaropsis ovoidea	CMW 22733; CBS 354.76; C 1375	Netherlands	Firewood	W. Gams 1976
Davidsoniella virescens	CMW 17339; CBS 17339	USA	*Acer saccharum*	D. Houston 1987
Endoconidiophora polonica	CMW 20930; CBS 100205	Norway	*Picea abies*	H. Solheim 1990
Huntiella moniliformis	CMW 36919; CBS 144008	Cameroon	*Theobroma cacao*	M. Mbenoun 2009
Thielaviopsis cerberus	CMW 36653; CBS 130763	Cameroon	*Elaeis guineensis*	M. Mbenoun & 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.

Table 2: Average diameter of cultures after 14 days of incubation on MEA of different concentrations of hygromycin

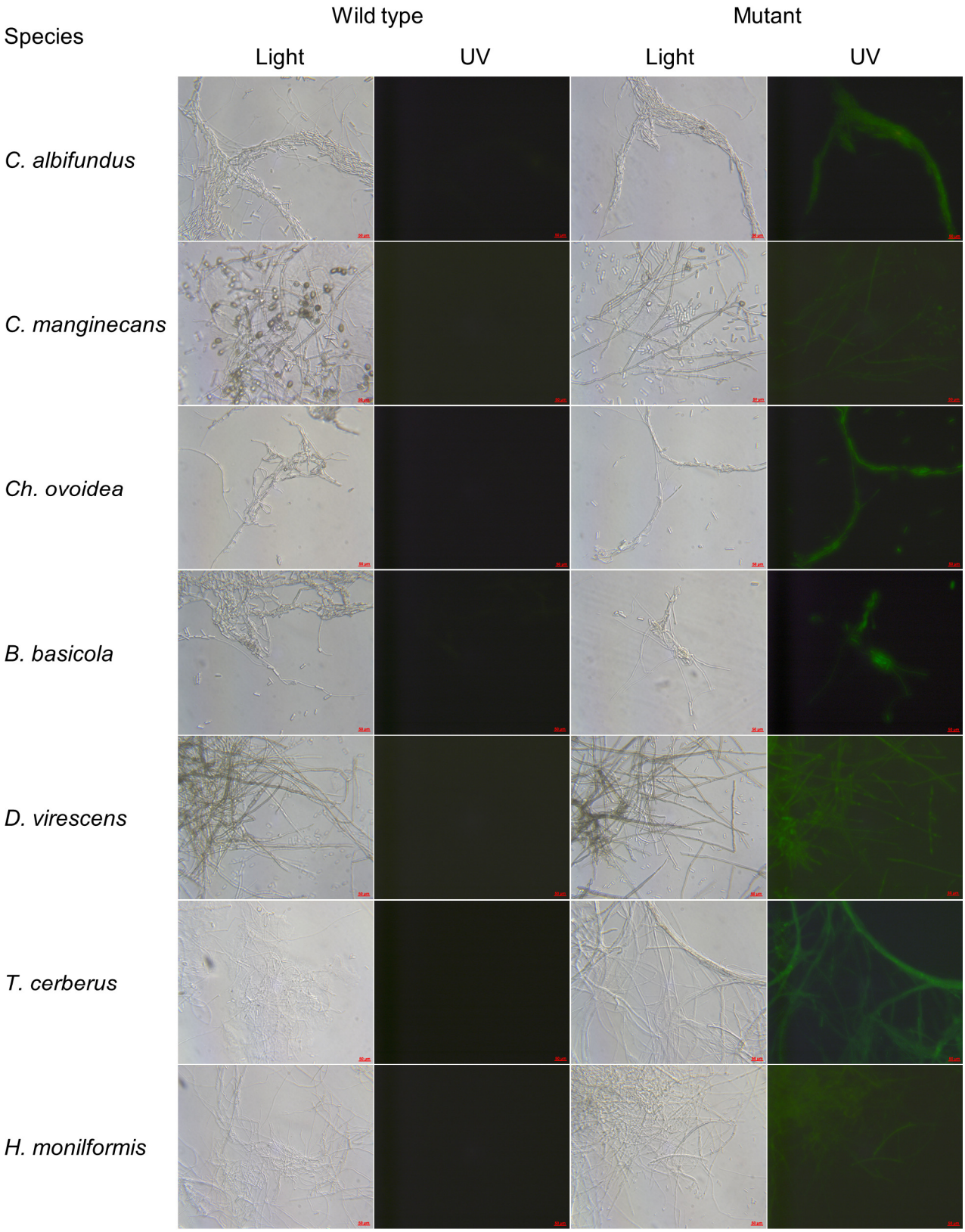
Species	Concentration of hygromycin		
	0 µg/ml	**25 µg/ml**	**50 µg/ml**
C. albifundus	38.7 mm	0 mm	0 mm
C. manginecans	36.0 mm	0 mm	0 mm
Ch. ovoidea	49.2 mm	0 mm	0 mm
B. basicola	55.0 mm	0 mm	0 mm
E. polonica	60 mm	0 mm	0 mm
D. virescens	60 mm	9.8 mm	0 mm
T. cerberus	60 mm	38 mm	0 mm
H. moniliformis	60 mm	0 mm	0 mm
A. cleistominuta	60 mm	0 mm	0 mm

Table 3: 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.* (2019).
[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.

Figure 1: Microscopy images of fungal isolates indicating fluorescence in mutant individuals. Images using a light field as well as under UV light to visualise GFP emissions are presented for comparisons. For each species, the wild type and a single transformant is shown.



Supplementary material

Suppl. Table 1: The primers used to amplify regions of the *hyg*^{R} and *GFP* genes in the fungal isolates.

Primer sets	Sequences (5' – 3')	Product size
hph-F	TAGCGCGTCTGCTGCTCCATACAAG	428 bp
hph-R	ACCGAACTGCCCGCTGTTCTC	
ZSG-F[1]	CCTGACCAAGGAGATGACCA	594 bp
ZSG-R[1]	GTCAGCTTGTGCTGGATGAA	

[1] Primers from Santana *et al.* (unpublished).

Chapter 3

Current progress in establishing a PEG/$CaCl_2$-protoplast transformation system in *Ceratocystis*

Abstract

Transformation systems are essential tools needed for characterisation of gene function. In fungi, the *Agrobacterium*-mediated transformation system is highly efficient and widely used, but the need to use plasmid DNA is a major drawback. Targeting cassettes are needed to direct exogenous DNA to a specific genomic region for targeted mutagenesis and inserting these cassettes into the plasmid requires many time-consuming and laborious cloning steps. The PEG/$CaCl_2$-protoplast transformation system is more flexible and can make use of linear DNA in addition to plasmid DNA. This makes targeting cassettes easier and faster to construct as PCR products can be used without further processing. Currently, an *Agrobacterium*-mediated transformation system is established in the economically important plant and tree pathogen *Ceratocystis albifundus*. Although this transformation system has successfully been used to produce random mutagenic strains, targeted genetic changes have yet to be attempted. This can be attributed, at least in part, to the difficulty of producing targeted knock-out cassettes in a plasmid. The aim of this study was to establish a PEG/$CaCl_2$-protoplast transformation system in *C. albifundus*, which could be used in future for targeted gene disruptions using linear cassettes. Two established protocols were used to produce *C. albifundus* protoplasts, one of these resulted in protoplast formation. The protoplasts were viable and regenerated their cell walls producing mature colonies of the fungus. The addition of DNA containing indicator genes to the protoplasts did not result in any transformation. This was despite a number of attempts and modifications to the protocol. While the PEG/$CaCl_2$ transformation protocol used here did not result in any transformations, the production of protoplasts provides a catalyst for the development of a targeted transformation system in *Ceratocystis*.

Introduction

Establishing a transformation system is the first step towards genome editing in a fungus. A transformation system is the process of introducing exogeneous DNA into a host cell, where in most cases, it can integrate into the chromosomal DNA to produce a genetically altered individual. By using transformation systems, one can begin to functionally study genes in a fungus of interest through random mutagenesis or more targeted approaches like knockout studies (Kück and Hoff 2010). Transformation systems have been well established in model filamentous ascomycetes, such as *Neurospora crassa* and *Aspergillus niger* (Roche *et al.* 2014; Cairns *et al.* 2018), making functional studies a common occurrence in these species. Although there has been a recent increase in the number of transformation systems being optimised in less studied ascomycetes (Díaz-Trujillo *et al.* 2018; Long *et al.* 2018; Liu *et al.* 2019; Sayari *et al.* 2019; Timofeev *et al.* 2019), there are still many species of interest lacking transformation protocols.

There are many different types of transformation systems, with the first and most commonly used being the PEG/$CaCl_2$-protoplast transformation system (Case *et al.* 1979). This system requires the use of protoplasts; fungal cells whose walls have been partially digested, making them more permeable to exogenous DNA (Fincham 1989). These protoplasts are then treated with polyethylene glycol (PEG) and calcium chloride which mediates the uptake of DNA into the cells. PEG/$CaCl_2$-protoplast transformation is widely used because it is a relatively simple and fast process, once the protoplasts have been made (Li *et al.* 2017). Both plasmids and linear DNA molecules can be used for transformation, overcoming the need to develop a plasmid in transformation systems such as *Agrobacterium*-mediated transformation (Weyda *et al.* 2017). The use of linear DNA, such as PCR products, circumvents tedious cloning steps that are required for Ti plasmids when attempting targeted genetic alterations.

The PEG/$CaCl_2$-protoplast transformation protocol has some shortcomings; which are mostly associated with the inherent difficulties of producing and working with protoplasts. The cell walls of filamentous ascomycetes are complex and variable between species (Bowman and Free 2006). This means that the types and concentrations of enzymes used to partially digest the cell wall must be tailored to the organism of study. Some species have proven recalcitrant to the production of protoplasts (Michielse *et al.* 2005), making the PEG/$CaCl_2$-protoplast transformation protocol unusable for these fungi. If protoplasts are produced, the fragile nature of the cells requires careful handling in an osmotically stable environment. This is very important, as the formation and recovery of viable protoplasts are the main factors that influence transformant yield. A recovery process following transformation is needed to regenerate the cell walls, allowing germination to proceed as normal (Olmedo-Monfil *et al.* 2004). Despite this, cell death is common in this protocol and can influence the efficacy of the process.

The establishment of transformation systems in non-model fungi has gained significant momentum recently. In the Ceratocystidaceae, a family consisting of many species that are important plant and tree pathogens (de Beer *et al.* 2014), established transformation systems are present in five species, including *Ceratocystis albifundus*, *Berkeleyomyces basicola*, *Endoconidiophora resinifera, Huntiella omanensis* and a *Thielaviopsis* species, previously known as *C. paradoxa* (Loppnau *et al.* 2004; Al-Jaaidi 2007; Tzima *et al.* 2014; Niu *et al.* 2019; Sayari *et al.* 2019; Wilson *et al.* 2019). These transformation systems include both *Agrobacterium*-mediated and PEG/$CaCl_2$-protoplast transformation systems. The first transformation system in this group was developed in *E. resinifera* using *Agrobacterium*-mediated transformation (Loppnau *et al.* 2004). This was later followed by a PEG/$CaCl_2$-protoplast transformation system in *B. basicola* and subsequently an *Agrobacterium*-mediated system. In a single year, PEG/$CaCl_2$-protoplast transformation systems were established in *H. omanensis* and a *Thielaviopsis* species (Niu *et al.* 2019; Wilson *et al.* 2019), while *Agrobacterium*-mediated transformation was reported in *C. albifundus* (Sayari *et al.* 2019).

The current study aimed to establish a PEG/$CaCl_2$-protoplast transformation system in *Ceratocystis albifundus*, an important pathogen native to Africa that infects endemic protea plants and introduced *Acacia mearnsii* trees used in plantation forestry (Roux *et al.* 2007). This species already has an *Agrobacterium*-mediated transformation system (Sayari *et al.* 2019), although it has only been used for random mutagenesis. The use of this system for functional studies would require modifying the Ti plasmid DNA (Hooykaas *et al.* 2018), a process that involves many laborious and costly cloning steps to make targeting cassettes for each individual target (Yu *et al.* 2004). A PEG/$CaCl_2$-protoplast transformation protocol for *C. albifundus* would allow for the construction of linear targeting cassettes via PCR, making the production of different knockout cassettes faster and more economical.

Materials and methods

Plasmid propagation and isolation

An AGL-1 strain of *Agrobacterium tumefaciens* carrying a helper plasmid and the Ti plasmid pC-HYG-GFP was provided by Sayari *et al.* (2019). AGL-1 has a genomic carbenicillin resistance gene while the pC-HYG-GFP plasmid carries a kanamycin and hygromycin resistance gene, as well as a *GFP* gene (Hellens *et al.* 2000; Sayari *et al.* 2019). *Agrobacterium tumefaciens* was grown in Luria-Bertani broth (10 g/l tryptone, Sigma-Aldrich, Johannesburg, South Africa; 10 g/l NaCl, 5 g/l yeast extract, Biolab, Germiston, South Africa) supplemented with 50 µg/ml carbenicillin and 100 µg/ml kanamycin (Sigma-Aldrich, Johannesburg, South Africa) and incubated at 28°C with 250 rpm shaking.

Plasmid isolations from *A. tumefaciens* were carried out using a standard alkaline-SDS plasmid miniprep (Barker 2005). To determine if both the helper and the pC-HYG-GFP plasmids were isolated, the extractions were subjected to gel electrophoresis using a 1% agarose gel (Lonza,

Basel, Switzerland) and 1 X Borax buffer (Sigma-Aldrich, Johannesburg, South Africa). The presence of the pC-HYG-GFP plasmid was further confirmed through PCR using primers hph-F and hph-R that amplify the hygromycin resistance gene (Sayari *et al.* 2019). PCR reactions were carried out in a volume of 25 µl using the KAPA *Taq* PCR kit (KAPA Biosystems, Boston, MA, USA). Each reaction consisted of 10 – 50 ng of plasmid DNA as template, 1 U KAPA *Taq* DNA Polymerase, 1 X KAPA *Taq* Buffer A, 0.2 mM dNTP mix and 0.2 µM of each primer. Initial denaturation was at 96°C for 5 min followed by 25 cycles of 96°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, after which a final elongation step of 72°C for 7 min was completed. The PCR products were then visualised and assessed using gel electrophoresis. These samples were purified with Sephadex G-50 columns (Sigma, Johannesburg, South Africa), sequenced using the BigDye cycle sequencing kit with Amplitaq DNA polymerase (Perkin-Elmer, Warrington, UK) and submitted for Sanger sequencing at the University of Pretoria Sequencing Facility (University of Pretoria, South Africa). The sequences were analysed via a BLASTp query to the NCBI database (www.ncbi.nlm.nih.gov), as well as to the translated coding regions from the published pC-HYG-GFP plasmid sequence (Sayari *et al.* 2019) using CLC Main Workbench software package (v12.0.4, CLC Bio, Denmark).

The plasmid isolation from AGL-1 was transformed into DHα5 *Escherichia coli* cells for selection and propagation of the pC-HYG-GFP plasmid. DHα5 cells were grown in LB broth and incubated overnight at 37°C with shaking. Competent *E. coli* cells were produced using a calcium chloride method and transformed with approximately 150 ng of plasmid DNA through heat-shock treatment (Chang *et al.* 2017). The cells were then plated on LB agar (2%, Biolab Germiston, South Africa) supplemented with 100 µg/ml kanamycin to select for transformants containing the pC-HYG-GFP plasmid which carries a kanamycin resistance gene. These plates were incubated overnight at 37°C to allow for colonies to emerge. Ten colonies were selected at random for a colony PCR using the primers specifically designed for the hygromycin resistance gene. Four random colonies that were shown to contain this fragment were collected and inoculated into LB broth supplemented 100 µg/ml kanamycin in preparation for plasmid isolation as described above. Plasmid DNA isolations showing a single band on an agarose gel, indicating that only the pC-HYG-GFP plasmid was present, was later used for transformations of *Ceratocystis albifundus*.

Growth conditions of the fungal isolate

Isolate CMW 4068 of *Ceratocystis albifundus* was obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. The isolate was maintained at room temperature (~25°C) on 2% MEA (2% malt extract, 2% agar, Biolab, Germiston, South Africa; 100 mg/l thiamine; 150 mg/l streptomycin, Sigma-Aldrich, Johannesburg, South Africa) through the sterile transfer of ascospore masses to fresh media. For the isolation of conidia, 200 ml of 1% ME broth was inoculated with blocks of mycelia and grown for 5 to 10 days at room temperature with 140 rpm shaking. Once grown, the conidia were isolated

by filtration through a single layer of Miracloth (Merck, New Jersey, USA) and centrifugation at 5 000 *g* for 30 min at 4°C. The pelleted conidia were used directly for protoplast production. Alternatively, the collected conidia were resuspended in fresh 1% ME broth and incubated overnight with shaking to allow for germination. The germlings were collected through centrifugation at 4°C for 20 min at up to 20 000 *g* and used as germinated conidia for transformations.

Protoplast production

Attempts to produce viable protoplasts were made by using two established protocols. In the first, a protocol for producing protoplasts in *Huntiella omanensis* (Wilson *et al.* 2019) was adapted and used to produce protoplasts from the germinated *C. albifundus* conidia. The germlings were resuspended in lysis buffer containing 1 M sorbitol and 1.25 – 15 mg/ml lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, Johannesburg, South Africa). These reactions were incubated at 25°C with 60 rpm shaking for up to 24 hours. The cells were checked regularly under an Axioskop microscope (Zeiss, Oberkochen, Germany) for the production of protoplasts.

Alternatively, ungerminated conidia of *C. albifundus* were used for protoplast production using an adjusted protocol for *Fusarium graminearum* (Moradi *et al.* 2013). The pelleted conidia were resuspended in 40 ml of lysis enzyme mixture containing 1.2 M KCl, 25 mg/ml driselase, 0.05 mg/ml chitinase and 5 mg/ml lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, Johannesburg, South Africa). The suspension was incubated at 25°C with 60 rpm shaking, and the digestion of the cells was monitored regularly until approximately 80% of all the cells were protoplasts.

When protoplasts were produced, the suspension was filtered through one layer of Miracloth before the protoplasts were harvested by centrifugation at 4°C, 3 000 *g* for 15 min. The supernatant was discarded, and the pelleted protoplasts were gently resuspended in 1 ml of STC buffer (20% sucrose, 50 mM Tris-HCl, pH 8.00, 50 mM $CaCl_2$). The protoplasts were counted using a haemocytometer and divided into 200 ul aliquots of 4.5×10^6 – 4.975×10^7 protoplasts per ml. The aliquots were then stored at -80°C until use.

PEG/$CaCl_2$-protoplast transformation

A standard PEG/$CaCl_2$-protoplast transformation protocol was used for transformation of the *C. albifundus* protoplasts (Sweigard *et al.* 1992). During the course of the study, numerous modifications were made to the protocol, targeting plasmid concentration, protoplast starting concentration, amount of PTC (60% PEG 4000 in STC buffer) used, as well as the inclusion of dimethyl sulfoxide (DMSO; Sigma, Johannesburg, South Africa) and KCl-Tris buffer in the PTC (Table 1). For the transformations, protoplasts were thawed on ice and transferred to a 50 ml falcon tube. Plasmid DNA was added and mixed with the protoplasts, followed by a 20 min incubation on ice. PTC was then slowly dripped onto the protoplasts to form a layer on top of the

protoplast-DNA mixture. This reaction was incubated at room temperature for a further 20 min. In some cases (Table 1), the PTC was substituted with 1 ml of PTC supplemented with 1.2% DMSO and/or KCl-Tris buffer (50 mM KCl, 10 mM Tris). After incubation, osmotic control media (3 g/l yeast extract, 200 g/l sucrose, 3 g/l casamino acids) was gently mixed into the contents of the falcon tube to a total volume of 6 ml, and incubated overnight or for two nights at 25°C with 60 rpm shaking. The recovery culture was evenly aliquoted (1 ml each) into six 60 mm sterile petri dishes. In all cases, one of the six aliquots were covered with two layers of ~10 ml TB3 agar (3 g/l yeast extract, 20 g/l sucrose, 3 g/l casamino acids, 0.8% agar) to serve as a control. The remaining five plates were first layered with ~10ml of TB3 agar supplemented with 25 µg/ml of hygromycin (Sigma, Johannesburg, South Africa). Once the agar had set, a second layer (~10 ml) of TB3 agar with 50 µg/ml hygromycin was poured and left to set. In transformation reactions where only 1 ml of PTC was used (Table 1), a 1 ml aliquot of the recovered culture was gently spread on the surface of a TB3 agar plate with 50 µg/ml hygromycin, in addition to the aliquots mixed into the TB3 agar. All plates were incubated at room temperature for a month and checked regularly for the emergence of colonies through or on the agar.

Results

Isolation of plasmid

Following plasmid extraction from *Agrobacterium tumefaciens*, gel electrophoresis yielded two bands of distinguishably different size (Figure 1). These were the two plasmids present in the *A. tumefaciens* starting culture, namely the helper plasmid and the Ti plasmid pC-HYG-GFP. Amplification of the hygromycin resistance gene confirmed that one of the plasmids was pC-HYG-GFP. The plasmid isolation from *A. tumefaciens* containing both plasmids was successfully used to transform *E. coli*. The presence of colonies showing resistance to kanamycin confirmed that these cells contained the pC-HYG-GFP plasmids, and four were randomly selected for plasmid extractions. Gel electrophoresis indicated the presence of a single plasmid in each (Figure 1). Amplification of the hygromycin resistance gene confirmed that this plasmid was the pC-HYG-GFP plasmid.

Protoplast production and PEG/$CaCl_2$-protoplast transformation

Initially, germinating conidia were targeted for use in enzyme digestions to yield protoplasts. The incubation of conidia overnight in fresh media resulted in a mixture of both germinated and ungerminated conidia. Germination was evident by the production of a germ tube of equal or longer length than that of the conidia. Attempts to collect the germlings through centrifugation at speeds of up to 20 000 *g* proved only moderately successful, with many germlings lost in the discarded supernatant. The pellet that resulted from centrifugation did not form a solid mass and was further disrupted when the supernatant was discarded by either pouring off or pipetting. Using the mixture of ungerminated conidia and germlings in a protoplast protocol established for

H. omanensis produced no protoplasts, even after 24 hours of digestion with the standard lysis buffer mixtures. Increasing the lysis enzyme concentrations up to three times the standard amount still failed to produce any protoplasts. Therefore, this approach was abandoned.

To address the low level of input material for the protoplast production protocol, the germination step for the conidia was omitted as ungerminated conidia could easily be collected in high numbers through centrifugation. The collected conidia were treated with a driselase, chitinase and *Trichoderma harzianum* lysis enzyme cocktail as per the *F. graminearum* protoplast protocol, resulting in the formation of protoplasts (Figure 2). Approximately 80% of the cells were protoplasts after 4 to 5 hours of digestion. The protoplasts were round in shape, a characteristic feature, as opposed to the cylindrical shaped conidia. The yield of the number of protoplasts produced ranged between $4.5 \times 10^6 – 5.175 \times 10^7$ protoplasts per attempt.

All attempts at transforming the *C. albifundus* protoplasts were unsuccessful despite modifications to a standard PEG/$CaCl_2$-protoplast transformation protocol (Table 1). Transformed colonies were expected to emerge through the two selective layers of media to the surface of the plates, however, even after a one-month incubation period, no colonies appeared in any of the attempts. Recovered protoplasts were also plated on the surface of the selective media to ensure that transformant-mortality was not influenced by the cells being encased in the agar, but no colonies were observed on these plates either. All control plates lacking the selective hygromycin resulted in mycelia emerging within the agar, later growing through to the surface of the plates. Single colonies on these control plates could not be distinguished as the growth was dense and resulted in a lawn of mycelia that covered the entire plate.

Discussion

In the family Ceratocystidaceae, PEG/$CaCl_2$-protoplast transformation has been successfully used in *B. basicola*, *H. omanensis* and a single species of *Thielaviopsis* (Al-Jaaidi 2007; Niu *et al.* 2019; Wilson *et al.* 2019). The development of this technology in *C. albifundus* would represent the next step in efforts to easily manipulate the genome of the fungus, providing the ability to produce cassettes for targeted alterations via PCR. This will improve the ease with which genetic engineering in this fungus can be done, circumventing the multiple cloning steps required in the established *Agrobacterium*-mediated protocol. Although protoplasts were successfully produced from *C. albifundus* conidia, these were not effective for subsequent transformation of a plasmid DNA into the cells. An inability to transform protoplasts with PEG/$CaCl_2$ has also been reported in *Endoconidiophora resinifera*, a relative of *C. albifundus* in the Ceratocystidaceae family (Loppnau *et al.* 2004). It appears the production of protoplasts in a species does not necessarily guarantee their transformation via the PEG/$CaCl_2$-protoplast transformation system. If the recalcitrance of these protoplasts to transformation are not caused by a currently unknown inherent resistance,

optimised conditions for transformation using PEG/$CaCl_2$ not tested here might yield transformants in future.

Viable protoplasts were produced from the ungerminated conidia of *C. albifundus* using an enzyme cocktail developed for *F. graminearum* protoplasts (Moradi *et al.* 2013). The formation of a mycelial lawn on the transformation control plates indicated the successful production of many viable *C. albifundus* protoplasts. Therefore, the *C. albifundus* protoplasts were able to survive and regenerate following the transformation attempts and subsequent recovery procedure, attesting to the viability of these cells. The use of a lysis buffer consisting of only *Trichoderma harzianum* lysing enzyme is sufficient for protoplast production in *H. omanensis* (Wilson *et al.* 2019), another relative of *C. albifundus*. This same mixture did not yield any protoplasts when applied to *C. albifundus* conidia, even when using three times the recommended concentration of enzyme. This could be attributed to a number of reasons. At 6 – 8 µm by 2 – 3 µm, the conidia of *H. omanensis* are markedly smaller than those of *C. albifundus* (8 - 24 µm by 3 – 4 µm; Wingfield *et al.* 1996; Liu *et al.* 2018), making it likely that even higher amounts of enzyme would be required to act on the much larger surface area of the *C. albifundus* conidia. Additionally, the cell wall composition of fungi are complex and can show great variation, even between related species (Bowman and Free 2006). Although the composition and structure of the *H. omanensis* and *C. albifundus* conidial cell walls has not been studied, these results could indicate a marked difference between the two species.

Many modifications were made to the PEG/$CaCl_2$-protoplast transformation protocol in an attempt to transform *C. albifundus,* although all these proved unsuccessful. Routine adjustments commonly made to the PEG concentration and the quantity of buffered PEG solution (PTC), as well as the amount of DNA added and the number of protoplasts used have all been shown to affect the transformation process (Fincham 1989; Li *et al.* 2017). Additionally, the inclusion of DMSO and KCl-Tris buffer to PTC has been reported to improve the efficacy of transformation (Fincham 1989). While most species require up to ten volumes of PTC for transformation, the use of five and ten volumes of PTC in *C. albifundus* did not result in a successful transformation. In *Aspergillus nidulans*, higher amounts of DNA used for transformation resulted in more transformants (Yelton *et al.* 1984). Usually 5 to 20 µg of plasmid DNA is sufficient (Fincham 1989; Li *et al.* 2017), however, even using 25 µg of pC-HYG-GFP did not yield any *C. albifundus* transformants. DMSO is commonly added in *Neurospora* PEG/$CaCl_2$-protoplast transformations (Yelton *et al.* 1984; Fincham 1989), while *Aspergillus nidulans* protocols supplement PTC with KCl-Tris buffer (Ballance *et al.* 1983; Ballance and Turner 1985). Neither DMSO nor KCl-Tris, added alone or together to the PTC, assisted in the transformation process for *C. albifundus*. To compensate for the slow growth rate of *C. albifundus* (reported as 2.2 – 5.6 cm of radial growth in 2 weeks on malt yeast extract agar; Lee *et al.* 2015), the recovery period was extended from an overnight incubation to two days. This would allow more time for the protoplasts to regenerate before being

subjected to hygromycin selection. These modifications still yielded no transformants, although the survival and regeneration of the protoplasts were confirmed on the control plates.

Based on the lack of successful transformations in this study, some modifications can be suggested to obtain transformants for future studies. Typically, the concentration of protoplasts used in most filamentous ascomycetes is between 10^8 and 10^9 protoplasts per ml (Fincham 1989), which is significantly higher than the amounts we were able to obtain. Although different protoplast concentrations were tested here, the number of protoplasts produced remained low, possibly due to a low number of conidia obtained from growing cultures. Future attempts at PEG/$CaCl_2$-protoplast transformation of *C. albifundus* could look at optimising the number of protoplasts produced by isolating more conidia for digestion. PEG of lower molecular weight is also thought to be more efficient than high molecular weight (Li *et al.* 2017), and so the use of PEG3000 instead of PEG4000 might be beneficial. Finally, the incubation period with the DNA could be increased to 30 min to ensure ample time for the DNA to adhere to the cell membrane of the protoplasts before the PTC is added.

The *C. albifundus* protoplasts produced here can act as the starting material for other studies. Many other transformation methods make use of protoplasts, including electroporation and liposome-mediated transformation. Although the use of intact cells has been successful in electroporation (Ozeki *et al.* 1994; Jin *et al.* 2008; Timofeev *et al.* 2019), most species have been recalcitrant (Ward *et al.* 1989; Goldman *et al.* 1990), including *C. albifundus* (current study, data not shown). Additionally, the PEG/$CaCl_2$-protoplast transformation system could be coupled with a CRISPR/Cas9 system to address the possibility that the plasmid was transferred but was not integrated into the genome. The Cas9 enzyme will create double-stranded breaks in the genome of the host, triggering the repair systems by either non-homologous end joining or homologous recombination repair (Deng *et al.* 2017). In this way, CRISPR/Cas9 may induce the integration of the exogenous DNA into the host chromosome, increasing the chances that transformants are produced and minimizing the potential for DNA loss after entry into the cells. Protoplasts are also required for pulse field gel electrophoresis to determine the chromosomal number of a fungus (Orbach *et al.* 1988; van Dam *et al.* 2017), something that has not been studied in *C. albifundus*. The protoplasts are lysed, allowing intact chromosomal DNA to be released and isolated before being run on a CHEF gel to separate the large molecules. Therefore, the ability to successfully produce protoplasts from *C. albifundus* would be beneficial to future studies that will explore different technologies not tested here.

References

Al-Jaaidi, S. S., 2007 Transformation of *Thielaviopsis basicola* to study host-pathogen interactions, pp. 73–122, University of New England, Armidale, Australia, PhD thesis.

Ballance, D. J., F. P. Buxton, and G. Turner, 1983 Transformation of *Aspergillus nidulans* by the orotidine-5'-phosphate decarboxylase gene of *Neurospora crassa*. Biochem. Biophys. Res. Commun. 112: 284–289.

Ballance, D. J., and G. Turner, 1985 Development of a high-frequency transforming vector for *Aspergillus nidulans*. Gene 36: 321–331.

Barker, K., 2005 Chapter 12 DNA, RNA, and protein, pp. 279–312 in *At the Bench A Laboratory Navigator*, Cold Spring Harbor Laboratory Press, New York.

Bowman, S. M., and S. J. Free, 2006 The structure and synthesis of the fungal cell wall. BioEssays 28: 799–808.

Cairns, T. C., C. Nai, and V. Meyer, 2018 How a fungus shapes biotechnology: 100 years of *Aspergillus niger* research. Fungal Biol. Biotechnol. 5: 13.

Case, M. E., M. Schweizer, S. R. Kushner, and N. H. Giles, 1979 Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc. Natl. Acad. Sci. 76: 5259–5263.

Chang, A. Y., V. W. Chau, J. A. Landas, and Y. Pang, 2017 Preparation of calcium competent *Escherichia coli* and heat-shock transformation. JEMI Methods 1: 22–25.

de Beer, Z. W., T. A. Duong, I. Barnes, B. D. Wingfield, and M. J. Wingfield, 2014 Redefining *Ceratocystis* and allied genera. Stud. Mycol. 79: 187–219.

Deng, H., R. Gao, X. Liao, and Y. Cai, 2017 CRISPR system in filamentous fungi: current achievements and future directions. Gene 627: 212–221.

Díaz-Trujillo, C., A. K. Kobayashi, M. Souza, P. Chong, H. J. G. Meijer *et al.*, 2018 Targeted and random genetic modification of the black Sigatoka pathogen *Pseudocercospora fijiensis* by *Agrobacterium tumefaciens*-mediated transformation. J. Microbiol. Methods 148: 127–137.

Fincham, J. R., 1989 Transformation in fungi. Microbiol. Rev. 53: 148–170.

Goldman, G. H., M. Van Montagu, and A. Herrera-Estrella, 1990 Transformation of *Trichoderma harzianum* by high-voltage electric pulse. Curr. Genet. 17: 169–174.

Hellens, R., P. Mullineaux, H. Klee, 2000 A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci. 5: 446–451.

Hooykaas, P. J. J., G. P. H. van Heusden, X. Niu, M. R. Roushan, J. Soltani *et al.*, 2018 *Agrobacterium*-mediated transformation of yeast and fungi, pp. 349–374 in Agrobacterium *Biology. Current Topics in Microbiology and Immunology, vol 418*, edited by S. B. Gelvin, Springer, Cham.

Jin, K., Y. Zhang, Z. Luo, Y. Xiao, Y. Fan *et al.*, 2008 An improved method for *Beauveria bassiana* transformation using phosphinothricin acetlytransferase and green fluorescent protein fusion gene as a selectable and visible marker. Biotechnol. Lett. 30: 1379–1383.

Kück, U., and B. Hoff, 2010 New tools for the genetic manipulation of filamentous fungi. Appl. Microbiol. Biotechnol. 86: 51–62.

Lee, D. H., J. Roux, B. D. Wingfield, and M. J. Wingfield, 2015 Variation in growth rates and aggressiveness of naturally occurring self-fertile and self-sterile isolates of the wilt pathogen *Ceratocystis albifundus*. Plant Pathol. 64: 1103–1109.

Li, D., Y. Tang, J. Lin, and W. Cai, 2017 Methods for genetic transformation of filamentous fungi. Microb. Cell Fact. 16: 1–13.

Liu, F., G. Li, J. Roux, I. Barnes, A. M. Wilson *et al.*, 2018 Nine novel species of *Huntiella* from southern China with three distinct mating strategies and variable levels of pathogenicity. Mycologia 110: 1145–1171.

Liu, L., Y. R. Cao, C. C. Zhang, H. F. Fan, Z. Y. Guo *et al.*, 2019 An efficient gene disruption system for the nematophagous fungus *Purpureocillium lavendulum*. Fungal Biol. 123: 274–282.

Long, L., Q. Lin, Y. Shi, J. Wang, and S. Ding, 2018 Highly efficient transformation of a (hemi-) cellulases-producing fungus *Eupenicillium parvum* 4–14 by *Agrobacterium tumefaciens*. J. Microbiol. Methods 146: 40–45.

Loppnau, P., P. Tanguay, and C. Breuil, 2004 Isolation and disruption of the melanin pathway polyketide synthase gene of the softwood deep stain fungus *Ceratocystis resinifera*. Fungal Genet. Biol. 41: 33–41.

Michielse, C. B., P. J. J. Hooykaas, C. A. M. J. J. van den Hondel, and A. F. J. Ram, 2005 *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr. Genet. 48: 1–17.

Moradi, S., F. Sanjarian, N. Safaie, A. Mousavi, and G. R. Bakhshi Khaniki, 2013 A modified method for transformation of *Fusarium graminearum*. J. Crop Prot. 2: 297–304.

Niu, X., M. Pei, C. Liang, Y. Lv, X. Wu *et al.*, 2019 Genetic transformation and green fluorescent protein labeling in *Ceratocystis paradoxa* from coconut. Int. J. Mol. Sci. 20: 2387.

Olmedo-Monfil, V., C. Cortés-Penagos, and A. Herrera-Estrella, 2004 Three decades of fungal transformation, pp. 297–313 in *Recombinant Gene Expression. Methods in Molecular Biology*, edited by P. Balbás, and A. Lorence, Humana Press, Totowa.

Orbach, M. J., D. Vollrath, R. W. Davis, and C. Yanofsky, 1988 An electrophoretic karyotype of *Neurospora crassa*. Mol. Cell. Biol. 8: 1469–1473.

Ozeki, K., F. Kyoya, K. Hizume, A. Kanda, M. Hamachi *et al.*, 1994 Transformation of intact *Aspergillus niger* by electroporation. Biosci. Biotechnol. Biochem. 58: 2224–2227.

Roche, C. M., J. J. Loros, K. McCluskey, and N. L. Glass, 2014 *Neurospora crassa*: looking back and looking forward at a model microbe. Am. J. Bot. 101: 2022–2035.

Roux, B. J., R. N. Heath, L. Labuschagne, G. K. Nkuekam, and M. J. Wingfield, 2007 Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus* on native South African trees. For. Pathol. 37: 292–302.

Sayari, M., M. A. van der Nest, E. T. Steenkamp, O. O. Adegeye, S. Marincowitz *et al.*, 2019 *Agrobacterium*-mediated transformation of *Ceratocystis albifundus*. Microbiol. Res. 226: 55–64.

Sweigard, J. A., F. G. Chumley, and B. Valent, 1992 Cloning and analysis of *CUT1*, a cutinase gene from *Magnaporthe grisea*. MGG Mol. Gen. Genet. 232: 174–182.

Timofeev, S., A. Tsarev, I. Senderskiy, E. Rogozhin, G. Mitina *et al.*, 2019 Efficient transformation of the entomopathogenic fungus *Lecanicillium muscarium* by electroporation of germinated conidia. Mycoscience 60: 197–200.

Tzima, A. K., E. J. Paplomatas, C. Schoina, E. Domazakis, S. Kang *et al.*, 2014 Successful *Agrobacterium* mediated transformation of *Thielaviopsis basicola* by optimizing multiple conditions. Fungal Biol. 118: 675–682.

van Dam, P., L. Fokkens, Y. Ayukawa, M. van der Gragt, A. ter Horst *et al.*, 2017 A mobile pathogenicity chromosome in *Fusarium oxysporum* for infection of multiple cucurbit species. Sci. Rep. 7: 9042.

Ward, M., K. H. Kodama, and L. J. Wilson, 1989 Transformation of *Aspergillus awamori* and *A. niger* by electroporation. Exp. Mycol. 13: 289–293.

Weyda, I., L. Yang, J. Vang, B. K. Ahring, M. Lübeck *et al.*, 2017 A comparison of *Agrobacterium*-mediated transformation and protoplast-mediated transformation with CRISPR-Cas9 and bipartite gene targeting substrates, as effective gene targeting tools for *Aspergillus carbonarius*. J. Microbiol. Methods 135: 26–34.

Wilson, A. M., P. M. Wilken, M. A. van der Nest, M. J. Wingfield, and B. D. Wingfield, 2019 The novel *H. omanensis* mating gene, *MAT1-2-7*, is essential for ascomatal maturation. Fungal Genet. Biol. Submitted.

Wingfield, M. J., C. de Beer, C. Visser, and B. D. Wingfield, 1996 A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. Syst. Appl. Microbiol. 19: 191–202.

Yelton, M. M., J. E. Hamer, and W. E. Timberlake, 1984 Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc. Natl. Acad. Sci. 81: 1470–1474.

Yu, J. H., Z. Hamari, K. H. Han, J. A. Seo, Y. Reyes-Domínguez *et al.*, 2004 Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. 41: 973–981.

Tables and figures

Table 1: Parameters tested for the transformation of *Ceratocystis albifundus* using a PEG/$CaCl_2$-protoplast transformation system

Amount of plasmid DNA (ng)	Protoplast concentration (protoplasts/ml)	Protoplast suspension used (µl)	PTC supplements	Amount of PTC used (ml)	Recovery incubation period (days)
15	1.6×10^7	200	-	2	1
18	1.6×10^7	200	-	2	1
20	1.6×10^7	200	-	2	1
25	1.6×10^7	200	-	2	1
25	4.975×10^7	400	-	1	1
25	4.975×10^7	200	-	1	1
25	4.975×10^7	200	-	1	1
25	4.975×10^7	200	DMSO	1	1
25	4.975×10^7	200	DMSO	1	2
25	4.975×10^7	200	KCl-Tris	1	1
25	4.975×10^7	200	KCl-Tris	1	2
25	4.975×10^7	200	DMSO + KCl-Tris	1	1
25	4.975×10^7	200	DMSO + KCl-Tris	1	2

Figure 1: Gel electrophoresis of plasmid DNA extractions from *A. tumefaciens* and *E. coli*. **A** Both the helper and Ti plasmid pC-HYG-GFP extracted from *A. tumefaciens*. **B – E** The pC-HYG-GFP plasmid that was isolated from the *E. coli* after transformation with the plasmid isolation seen in **A**.

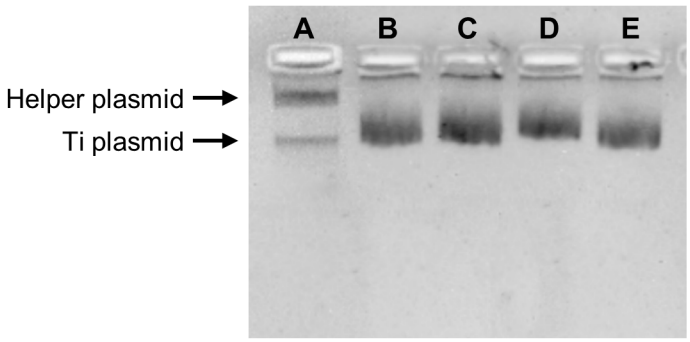
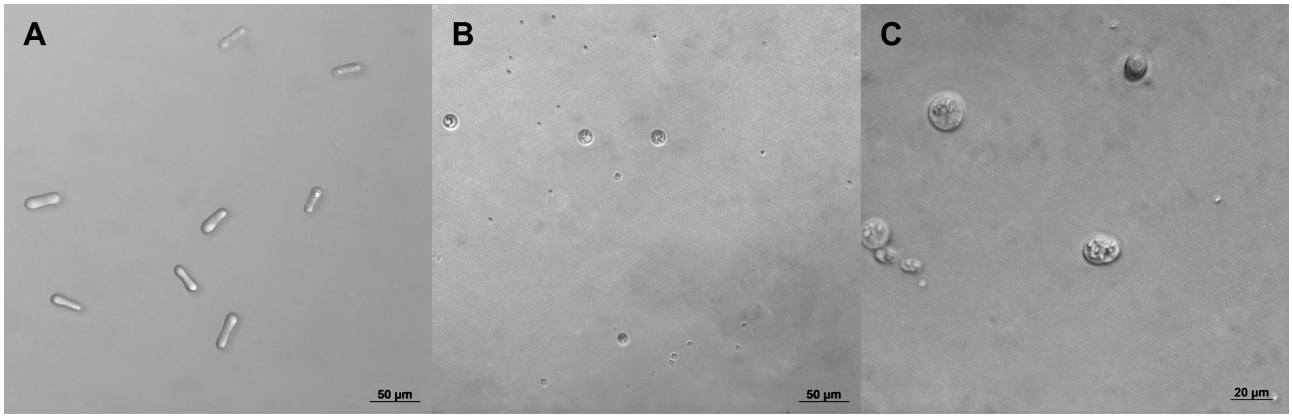


Figure 2: Microscope photographs of the conidia and protoplasts of *Ceratocystis albifundus*. **A** The ungerminated conidia before enzyme digestion. **B** & **C** Protoplast produced from conidia through enzyme digestion.



Summary

The usefulness of transformation systems in fungal pathogens has been well described. Although functional studies in the Ceratocystidaceae is lagging behind model fungi from *Aspergillus* and *Neurospora*, the economic impact of many species in this family on both the agricultural and forestry industries speaks to the need to address this. Developing reliable transformation systems is the first step towards functionally studying various aspects related to the biology of these important fungi, including the systems underlying pathogenicity. This dissertation aimed to address this need for transformation systems by using a two-pronged approach. The first explored the viability of transferring an *Agrobacterium*-mediated transformation system, that has been established in *Ceratocystis albifundus*, across species and generic boundaries. During the course of this study, the existing protocol was successfully optimised to improve the yield of transformants from *C. albifundus*. When this protocol was applied as is to eight other species representing eight Ceratocystidaceae genera, successful transformation was possible in six of the species representing six genera. This finding indicates that the technology is highly transferable, even among distant relatives from the same family. It should be noted that the transformation efficiency was markedly lower in these species when compared to *C. albifundus*. Several suggestions were made for adjustments that should improve these efficiencies for future studies. The current study produced a small collection of random transformants expressing green fluorescent protein, making them potentially useful for studies looking at the infection pathway of these pathogens. Although the *Agrobacterium* system is effective for transformations in *C. albifundus*, this approach requires the use of the Ti plasmid which necessitates multiple cloning steps when doing targeted mutagenesis. As a means of addressing these expensive and time-consuming cloning steps, the second approach was an attempt to establish a PEG/$CaCl_2$-protoplast transformation protocol in this species. This system can make use of linear DNA, such as PCR products, making it ideal for high-throughput gene editing. The use of a PEG/$CaCl_2$-protoplast transformation system relies on the successful production of viable protoplasts, and a protocol was created to produce these from *C. albifundus* conidia. When these protoplasts were used for transformation with PEG/$CaCl_2$-protoplast protocols, no transformants were obtained in spite of the many alterations that were made to various parts of the protocol. However, the study still provides a tangible starting point for future attempts at optimizing the system. Additionally, the ability to produce protoplasts from *C. albifundus* will allow other transformation systems to be tested that also depend on the use of these specialized cells. Protoplasts are also useful beyond transformation and could be used to determine the chromosome number in this important pathogen, something that has previously not been possible. The work presented in this dissertation provides a benchmark for the transformation of other Ceratocystidaceae species that has not yet been investigated. It is envisioned that the findings and technologies presented here may serve to initiate future studies characterising gene function in these globally important plant pathogens.