CHAPTER 2

Parameters Affecting the Efficiency of *Agrobacterium tumefaciens*-Mediated Transformation of *Colletotrichum graminicola*

Introduction

The goal of work described in Chapters 2 and 3 of my dissertation was to develop a transformation system for *Diplodia pinea* that would allow introduction into the genome of a foreign (jellyfish) gene encoding a protein that fluoresces green when exposed to UV light. This would create a "marked" transgenic strain of *D. pinea* that could be used for inoculation studies in the greenhouse in order to investigate colonization (both latent and pathogenic) of host tissues. The GFP-marked strain could be differentiated from any endophytic or latent fungal inhabitant of the tissues, and could be easily visualized under an epifluorescence or confocal microscope without the need for fixation, thin-sectioning, or other manipulations. Initially, standard PEG-mediated methods for transformation were attempted, but without success. These are described in chapter 3. Later, my focus shifted to a newer method for transformation involving the crown gall bacterium *Agrobacterium tumefaciens*. *A. tumefaciens*-mediated transformation (ATMT) has sometimes been described in the literature as a method that could work for transformation of recalcitrant fungi, when more traditional methods had failed. Parameters for ATMT were first tested by using a different filamentous fungus, *Colletotrichum graminicola*, which is a pathogen of corn. This seemed wise since *D. pinea* has never been subjected to molecular manipulations, while *C. graminicola* had been the subject of molecular studies for many years in our laboratory, and so numerous molecular tools and protocols were already available for it. Optimized parameters for ATMT of *C. graminicola* are presented in this chapter of my dissertation. This work is significant because ATMT had not been used for this fungal species before, and furthermore very few investigations of the effect of experimental parameters on transformation efficiency in fungi have been published. Because of this, this chapter has been written up separately and submitted for publication. Attempts to apply the same methods to *D. pinea* are presented in Chapter 3. Although ultimately ATMT failed to

produce GFP-transformants of *D. pinea*, the work presented in Chapter 3 serves as a useful foundation for future attempts to transform the fungus by this or other means.

The green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* was cloned in 1992 (Prasher 1992) and has revolutionized microscopy studies in many fields, including studies of host/pathogen interactions (Lorang et al. 2001). Unlike other reporter genes, such as GUS, that typically require killing and fixing the sample tissue for detection of expression, GFP fluoresces in living tissues in the presence of UV or blue light and oxygen. GFP facilitates continuous observations of fungal pathogens *in vivo* (Lorang et al. 2001). Mutant forms of the GFP protein have removed disadvantages of the wild type protein for microscopy studies, including improper folding at high temperatures, and photobleaching (Cubitt et al. 1995; Lippincott-Schwartz and Patterson 2003). The mutant SGFP protein (serine to threonine substitution at amino acid 65) has become popular because of the particularly strong fluorescence it confers to filamentous fungi (Fernandez-Abalos et al. 1998). SGFP produces a "red shift" in excitation, with a maximum of 488 nm (Lorang et al. 2001). This makes SGFP ideal for confocal or light microscopy studies of pathogenic fungi *in planta*.

GFP has been utilized for many host-fungal pathogen interaction studies. Some common applications of GFP include using strains expressing the protein constitutively throughout the cytoplasm to examine fungal infection and colonization habits *in planta* (Maor et al. 1998; Gold et al. 2001; Lorang et al. 2001; Lee et al. 2002) and studying the expression of GFP-fusions with various plant and fungal genes during pathogenic interactions (Lorang et al. 2001; Lippincott-Schwartz and Patterson 2003). Recently, several reports have used GFP-expressing pathogens to provide new insights into some very important diseases of trees, including Dutch elm disease and pitch canker (Royer et al. 1991; Wang et al. 1999; Balint-Kurti et al. 2001; Covert et al. 2001). One of the greatest advantages of using GFP-expressing pathogens for tree pathology is that the pathogen can be distinguished from the numerous other epiphytic and endophytic fungi that are typically present in tree tissues (Chapela 1989; Farr et al. 1989; Carroll 1995; Lee et al. 2002; Flowers et al. 2003).

Molecular transformation is a process in which foreign DNA is introduced, replicated, and expressed in a recipient cell. Transformation systems have been

developed for many prokaryotes and eukaryotes, including filamentous fungi. The most commonly used methods for transformation of filamentous fungi have been polyethylene glycol (PEG)-mediated transformation, and ATMT. Other methods, including electroporation and biolistic bombardment have also been successful, although these often result in lower transformation efficiencies (Timberlake and Marshall 1989; Gold et al. 2001; Mullins and Kang 2001). PEG-mediated transformation systems require the production of fungal protoplasts by removal of the fungal cell walls with a cocktail of cell-wall degrading enzymes. Production of viable, competent protoplasts has been considered to be a major obstacle in PEG-mediated protocols, in large part because different batches of commercially-available cell-wall degrading enzymes tend to be inconsistent in their performance (Timberlake and Marshall 1989; Gold et al. 2001). Traditional PEG-mediated transformation techniques also do not seem to work for all filamentous fungi. Thus, some fungi, including *Calonectria morganii* (Malonek and Meinhardt 2001), and *Fusarium circinatum* (Covert et al. 2001) have only been successfully transformed with the newer ATMT approach. Other advantages of ATMT include generally higher transformation efficiencies, and the ability to transform a variety of fungal tissues other than protoplasts (de Groot 1998; Gold et al. 2001; Mullins and Kang 2001).

I developed an ATMT protocol for the plant pathogenic fungus *Colletotrichum graminicola*, the cause of anthracnose leaf blight and stalk rot of corn. ATMT results in higher transformation efficiencies than previously available PEG-mediated protocols, and falcate spores can be used instead of protoplasts. Various experimental parameters were tested for their effects on transformation efficiencies. The parameters with the greatest influence were the *A. tumefaciens* strain used and the Ti-plasmid it carried, the ratio of bacterium to fungus during co-cultivation, and the length of co-cultivation. Southern analysis demonstrated that most transformants (80%) contained tandem integrations of plasmid sequences, and at least 36% had integrations at multiple sites in the genome. In a majority of cases (70%), the whole Ti-plasmid, and not just the T-DNA, had integrated as a series of tandem repeats. This surprising result suggests that ATMT may occur by a different mechanism in fungi than in plants, where only the T-DNA becomes integrated. Tandem integrations, especially of the whole plasmid, make it difficult to rescue DNA

from both flanks of the integrations with standard PCR-based approaches. Thus, ATMT may be unsuitable for insertional mutagenesis of *C. graminicola* without further modification.

Colletotrichum graminicola (Ces.) G.W. Wils. (telomorph, *Glomerella graminicola*) is the causal agent of anthracnose stalk rot (ASR) and anthracnose leaf blight (ALB) of corn. Although epidemics were reported in many parts of the world during the 1950s and 1960s, anthracnose was not considered important in the United States until the early 1970's, when severe outbreaks of ALB and ASR destroyed many acres of corn (Warren et al. 1973; Hooker and White 1976). With the elimination of highly susceptible germplasm, widespread anthracnose epidemics have not recurred. However, corn breeders and growers must remain vigilant because anthracnose is still very common, and causes yield losses annually in the United States and worldwide (Byrnes and Carroll 1986; Anderson and White 1987; Bergstrom and Nicholson 1999).

C. graminicola is a member of an experimentally tractable genus and has been extensively investigated as a model plant pathogen (Perfect et al. 1999; O'Connell et al. 2004). Most *Colletotrichum* fungi have an interesting hemibiotrophic habit, in which the pathogen initially colonizes the host biotrophically before switching to a necrotrophic phase (Perfect et al. 1999). During infection and colonization, *Colletotrichum* fungi produce highly specialized infection structures including appressoria, penetration pegs, and primary (biotrophic) and secondary (necrotrophic) hyphae (Bergstrom and Nicholson 1999; Perfect et al. 1999). The ability to introduce foreign DNA (transformation) is an important tool for a model fungus. *C. graminicola* can be transformed by a PEG-mediated strategy, and PEG-mediated transformation has been used to produce targeted gene disruptions (Vaillancourt and Hanau 1994; Fang et al. 2002) and collections of restriction-enzyme mediated random insertional mutants (Epstein et al. 1998; Thon et al. 2000).

A. tumefaciens, which causes crown gall disease on many dicotyledonous plants, has become an essential tool for plant research. In 1998, de Groot *et al.,* established a protocol for ATMT of filamentous fungi. Whereas only protoplasts can be transformed using PEG, a major advantage of ATMT is that various fungal tissues, including protoplasts, conidia, hyphal fragments, and basidiocarp pieces, can be successfully

transformed (de Groot 1998). ATMT can be used to produce targeted gene disruptions and random insertional mutations, often at efficiencies significantly higher than those achieved with PEG-mediated transformation (de Groot 1998; Mullins et al. 2001; Dobinson et al. 2004).

I have developed an ATMT protocol for *C. graminicola*. Although ATMT has been used previously to transform two other *Colletotrichum* species (*C. gloeosporioides* and *C. higginsianum*) (de Groot 1998; O'Connell et al. 2004), I report for the first time the effect of specific experimental parameters on transformation efficiency for a member of this genus. The ATMT protocol described here has important advantages over previously available PEG-mediated methods for transformation of *C. graminicola,* including much higher transformation efficiencies and the ability to use falcate spores instead of protoplasts. I produced a novel Ti plasmid that confers hygromycin B resistance and green fluorescent protein (GFP) expression for this work, and a new *A. tumefaciens* strain was found to be capable of transforming filamentous fungi. Analysis of the integration events in a sample of transformants demonstrated that the current protocol, while satisfactory for most applications, might be less suitable for production of random insertional mutations due to potential difficulties with rescuing DNA flanking plasmid integration sites.

Materials and Methods

Fungal cultures and production of spore suspensions: *C. graminicola* wild-type strain M1.001 (also known as M2) (Forgey et al. 1978) was grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) for between 10 and 15 days at 23˚C in continuous light. Falcate spore suspensions were prepared by adding 1 ml of sterile distilled water to the culture and gently rubbing the surface with a sterile pestle. The suspensions were recovered from the plates, filtered through several layers of sterile cheesecloth, washed 3 times in sterile distilled water, and adjusted to a concentration of $1X10^6$ spores per ml for immediate use in transformation experiments.

Construction of plasmid pJF1: The *Sal* I/*Eco* RI fragment from pCT74 (Lorang et al. 2001) containing the *sgfp* gene under the regulation of the *ToxA* promoter and the *nos*-

terminator was ligated into the multiple cloning site of pBHt2 (Mullins et al. 2001), resulting in pJF1 (FIGURE 2.1). pJF1 was cloned in *E. coli* strain DH5α and purified using the Qiagen mini-prep kit (Qiagen Sciences, Maryland). The region between the T-borders was sequenced by primer walking using the Big Dye terminator sequencing kit (ABI Prism, Warrington, WA).

Fungal transformation: All transformation experiments were done according to the following protocol unless otherwise noted. *A. tumefaciens* strain AGL-1 (provided by Dr. P. Romaine, The Pennsylvania State University), containing either pBin-GFP-hph (O'Connell et al. 2004), or pJF1, was grown in 5 ml minimal media (MM) (Hooykaas et al. 1979) supplemented with 50 μg/ml kanamycin 9 with shaking at 250 rpm for 2 days at 28˚C. Two hundred microliters of this culture were transferred to 5 ml of initiation media (IM) (Bundock et al. 1995) containing 200 μM acetosyringone (AS; Aldrich), and incubated for 6-7 hours with shaking at 250 rpm at 28˚C until OD_{660}= 0.28-0.35. *C. graminicola* spores (100 μl, containing $1X10^5$ spores) were mixed with an equal volume of the IM *Agrobacterium* culture and the entire volume was spread onto sterile nitrocellulose membranes (Whatman, Hillsboro, OR) that were placed on IM co-cultivation agar (Mullins et al. 2001) amended with 200 μM AS. Following co-cultivation under ambient laboratory conditions for 3 days, the membranes were transferred to potato dextrose agar selection media supplemented with 200 μg/ml cefotaxime, 250 μg/ml carbenicillin, and 250 μg/ml hygromycin B. Transformants were transferred after 5-10 days to PDA supplemented with 250 μg/ml hygromycin B. In all experiments, *C. graminicola* spores co-cultivated with uninoculated IM were included as a negative control.

Analysis of transformant stability: Fifty individual randomly-selected transformants (25 transformed with pJF1, and 25 with pBin-GFP-hph) were single-spored and subcultured 8 times on PDA without hygromycin B before transfer to PDA supplemented with 250 μg/ml hygromycin B. Transformants containing either pBin-GFP-hph or pJF1 were observed for GFP expression using epifluorescence microscopy. Twenty spores from each of 3 transformants growing on non-selective medium were collected and germinated

on PDA. After 1 day of growth, these germlings were transferred to PDA selection media (250 μg/ml hygromycin B). Germling growth on selective medium and GFP expression was observed.

DNA extraction and Southern blot analysis: Thirty-three individual, randomly selected single-spored transformants (25 transformed with pJF1, and 8 with pBin-GFP-hph) were grown for 6-7 days in potato dextrose broth standing cultures (PDB; Difco Laboratories, Detroit, MI, USA) at 23˚C under continuous light. Mycelia were collected, frozen, and lyophilized before genomic DNA was extracted according to the protocol of Thon et al. (2000). The products that resulted from digestion of 1 μg DNA with *Bam*HI, *Hind*III, *Xmn*I, or *Sma*I were separated by electrophoresis on a 0.8% agarose gel. The DNA was transferred to a positively charged nylon membrane (Roche Diagnostics Cooperation, Indianapolis, Indiana) according to the Turboblotter protocol (Schleicher and Schuell, Germany). Prehybridization, hybridization, and high stringency washes at 65˚C were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Germany). A 544 bp PCR product was amplified from the hph gene in pCT74 using the following primers: hphFOR (5'-gctgcgccgatggtttctaca-3') and hphREV (5'-gcgcgtctgctgctccat-3'). The amplified DNA fragment was purified with the Qiagen PCR purification kit (Qiagen Sciences, Maryland) and labeled with digoxigenin according to the DIG High Prime DNA Labeling and Detection Starter Kit II protocol for use as a probe in the Southern experiments.

Calculations of A. tumefaciens culture density: MM cultures of *A. tumefaciens* strains C58C1 and AGL-1, containing either pBin-GFP-hph or pJF1, were shaken at 250 rpm for 2 days at 29˚C. One hundred and fifty microliters of this culture were then transferred to IM supplemented with 200 μM AS and shaken at 250 rpm at 29˚C for 6 h. OD_{660} readings were taken, and dilutions were spread on Luria-Bertani agar (LB, Difco, Sparks, MD) supplemented with 50 μg/ml kanamycin. Colonies were counted after the plates had been incubated for 2 days at 29˚C.

Results

Effects of experimental parameters on transformation efficiency: As is typical for transformation protocols, the absolute number of transformants produced with a given set of parameters varied among experimental replications in this study. Nonetheless, I was able to conclude that some parameters had a reproducible effect on transformation efficiencies after observing trends in multiple experiments.

Transformation efficiencies resulting from the use of *A. tumefaciens* strain C58C1 (provided by Dr. M. Goodin, University of Kentucky) versus AGL-1 were compared. Regardless of which plasmid it contained, *A. tumefaciens* strain AGL-1 consistently produced more *C. graminicola* transformants than strain C58C1 (TABLE 2.1). The particular combination of bacterial strain and plasmid was also important, in that *A. tumefaciens* strain AGL-1 containing plasmid pBin-GFP-hph consistently gave higher transformation efficiencies than other combinations, while strain C58C1 containing plasmid pJF1 consistently gave lower efficiencies. I tested the possibility that this difference could be related to differences in the culture densities reached by the various bacterial strains during initial IM cultivation. I found that strain C58C1 transformed with pBin-GFP-hph produced much higher CFUs per OD unit than the other combinations, which were all similar to one another (TABLE 2.2). Thus, although there were differences in culture density, they did not correlate with the variation I saw in transformation efficiency among strains.

To test the effect on transformation efficiency of the ratio of bacterium to fungus during co-cultivation, 50, 100, 200, or 300 μl of *A. tumefaciens* IM culture were co-cultivated with 100 μl (1×10^5) of *C. graminicola* spores. The difference in volume in each case was made up with uninoculated IM medium. Transformation efficiency was consistently highest at the highest ratio of 3 times the volume of *A. tumefaciens* culture to *C. graminicola* (FIGURE 2.2).

C. graminicola spores were co-cultivated with *A. tumefaciens* cells for 2 or 5 days before transfer to selection media. Transformation efficiency was consistently increased after a longer co-cultivation (TABLE 2.1).

Transformation efficiencies were compared in experiments in which AS was omitted from the liquid IM and the IM co-cultivation media. In agreement with most

previous studies (Gouka et al. 1999; Malonek and Meinhardt 2001), inclusion of AS in the IM media was essential for transformation of *C. graminicola* (data not shown).

Other experimental parameters that were tested appeared to have relatively little effect on transformation efficiency.

Nylon membranes (Roche Chemicals, Mannheim, Germany) were tested as an alternative to nitrocellulose membranes for co-cultivation. *C. graminicola* transformants were recovered from both the nitrocellulose and nylon membranes, and no significant difference in transformation efficiency was observed (TABLE 2.3).

The standard IM co-cultivation agar recipe contains 5 mM glucose. I tested other glucose concentrations including 0, 1.25, and 2.5 mM glucose. The concentration of glucose in the IM co-cultivation agar appeared to have no consistent effect on transformation efficiency (FIGURE 2.3).

Transformant stability: A total of 50 individual single-spored transformants retained hygromycin B resistance and GFP expression after subculturing them 8 times on PDA in the absence of antibiotic selection. Twenty germlings collected from 3 of these subcultured transformants all grew when transferred onto selection media, and retained GFP expression. These results demonstrate that the ATMT transformants are mitotically stable.

Characterization of integration events: To determine the types of integrations that had occurred, 33 individual single-spored transformants (25 transformed with pJF1 and 8 with pBin-GFP-hph) were analyzed by Southern hybridization (FIGURE 2.4). The pJF1 transformant DNA was digested separately with *Hind*III and *Xmn*I. Each of these restriction enzymes cuts once just inside the T-borders, at opposite ends of the T-DNA (FIGURE 2.1). Lane 2 in Figure 2.4 is an example of a transformant containing multiple head-to-tail tandem insertions of the T-DNA. A 3.6 Kb band, the same size as the T-DNA, is visible in both digestions. Analysis of the pJF1 transformants indicated that 30% had tandem integrations of the T-DNA. Lane 3 in Figure 2.4 is an example of integration of Ti-plasmid sequences into multiple sites in the genome. A single 3.6 Kb band is visible, indicating that at least one integration consists of tandem repeats of the T-DNA,

and there are three additional bands in the *Xmn*I digest. Two of these could represent DNA flanking the tandem insertion, but the third must represent an independent insertion of plasmid sequences. At least 36% of the pJF1 transformants contained insertions at multiple sites (it is not possible to be more precise because if only one or two faint additional bands were seen they could be interpreted as either flanking DNA or independent insertions). Lane 4 in Figure 2.4 is an example of a transformant in which T-DNA sequences appear to have integrated as a single copy at a single site. Only 16% of the pJF1 transformants displayed this pattern. Lane 8 in Figure 2.4 is an example of a transformant in which the entire Ti-plasmid appears to have integrated as a series of tandem duplications. A 10 Kb band, the same size as the linearized plasmid, is visible in both digestions. Surprisingly, a large majority (70%) of the pJF1 transformants exhibited banding patterns like this. Analysis of 8 pBin-GFP-hph transformants demonstrated that the same types of integrations seen in the pJF1 transformants were also represented there, including tandem integration of the entire plasmid in five out of eight transformants (73%) (data not shown). Thus, the type of integration obtained doesn't appear to depend on the plasmid used.

Discussion

PEG-mediated protoplast transformation protocols have been published previously for *C. graminicola* (Panaccione et al. 1988; Epstein et al. 1998; Thon et al. 2000), but the ATMT protocol I have developed improves upon those methods in two important ways: first, I can obtain much higher transformation efficiencies than with the PEG-mediated methods; and second, it is possible to use falcate spores rather than protoplasts for transformation. This is a significant advantage since protoplasts are relatively expensive and time-consuming to produce, and the lysing enzymes used for protoplast production often vary from batch to batch, affecting the reproducibility of the PEG-mediated protocol.

ATMT of two other *Colletotrichum* species has been reported previously (de Groot 1998; O'Connell et al. 2004). In these other reports, the transformation efficiency was much lower than was achieved for *C. graminicola* in my study. Thus, only 130 transformants per 10^6 conidia were reported for *C. gleosporioides* (de Groot 1998) and

250 transformants per 10^6 conidia for *C. higginsianum* (O'Connell et al. 2004). In contrast, the transformation efficiency achieved with AGL-1 transformed with pBin-GFP-hph, and my standard experimental parameters, ranged from a low of 300 to a high of 1706 transformants per 10^5 conidia. When considered on a per spore basis, this represents up to a 130-fold and a 70-fold increase, respectively, over the previous studies.

A. tumefaciens strain AGL-1 was used successfully to transform *C. graminicola*. Other fungal species that have been transformed with this strain include *Agaricus bisporus* (Chen et al. 2000), *Fusarium oxysporum* (Mullins et al. 2001), *F. circinatum* (Covert et al. 2001), *Hebeloma cylindrosporum* (Combier 2003), *Helminthosporium turcicum* (Degefu and Hanif 2003), and *Verticillium dahliae* (Dobinson et al. 2004). AGL-1 transformation efficiencies for *C. graminicola* were higher than the reported efficiencies for these other fungi.

Mine is the first use for fungal transformation of *A. tumefaciens* C58C1, a strain that is commonly used for ATMT of plants (Molnar et al. 2002). Strain C58C1 did not transform *C. graminicola* as effectively as AGL-1, even though C58C1 had equal or greater culture density. However, transformation efficiencies achieved with C58C1 in my study were still higher than those published for other *Colletotrichum* species.

More transformants were always obtained when pBin-GFP-hph versus pJF1 was used in the ATMT protocol. Rogers, *et al.* (2004) also reported that the use of different binary vectors could result in different transformation efficiencies. I hypothesized that variation in the titer of the strains carrying the various plasmids might explain this disparity, and so I calculated the culture density for the various combinations of strain and plasmid after growth to a standard OD_{660} in liquid IM. Although there was variation in the culture density, specifically for the C58C1 strain transformed with the pBin-GFP-hph plasmid, there was no obvious correlation between this variation and the transformation efficiency. In the pBin-GFP-hph plasmid, the *hph* gene is driven by the *Aspergillus nidulans gpdA* promoter, while pJF1 contains the *A. nidulans trpC* promoter. It is possible that the *gpdA* promoter gives a higher level of expression of the *hph* gene in *C. graminicola*. Further evidence in support of this idea is that pJF1 transformants appeared 1-7 days after pBin-GFP-hph transformants on selection media. Even though

transformation efficiencies were lower with pJF1 than with pBin-GFP-hph, they were still comparable to ATMT efficiencies reported for other *Colletotrichum* species.

In agreement with other reports, more *C. graminicola* transformants were obtained when a higher ratio of *A. tumefaciens* culture to fungal spores was used for co-cultivation (Mullins et al. 2001; Rho et al. 2001; Combier 2003; Meyer et al. 2003). Meyer *et al.*, 2003, found that transformation efficiency began to drop with very high *A. tumefaciens* titers, presumably due to nutrient limitations. However, I observed no decrease in transformation efficiency at the highest bacterial titer I tested, thus it is possible that further increasing the titer would improve transformation efficiency for *C. graminicola* even more.

AS interacts with glucose levels to regulate expression of *A. tumefaciens* virulence (*vir*) genes that aid in Ti-plasmid transfer (Shimoda et al. 1990). The addition of AS to IM liquid and co-cultivation media has been reported to have variable effects on transformation efficiencies in fungi. Typically, AS is essential for transformation, but in at least one case addition of AS appeared to inhibit transformation, and it has been reported to decrease the number of transformants with integrations at a single site, a particularly useful class for random mutagenesis applications (Malonek and Meinhardt 2001; Mullins et al. 2001; Combier 2003; Degefu and Hanif 2003; Leclerque et al. 2004). I found that AS in the IM was essential for successful ATMT of *C. graminicola*. On the other hand, I found that glucose levels in the medium appeared to be irrelevant.

Others have reported that more fungal transformants can be obtained with longer periods of co-cultivation prior to selection (Mullins et al. 2001; Rho et al. 2001; Combier 2003; Meyer et al. 2003; Leclerque et al. 2004; Michielse et al. 2004). In agreement with these prior studies, *C. graminicola* transformation efficiency was improved with a 5-day versus a 2-day co-cultivation period. However, a thick mycelium developed on filters after 5 days of co-cultivation, and made the observation of transformants more difficult. A 3-day co-cultivation period was chosen for subsequent experiments. This resulted in acceptable transformation efficiencies without significant background growth.

Most ATMT reports use nitrocellulose or other cellulose membranes for co-cultivation (Bundock et al. 1995; Piers et al. 1996; de Groot 1998; Gouka et al. 1999; Mullins et al. 2001; Zwiers and De Waard 2001; Zhang et al. 2003; Leclerque et al. 2004;

O'Connell et al. 2004). ATMT transformation of other fungi has also been successful using glass microfiber (Combier 2003), cellophane (Loppnau et al. 2004), filter paper (Covert et al. 2001) and nylon Hybond N-filters (Malonek and Meinhardt 2001). In my experiments, no difference in transformation efficiency was observed between nitrocellulose and nylon membranes. Nylon membranes autofluoresced more than the nitrocellulose membranes under the epifluorescence microscope, making GFP-expressing transformants more difficult to detect. On the other hand, the nylon membranes were much easier to handle because they were more flexible and less electrostatic than nitrocellulose, and thus are a better choice for selection of non-fluorescent transformants.

One popular application of ATMT is to produce a collection of random insertional mutants that can be screened for a phenotype of interest (Mullins et al. 2001; Rho et al. 2001; Rogers et al. 2004; Li et al. 2005). Ideally, the genomic DNA flanking integrated plasmid sequences can be rescued by an inverse PCR approach, and rescued portions of a mutated gene can be rapidly sequenced and identified based on similarity to DNA databases. The rescued DNA can be used directly to recreate the mutant via targeted integration. For *Agrobacterium*-mediated random mutagenesis, it is important that the majority of transformants contain an integration of the plasmid sequences at only one site in the genome, and that there be only a single copy of the integrated T-DNA so that both flanks may be easily rescued. A majority of the *C. graminicola* transformants analyzed in my study appeared to have integrations of plasmid DNA at single sites; however most of these integrations were tandem, head-to-tail insertions of plasmid sequences. It will not be possible to use inverse PCR to rescue both flanks for this type of integration. Even more troublesome is the very large number of cases in which the entire plasmid appears to have integrated as a series of tandem repeats. This observation suggests the possibility that *Agrobacterium*-mediated transformation does not occur by the same mechanism in fungi as it does in plants, where only the T-DNA is transferred into the plant genome. A similar result was described for *A. tumefaciens*-mediated transformation of *Saccharomyces cerevisiae* (Bundock et al. 1995). In transformants containing the entire plasmid, it will be difficult or impossible to use T-DNA primers to amplify and rescue flanking DNA. Because of these potential difficulties, the use of my protocol without further modifications may prove problematic for insertional mutagenesis. For other

purposes, however, the ATMT protocol presented here should be highly suitable. For example, because of its high transformation efficiency, this protocol could be particularly useful for complementation of mutant phenotypes with clone libraries.

ATMT of *C. graminicola* falcate conidia is a valuable alternative to PEG-mediated transformation of protoplasts for most transformation applications. ATMT is highly efficient, resulting in large numbers of mitotically stable transformants. Attempts to optimize parameters for *Colletotrichum* fungi have not been reported before. In my experiments, the protocol parameters that had the greatest influence on transformation efficiency were the *A. tumefaciens* strain used and the Ti-plasmid it carried, the ratio of bacterium to fungus, and the length of co-cultivation. I was surprised to find that tandem integrations of the entire plasmid, and not just the region between the T-borders, were very common, suggesting that the mechanism of integration may be different in fungi and plants. Although it is likely that further efforts will be required to optimize the protocol for production of random insertional mutants, the current protocol should be a useful addition to the repertoire of molecular tools for this important pathogenic fungus.

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Table 2.1: The effect of different co-cultivation times and of different *A. tumefaciens* strains on transformation efficiency. Total pBin-GFP-hph and pJF1 transformants per 10^5 conidia obtained with 2 and 5 day co-cultivation periods with *A. tumefaciens* strains C58C1 and AGL-1. Results from 2 replicate experiments are reported.

		2 day co-cultivation		5 day co-cultivation	
Strain	**Ti-plasmid**	**Experiment 1**	**Experiment 2**	**Experiment 1**	**Experiment 2**
AGL-1	pBin-GFP-hph	16	89	280	581
C58C1	pBin-GFP-hph	3	6	183	286
AGL-1	pJF1	1	9	18	126
C58C1	pJF1	0	0	12	58

Table 2.2: OD_{660} reading and colony forming units per ml of *A. tumefaciens* strains C58C1 and AGL-1 with Ti-plasmid pBin-GFP-hph and pJF1.

***A. tumefaciens* strain**	**Ti-plasmid**	**OD_{660} reading**	**CFU/ml (X 10^8)**
C58C1	pBin-GFP-hph	0.333	4.6
C58C1	pJF1	0.328	0.8
AGL-1	pBin-GFP-hph	0.272	0.6
AGL-1	pJF1	0.262	0.9

Table 2.3: Number of transformants per 10^5 conidia obtained on nylon and nitrocellulose membranes in 3 different transformation experiments

	1	2	3
pBin-GFP-hph			
Nitrocellulose	166	362	288
Nylon	345	392	236
pJF1			
Nitrocellulose	15	12	20
Nylon	13	15	17

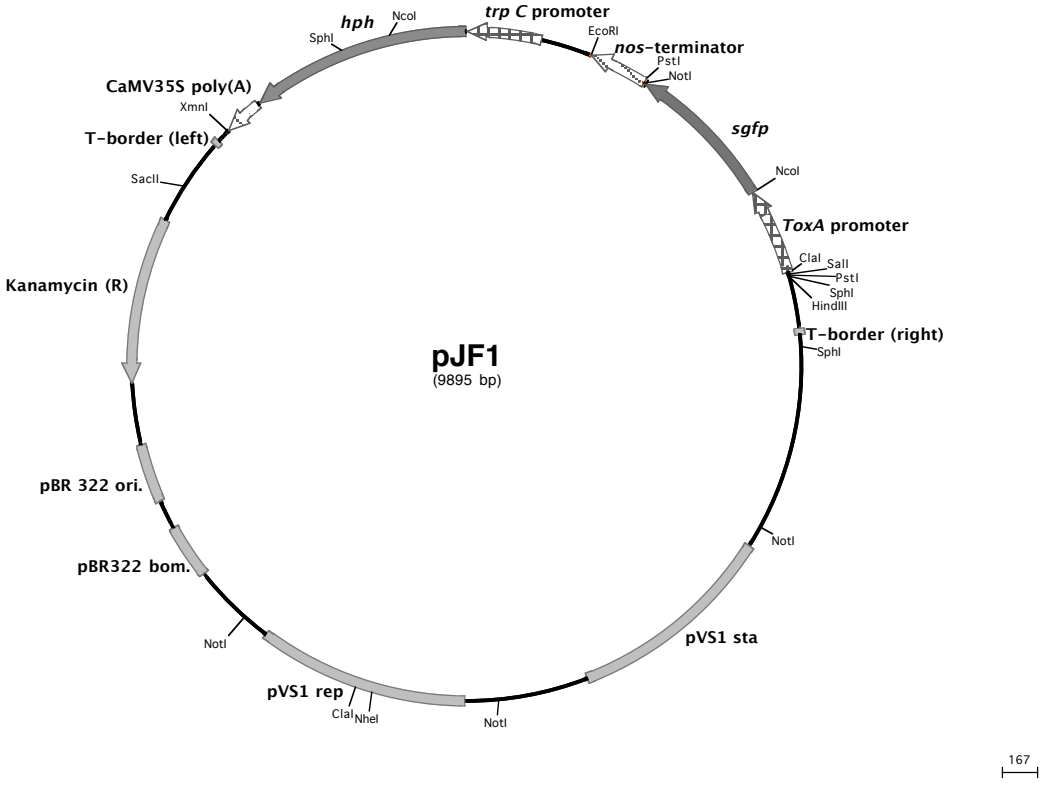
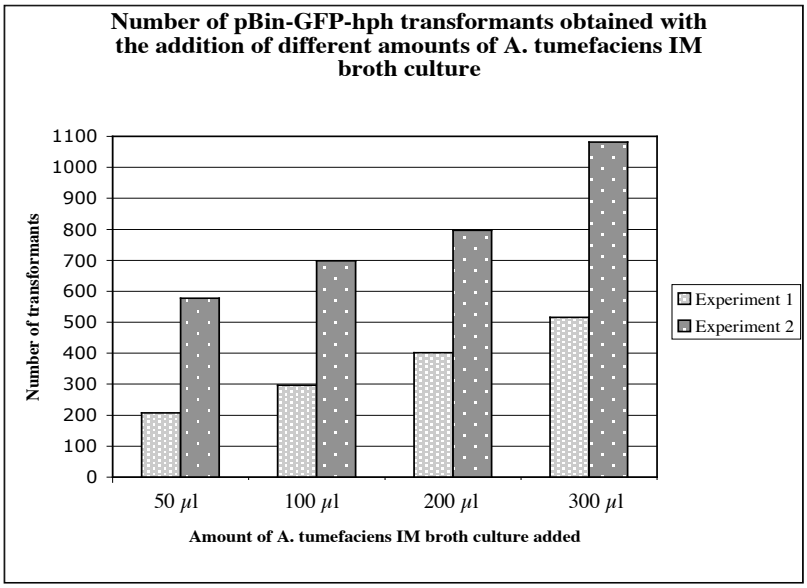


Figure 2.1: Map of novel binary vector pJF1. pJF1 was constructed by inserting the *Sal* I/*Eco* RI fragment of pCT74 containing the *ToxA* promoter, *sgfp*, and *nos*-terminator (Lorang et al. 2001) into the *Sal* I/*Eco*RI sites in the multiple cloning site of pBHt2 (Mullins et al. 2001). pBHt2 was constructed on the pCAMBIA1300 backbone (CAMBIA, Canberra, Australia). *ToxA* promoter: from a host-selective toxin protein gene from *Pyrenophora tritici-repentis*; *sgfp*: SGFP-TYG gene; *nos*-terminator; *trp C* promoter: derived from *Aspergillus nidulans; hph*: hygromycin B resistance gene; CaMV35S poly(A): *Cauliflower mosaic virus* 35S terminator

A



B

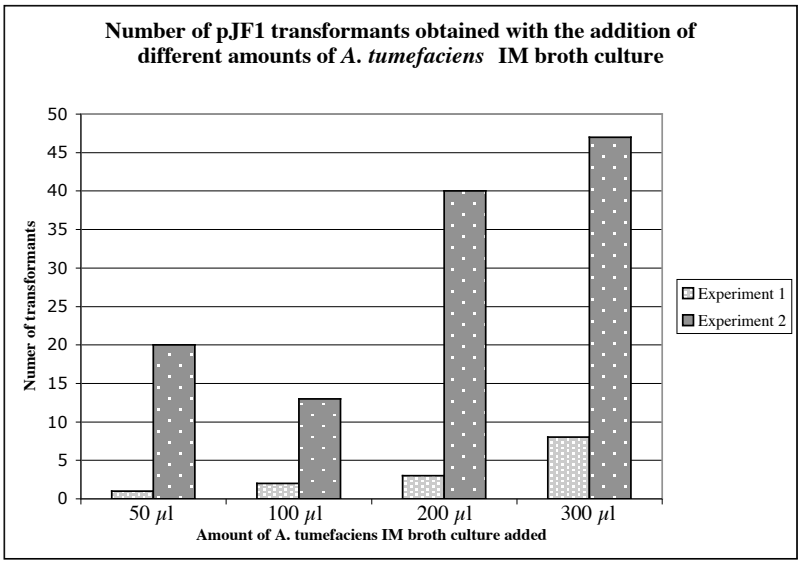


Figure 2.2: The effect of different amounts of *A. tumefaciens* IM broth culture co-cultivated with *C. graminicola* spores on transformation number. Total pBin-GFP-hph transformants per 10^5 conidia (A) and pJF1 transformants per 10^5 conidia (B) obtained with either 50, 100, 200, or 300 µl of *A. tumefaciens* IM broth culture co-cultivated with 100 µl of *C. graminicola* falcate spores. Results from 2 replicate experiments are reported. (OD_{660} for AGL-1 with pBin-GFP-hph = 0.33 in experiment 1 and 0.35 in experiment 2; with pJF1 = 0.30 in experiment 1 and 0.31 in experiment 2)

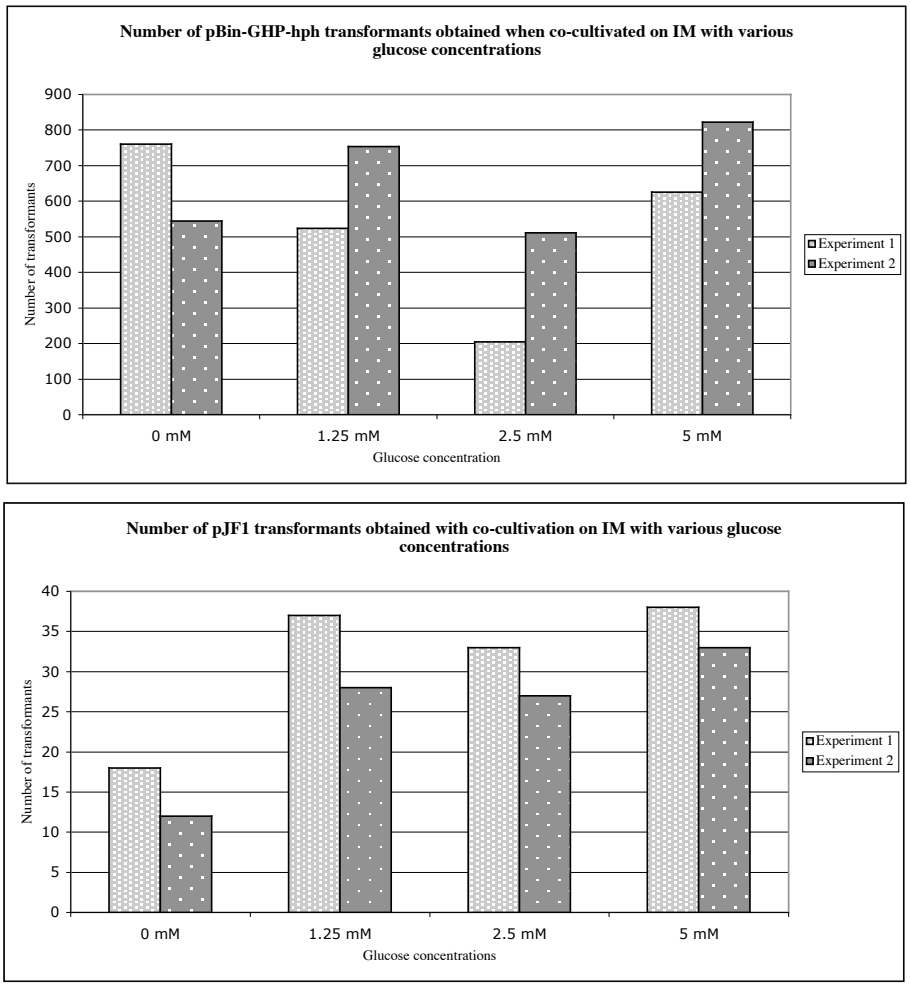


Figure 2.3: Number of transformants per 10^5 conidia obtained when different glucose concentrations are added to the IM co-cultivation medium. Results of 2 replicate experiments are reported.

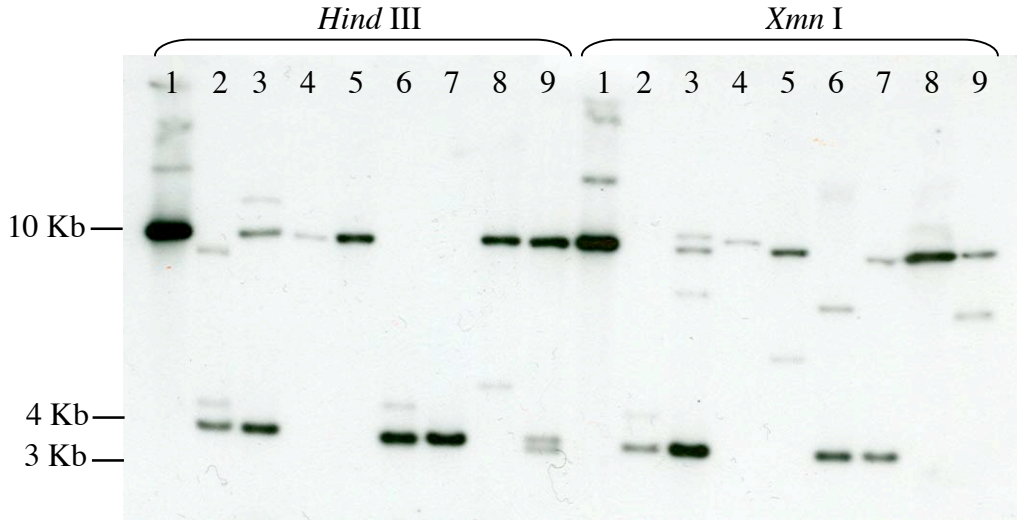


Figure 2.4: Southern hybridization of *C. graminicola* pJF1 transformants. Genomic DNA of pJF1 transformants digested with *Hind* III and *Xmn* I was hybridized with a 544 bp DIG-labeled fragment of the *hph* gene. Lane 1 contains digested pJF1 Ti-plasmid DNA, and lanes 2-9 contain *C. graminicola* pJF1 transformant DNA. (Untransformed *C. graminicola* does not hybridize with the *hph* probe, data not shown). A representative subset of the 25 transformants analyzed is shown in this figure.