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Technical Protocol for the Transformation of Filamentous Fungi Using *Agrobacterium*-Mediated Transformation

by Jordan Baumbach, Sean Halper, Gabriel Schuler, and Matthew Servinsky

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14. ABSTRACT
This report contains the standard protocol used for fungal transformations using the *Agrobacterium*-mediated transformation (AMT) method. Users should be able to conduct AMT experiments using the following standard protocols. Recipes for media used for transformation are included in this role. A brief background to AMT and the different *Agrobacteria* strains are also described to help end users in selecting the correct strains for their research. Discussion includes considerations for AMT use in fungal research. Each fungal isolate may have its own peculiarities, and some tend to be recalcitrant. The following protocol has been used at ARL for the chromosomal integration of DNA into 16 different *Ascomycete* fungal strains.

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filamentous fungi, *Agrobacterium*-mediated transformation, genetic engineering, co-culture protocol, competent cell preparation, Biological and Biotechnology Sciences

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1. Introduction

Filamentous fungi are a diverse group of organisms that include those of biotechnology interest due to their ability to cause plant, animal, or human disease, their ability to synthesize biomolecules of interest including antibiotics and other medications, and their ability to produce degrading enzymes. Transformation of filamentous fungal organisms is more difficult than most bacterial transformations due to their complex cell walls. Typical transformation methods for filamentous fungi include protoplast-based transformation, *Agrobacterium*-mediated transformation (AMT), and electroporation. For protoplast transformation, enzymes are used to digest the fungal cell wall. The success of this method varies by fungal strain due in part to variations in the fungal cell wall composition. Generally, the preparation of protoplasts can be tedious and requires gentle handling and micromolar quantities of DNA for transformations. Electroporation methods are much quicker but again vary based on fungal strains and cell wall composition and require the preparation of micromolar quantities of DNA. AMT requires routine culturing of the *Agrobacterium* and only small amounts of plasmid DNA for transformation. Once the *Agrobacterium* contains the plasmid with the DNA of interest, known as the binary vector, the *Agrobacterium* can be used repeatedly for transformations if properly stored.

AMT uses the plant pathogenic gram-negative *Agrobacterium tumefaciens* to insert target DNA into host genomes. AMT was first developed for plant transformation but since then has been used in a wide range of organisms including algae, fungi, sea urchin, and HeLa cells.[1–4] In nature the bacteria infect plants through wounds or through their stomatal openings and transfer a segment of their tumor-inducing (Ti) plasmid DNA, known as T-DNA, into the plant's genome, leading to the formation of tumors that produce opines, which can be used for carbon or nitrogen sources.[5] The infection process, including the transfer of the DNA, is induced by the chemical signals from the plant including glucose presence, pH, and the presence of secondary metabolites such as acetosyringone.[6] The proper maintenance of these conditions is important for inducing transformation, especially in non-host organisms such as fungi. There has been much work on domesticating *Agrobacterium* and optimizing the plasmids for biotechnology uses. Wildtype *Agrobacterium* contains a plasmid, known as the Ti plasmid, that houses the *Vir* genes, responsible for both the transfer of DNA into the host plant, as well as genes responsible for tumor and opine production. The Ti plasmid has been split in two for laboratory purposes. One plasmid known as the helper plasmid contains the genes needed for the transfer of the DNA into the host genome. The helper plasmid is retained in the *Agrobacterium* strains and are generally not manipulated.

The second plasmid, known as a binary plasmid, is manipulated for genetic engineering purposes. This plasmid contains either a single origin of replication for both *Escherichia coli* and *Agrobacterium* or two separate origins of replication, bacterial selection markers, and the left and right border sequences (Figure 1). The left and right border sequences designate the T-DNA that is going to be transferred into the target organism. These sequences are 25 base pairs in length.[7] AMT was shown to be effective in filamentous fungi in 1998 when used to transform seven fungal species including common laboratory strains such as *Neurospora crassa* and *Aspergillus niger* and a Basidiomycete.[1] Since then, it has been used in a wide range of filamentous fungal organisms. Binary plasmids are available that are specifically designed for use in fungal organisms include those for overexpression and knockout generation.[8,9] The following is a detailed protocol for the transformation of filamentous fungi using the AMT method as commonly used at the U.S. Army Combat Capabilities Development Command (DEVCOM) Army Research Laboratory (ARL) as well as a description of the results from test transformations done using this method.

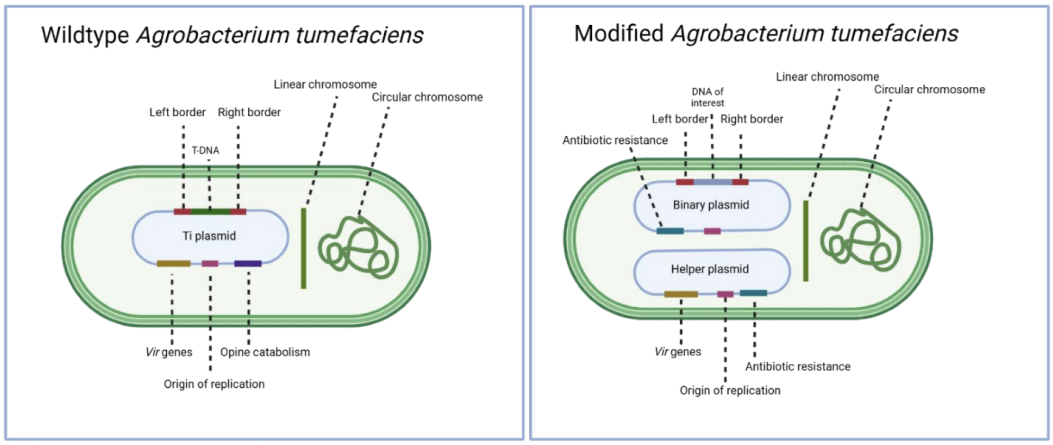

Figure 1. Illustrative comparison of *A. tumefaciens* genetic components. The Wildtype *A. tumefaciens* has a Ti plasmid that contains the T-DNA and the border sequences surrounding it as well as the *Vir* genes that are required for the transfer of the T-DNA into the host. The modified *Agrobacterium* strain contains two engineered plasmids. The helper plasmid contains the *Vir* genes as well as an added antibiotic resistance gene. In the binary plasmid, the T-DNA left and right border sequences are conserved and surround the DNA of interest, which is to be transformed into the target organism. This plasmid also contains antibiotic resistance for selection and either an origin of replication recognized by both *E. coli* and *Agrobacterium* or two separate origins of replication.

2. Materials

Agrobacterium culture, competent cell preparation, and fungal transformation protocols can be conducted in laboratories prepared for general microbiological work. The following is a list of materials needed or recommended for the transformation protocols.

2.1 General Laboratory Materials

- Benchtop centrifuge
- Chilled centrifuge
- –80 °C freezer
- –20 °C freezer
- 4 °C refrigerator
- Autoclave
- Vacuum pump (optional)
- Biosafety cabinet
- Electroporator (optional)
- Temperature-controlled shaker
- Incubator (optional)
- Micropipettes and pipette tips
- Petri dishes
- Cell spreaders
- Flameless sterilizer (optional)
- Heat block/water bath
- Thermocycler and polymerase chain reaction (PCR) reagents

2.2 *Agrobacteria* Competent Cell and Growth Materials

- Binary vector plasmid for transformation
- Yeast extract peptone (YEP) media
- Streptomycin
- Electroporation cuvettes
- Kanamycin (or other selection antibiotic)
- 25–50-mL centrifuge tubes
- Ice-cold sterile 20-mM calcium chloride
- Ice-cold 10% sterile glycerol
- 50% sterile glycerol
- Ice-cold autoclaved deionized (DI) water
- 1.5-mL Eppendorf tubes

2.3 Fungal AMT Co-Culture and Selection Materials

- Induction medium with acetosyringone (IMAS) media
- Acetosyringone
- 2-morpholinoethanesulfonic acid (MES) hydrate
- Streptomycin
- Kanamycin (or other selection antibiotic)
- Filter paper
- Spreaders
- Forceps
- Ethanol
- Hemocytometer
- Cell strainers or Miracloth
- 15-mL conical tubes
- 1.5-mL Eppendorf tubes

2.4 Cultures and Strains

A. tumefaciens strains (AGL-1, EHA105, GV3101, and LBA4404) were obtained from GoldBio (St Louis, Missouri) (Table 1). Strains were grown in liquid YEP + streptomycin for 24 h and stored in –80 °C as a 25% glycerol stock. Fungal strains used were obtained from the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) Northern Regional Research Laboratory (NRRL) culture collection (Peoria, Illinois), American Type Culture Collection (ATCC) (Manassas, Virginia), or were from environmental sampling. Fungal isolates were grown on potato dextrose agar (PDA) and spores were harvested in sterile water and stored in 4 °C, or in –20 °C in a 25% glycerol stock. The binary plasmid pRF-HUE[8] was obtained from the Fungal Genetic Stock Center (Manhattan, Kansas). Additional plasmids expressing green fluorescent protein (GFP) were built using the ARL modular cloning fungal genetic toolkit utilizing the pRF-HUE backbone including antibiotic resistance, origin of replication, and left and right border sequences.

Table 1. ***A. tumefaciens*** **strains.**[7]

Agrobacteria **strain**	**Chromosome background**	**Helper plasmid**	**Antibiotic resistance**[a]
GV3101	C58C1	C58C1	Rif, Strep
EHA1015	C58C	pTiEHA105	Rif, Nal, Strep
AGL-1	C58C *recA*	pTiBo542ΔT	Rif, Cb, Nal, Strep
LBA4404	Ach5C	pAL4404	Strep

[a] Antibiotics: Rif = rifampicin; Strep = streptomycin; Nal = nalidixic acid; Cb = carbenicillin.

3. Methods

The methods for culturing the *A. tumefaciens* strains with and without binary plasmids assumes that the strains being used have the streptomycin selection of the helper plasmid, which maintains the genes needed for the transformation process, and kanamycin is being used as the selection markers for the binary vector. If different strains of *Agrobacteria* are used, or a binary plasmid that does not use kanamyacin is used, the protocol should be adjusted to reflect those differences. Compositions for the YEP and IMAS media can be found in the Appendix.

3.1 Preparation of Electrocompetent *Agrobacteria* Competent Cells

The preparation of electrocompetent *Agrobacteria* competent cells is as follows:

- In the morning, prepare 5 mL of YEP + streptomycin (50 mg/L) and inoculate with a single colony of *Agrobacteria* strain.
- Place in 28 °C incubator shaker at 250 rpm and grow for 24 h.
- In the morning, inoculate 250 mL of YEP + streptomycin (50 mg/L) with the 24-h culture. Place in 28 °C incubator shaker and grow for approximately 24 h until cells reach optical density at 600 nm (OD_{600}) of approximately 1.0–1.5.
- Place cells on ice and transfer to prechilled centrifuge tubes.
- Centrifuge cells at 4000 rpm for 10 min at 4 °C.
- Remove supernatant and suspend cells in 250 mL of ice-cold DI H_2O.
- Centrifuge cells at 4000 rpm for 10 min at 4 °C.
- Remove supernatant and suspend cells in 125 mL of ice-cold DI H_2O.
- Centrifuge cells at 4000 rpm for 10 min at 4 °C.
- Remove supernatant and suspend cells in 50 mL of ice-cold 10% glycerol.
- Centrifuge cells at 4000 rpm for 10 min at 4 °C.
- Remove supernatant and suspend cells in 250 µL of ice-cold 10% glycerol.
- Aliquot 50 µL into prechilled Eppendorf tubes.
- Store in –80 °C freezer.

3.2 Preparation of Freeze–Thaw *Agrobacteria* Competent Cells

The preparation of freeze–thaw *Agrobacteria* competent cells is as follows:

- In the morning, prepare 5 mL of YEP + streptomycin (50 mg/L) and inoculate with a single colony of *Agrobacteria* strain.
- Place in 28 °C incubator shaker at 250 rpm and grow for 24 h.

- Inoculate 50 mL of YEP + streptomycin (50 mg/L) with 2 mL of the 24-h culture. Place in 28 °C incubator shaker and grow for approximately 24 h until OD_{600} is between 0.5 and 1.0.
- Place cells on ice and transfer to prechilled sterile centrifuge tubes.
- Centrifuge cells at 10,000 g's for 8 min at 4 °C.
- Remove supernatant and suspend cells in 5 mL of ice-cold sterile 20-mM $CaCl_2$.
- Centrifuge cells at 10,000 g's for 8 min at 4 °C.
- Remove supernatant and suspend cells in 1 mL of ice-cold sterile 20-mM $CaCl_2$.
- Aliquot 50 µl into pre-chilled 1.5 mL tube.

3.3 Transformation of Electrocompetent *Agrobacteria* Competent Cells

The transformation of electrocompetent *Agrobacteria* competent cells is as follows:

- Place cells on ice to thaw.
- Place cuvette on ice to chill.
- Add 20 µL of cells to the cuvette.
- Add approximately 10 ng of binary vector to the cells in the cuvette.
- Tap the cuvette gently to get cells and DNA to the bottom of the cuvette.
- Electroporate at 2.5 kV or use preset *Agrobacteria* setting available on most electroporators.
- Add 1 mL of YEP to cells and transfer to a culture tube.
- Place in 28 °C incubated shaker and shake for 2–4 h.
- Plate 100 µL of culture onto a YEP + streptomycin (50 mg/L) and kanamycin (50 mg/L) plates.
- Incubate at 28 °C for 2 days or at room temperature for 3–4 days.
- Select colonies from transformation plate and screen by colony PCR for presence of binary vector.

3.4 Transformation of Freeze–Thaw *Agrobacteria* Competent Cells

The transformation of freeze–thaw *Agrobacteria* competent cells is as follows:

- Transfer cells to ice.
- Add 0.1 to 1 µg of DNA to cells (keep cells frozen).
- Place in 37 °C heat block or water bath for 5 min.
- Add 1 mL of YEP to cells and transfer to a culture tube.
- Place in 28 °C incubated shaker and shake for 2–4 h.

- Plate 100 µL of culture onto a YEP + streptomycin (50 mg/L) and kanamycin (50 mg/L) plates.
- Incubate at 28 °C for 2 days or at room temperature for 3–4 days.
- Select colonies from transformation plate and screen by colony PCR for presence of binary vector.

3.5 Preparation of Fungal Spores for Transformation

The preparation of fungal spores for transformation is as follows:

- Grow fungal isolates in spore production media of choice at appropriate conditions for strains being used.
- Wash spore plate with 10 mL of sterile 0.1% Tween 80. Try not to aerosolize the spores to reduce contamination issues in the biosafety cabinet.
- Pass the spores through a cell strainer or four layers of Miracloth to remove mycelial chunks.
- Prepare a serial dilution of spores 1:10 and 1:100 with final volume of 1 mL. Make sure to mix spores well between pipetting.
- Clean the hemocytometer with 70% ethanol and dry before use.
- Place cover slip on hemocytometer and load 10 µl of spore solution into each side of the hemocytometer.
- Count the spores in the four corners of the hemocytometer. Record the number of spores for each of the corners from both sides of the hemocytometer. You should finish with eight spore counts. If there are too many spores to count, dilute spores 1:10 again (final dilution of 1:1000). If there are very few spores, use the 1:10 dilution of spores instead to get a more accurate spore count.
- Calculate the average of the eight corners.
- Calculate the spore concentration per milliliter using the following formula:
    - Spore concentration = dilution factor × spore count average × 1×10^4
    - Some fungal spores can be stored in 4 °C and used repeatedly for months without losing too much transformation efficiency.

3.6 Fungal *Agrobacteria* Co-culture and Selection

The fungal *Agrobacteria* co-culture and selection is described as follows and shown graphically in Figure 2.

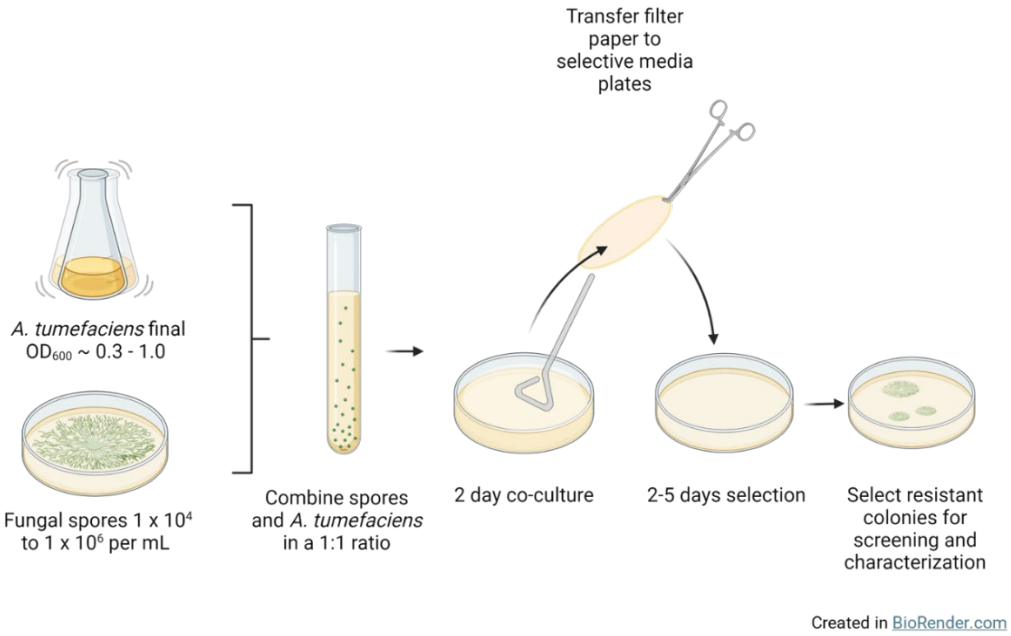

Figure 2. Graphic depiction of the steps involved in the fungal *Agrobacteria* co-culture and selection during AMT. Fungal spores will be grown based on individual species preferences.

- Inoculate 2 mL of YEP + streptomycin* (50 mg/L) and kanamycin† (50 mg/L) with a single colony of *Agrobacteria* containing the binary plasmid.
- Place in 28 °C incubator shaker at 250 rpm and grow for 24 h.
- Check the growth of the culture and make sure that the growth is not clumpy. Clumpy cultures will reduce transformation efficiencies.
- Start IMAS cultures and incubate for at least 4 h prior to co-culture.
    - For same-day co-cultures: inoculate 5 mL of IMAS media with 400 µL of 24-h culture (it will take about 4–6 h of growth to get the proper OD_{600}).
    - For next-day co-cultures: start overnight culture with 5 mL of IMAS and 200 µL of 24-h culture.
- Place in 28 °C incubator shaker at 250 rpm and grow until OD_{600} is between 0.3 and 1.0.
- Prepare the IMAS plates no longer than 24 h before use.
- Autoclave filter paper and place a filter paper on each of the IMAS plates.
- Dilute spores to a final concentration of 2×10^6 spores/mm in sterile water.
- Prepare a control for each spore type being transformed. *Agrobacteria* carrying no binary vector is the best control, but IMAS will work as well.

* Dependent on *Agrobacteria* strains.
† Dependent on binary plasmid.

Mix *Agrobacteria* or IMAS and spores in a 1:1 ratio. Prepare 200 µL of mix for each transformation plate.

- Prepare transformations by mixing *Agrobacteria* with binary vector and spores in a 1:1 ratio. Prepare 200 µL of mix for each transformation plate.
- Spread 200 µL of *Agrobacteria* spore mixture on filter paper and spread well using spreader.
- Wrap plates in parafilm and place in dark at 24 °C. Incubate the co-culture for 2–3 days.
- After 2–3-day co-culture, transfer filter paper from IMAS plates to selection plates using sterilized forceps. Selection plates should contain cefoxitin (300 mg/L) to kill off the *Agrobacteria*. Avoid creating air bubbles when placing the filter paper onto the new plates as this can result in false-positive growth.
- Wrap plates in parafilm and incubate 2–5 days in growth conditions favorable to the fungal strain used.
- After 2–5 days growth, remove filter paper using sterilized forceps and place filters in autoclave waste.
- Wrap plates and observe for colonies. Plates may need additional growth time for colonies to be visible.
- Compare control plates to transformation plates. Control plates should not have any colonies present on them.
- Colonies should be transferred to new plates containing cefoxitin (300 mg/L) to kill off any residual *Agrobacteria* before using PCR to confirm T-DNA presence in the fungus.

4. Results

4.1 *A. tumefaciens* Competent Cell Preparation

Both methods of the competent cell preparations resulted in successful transformation of the binary vector into the *Agrobacteria*. The electroporation-based transformation had a higher transformation efficiency and requires minimal amounts of DNA (<10 ng/transformation). Positive colonies were checked with PCR for the presence of the hygromycin resistance gene. With both methods, each colony selected tested positive for the hygromycin resistance gene from the binary vector. *Agrobacteria* containing the binary plasmids can be stored in 25% glycerol in the –80 °C freezer for future use.

4.2 Fungal Transformation

Table 2 lists 23 fungal species that were used for transformation experiments; of these, 16 *Ascomycete* fungal strains were capable of being transformed using hygromycin B selection with the binary vectors pRF-HUE or plasmids built at ARL using the pRF-HUE backbone.[8] Figure 3 shows examples of clean control plates and transformation plates from AMT of eight different fungal strains. Some of the strains (specifically black *Aspergillus* species) produced background colonies in the control experiments at hygromycin levels that killed off all the spores during minimal inhibitory concentration (MIC) testing. Individual colonies were transferred onto new selection plates and tested with PCR for the presence of the transgene and were positive for the transgene. *Hormoconis resinae* was used to test the transformation ability of four *Agrobacteria* strains. In the first replication there were no positive colonies for GV3101. Replication experiments produced low colony formation (approximately one colony per plate) when compared to the other three strains (30–100 colonies per plate).

Transformation of fungal spores with the binary vector expressing yeast codon optimized *eGFP* under the control of *A. niger GAPDH* promoter was conducted with selected fungal strains. GFP was visualized under the fluorescent microscope in *H. resinae*, *Trichoderma reesei*, and an environmental isolate. This additional evidence confirms that transformation was a success in these organisms. On occasion background growth has been observed on the control selection plate; in those cases some colonies on the transformation plates also did not express GFP, suggesting that these were survivors of the antibiotic selection and not transformation events. When the control plates containing hygromycin were free of colonies, the plates of transformants had colonies that all produced GFP.

Table 2. Fungal species tested for transformation.

Isolate	Phylum	Transformation
Alternaria alternata	Ascomycota	No
Armillaria mellea	Basidiomycota	No
A. niger	Ascomycota	Yes
Aspergillus terreus	Ascomycota	Yes
Aspergillus tubingensis	Ascomycota	Yes
Aspergillus versicolor	Ascomycota	Yes
Aureobasidium pullulans	Ascomycota	No
Chaetomium globosum	Ascomycota	No
Curvularia lunata	Ascomycota	Yes
FRI Aspergillus environmental isolate	Ascomycota	Yes
FRI Trichoderma environmental isolate	Ascomycota	Yes
Fusarium moniliforme	Ascomycota	Yes
Fusarium oxysporum	Ascomycota	Yes
Ganoderma lucidum	Basidiomycota	No
H. resinae	Ascomycota	Yes
Mortierella verticillata	Glomeromycota	No
Paecilomyces variotii	Ascomycota	Yes
Penicillium simplicissimum	Ascomycota	Yes
Pennicillium funiculosum	Ascomycota	Yes
Pennicillium matensii	Ascomycota	Yes
R020 - Bioprospected isolate	Ascomycota	Yes
T. reesei	Ascomycota	Yes

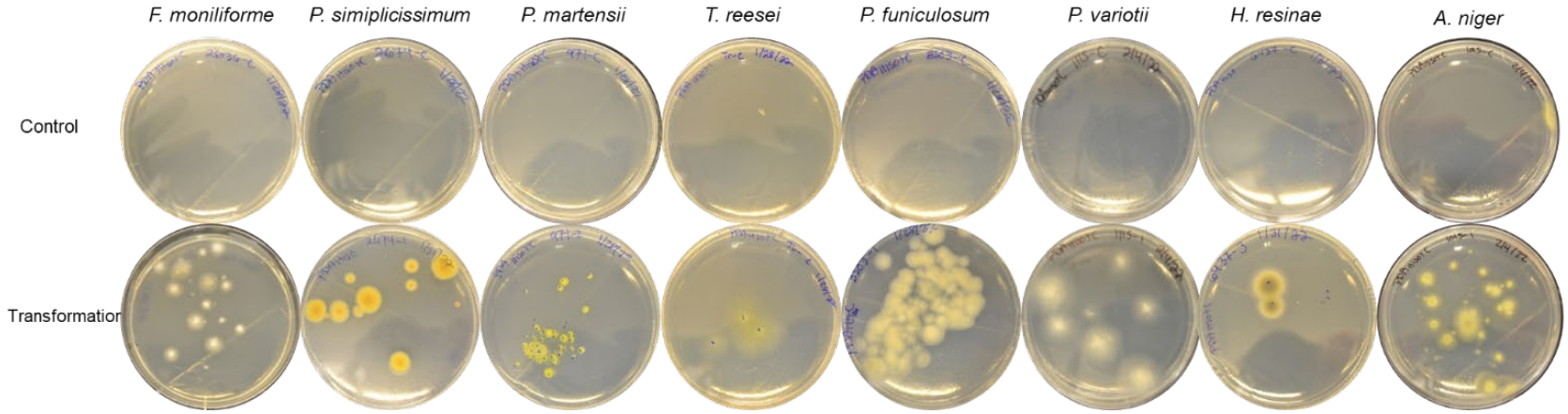

Figure 3. AMT of eight different fungal strains. All eight strains were transformed using the protocol presented and selected on PDA plates containing cefoxitin and hygromycin B. Control plates were inoculated with spores co-cultured with AGL-1 without a binary plasmid. Transformation plates were inoculated with fungal spores co-cultured with AGL-1 carrying a binary plasmid containing hygromycin B resistance cassette.

4.3 PCR Confirmation

PCR tests were used to confirm antibiotic-resistant colonies contained the transgenes. Primers to amplify the fungal internal transcribed spacer (ITS) were used as a control for PCR success and primers to amplify the hygromycin resistance gene was used to identify transformants. Multiple quick PCR methods were tested with varying success. The most successful colony preparation method was to select a chunk of mycelia from the plate and suspend in 50-µL Qiagen elution buffer (EB) (Qiagen, Hilden, Germany). It was heated for 20 min at 95 °C in thermocycler, then centrifuged and the supernatant used for PCR. Colonies that were transferred to new selection plates and screened with PCR were consistently positive for the hygromycin resistance gene. ITS primers were run as a fungal DNA control. In cases where there was no amplification of the hygromycin gene there was generally no ITS amplification as well. The ITS primers used for a broad range of fungal species were ITS1F: TCCGTAGGTGAACCTGCGG and ITS4R: TCCTCCGCTTATTGATATGC.[10]

5. Discussion

There are multiple methods reported for the nuclear transformation of filamentous fungal species. The most common is protoplast mediated transformation, which requires the digestion of the fungal cell wall with lysing enzymes.[11] This method can be tedious and requires special handling and accurate timing for the cell stage and lysing. Electroporation and ultrasonication have also been reported and are relatively quick and simple methods, if they work.[12] The previous three methods were all attempted at ARL with little to no success. ARL scientists had the most success with AMT using the protocol presented here.

Four different *A. tumefaciens* strains were used for fungal transformations. Preparations for competent cells—both heat-shock and electrocompetent—were similar and produced colonies positive for the binary plasmid. Electrocompetent cells require a smaller quantity of DNA to produce many colonies. For binary plasmids, which are low copy numbers such as those used at ARL, it is preferred to use electroporation for transformation of binary plasmids into the *Agrobacteria*. The four strains were investigated for their ability to transform filamentous fungi. The number of hygromycin-resistant colonies was greatly reduced when *Agrobacteria* strain GV3101 was used for transformation. Therefore, the recommendation would be to avoid using GV3101 or use an increased starting spore concentration if using GV3101.

Using the protocol shown here, ARL demonstrated transformation in 16 different fungal isolates from the *Ascomycota phyla*. This includes four strains that were environmental isolates and additional strains not yet reported to be transformed in the literature. Modifications of the protocol presented in DeGroot et al. include removing the second selection step, as the second selection did not enhance the number of GFP-positive colonies when compared to hygromycin-B-resistant colonies.[1] Transformations were confirmed by a colony PCR method developed for rapid PCR of fungal colonies. It is best for these colonies to be checked after moving the fungus to a new plate containing cefoxitin to kill off the *Agrobacteria* as there can be amplification from residual *Agrobacteria* on the original plates and the risk is lessened by moving colonies to a second plate before screening. In addition, PCR primers from the backbone of the plasmid can be used to check for carryover.

While AMT is an efficient method for transformations, there are some needed considerations. First, AMT only works for chromosomal integrations and will not work for transformation of episomes. Secondly, AMT has been reported in plants to result in multiple insertions per genome. There is little information on T-DNA insertion landscape in fungi. In *Magnaporthe oryzae* a study of T-DNA random insertion events identified that 81% of the fully characterized colonies have a single T-DNA insertion event.[13] If single insertions are needed, additional screening may be necessary to find a transformation event with a single insertion, or there is a potential for checking a lower-efficiency *Agrobacteria* strain for reduced insertion sites. Targeted insertion with AMT is possible in fungi, but the efficiency varies by fungal strain. Mutation of KU genes can result in increased homologous repair-driven targeted insertions.[14] It is also important to note that there can be loss of DNA around the border sites, and sometimes insertion of plasmid DNA following the left border.[15] Duplication of the left border repeat has reduced excess DNA insertion following the left border.[7] Current binary plasmids contain the selection marker to the left of the transcriptional unit to increase the likelihood of antibiotic-resistant colonies containing the gene of interest. If there are crucial DNA sequences close to the border, sequencing would be recommended.

Before starting any transformation experiments, it is important to identify a selection that will work for the fungus of interest. The most common selective agent with fungal transformations is hygromycin B. This is a negative selection that works in a wide range of eukaryotic cells. It is important to identify the correct dosage for selection before starting. While hygromycin B kills off many fungal cells, there are still many isolates that are resistant to it. There are additional chemicals that can provide selection including geneticin, bleomycin, zeocin, etc. Additionally, there is the benefit of developing auxotrophic mutants that do not

require the use of antibiotics and may have positive selection available such as is seen with *pyrG* and *amdS* mutations. There are binary plasmids available for fungal transformation with these different selective agents.[9]

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Appendix. Media Recipes

Table A-1. Yeast extract peptone (YEP) 1 L.

Component	Amount
Yeast extract	10 g
Sodium chloride	5 g
Peptone	10 g

Table A-2. Induction medium with acetosyringone (IMAS) 1 L.

Component	Amount	
2.5× salt solution	400 mL	. . .
20% glycerol	25 mL	. . .
20% glucose	9 mL	. . .
1-M MES	40 mL	Add after autoclaving
100-mM acetosyringone	2 mL	Add after autoclaving

- 1-M 2-morpholinoethanesulfonic acid (MES) hydrate (100 mL). Dissolve 19.52 g in 100-mL deionized (DI) H_2O, check pH and adjust to 5.3 if needed. Sterile-filter, aliquot, and store in –20 °C.
- 100-mM acetosyringone (10 mL). Dissolve 0.1962 g in 10-mL ethanol. Store in –20 °C in aliquots. Acetosyringone may precipitate during storage at –20 °C. Make sure to fully dissolve the acetosyringone before adding to solid media.

Table A-3. 2.5× salt solution 1 L.

Component	Amount
KH_2PO_4	3.625 g
K_2HPO_4	5.125 g
NaCl	0.375 g
$MgSO_4$ - $7H_2O$	1.250 g
$CaCl_2$ - $2H_2O$	0.165 g
$FeSO_4$ - $7H_2O$	45 µL of 0.5-mM solution
$(NH_4)_2SO_4$	1.250 g

- Dissolve each in order in 800 mL of DI H_2O. Dissolve one at a time or precipitate will form. Bring final volume up to 1 L. Filter-sterilize and store at room temperature.

List of Symbols, Abbreviations, and Acronyms

AMT	*Agrobacterium*-mediated transformation
ARL	Army Research Laboratory
ARS	Agricultural Research Service
ATCC	American Type Culture Collection
DEVCOM	U.S. Army Combat Capabilities Development Command
DI	deionized
DNA	deoxyribonucleic acid
EB	elution buffer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
H_2O	water
IMAS	induction medium with acetosyringone
ITS	internal transcribed spacer
MES	2-morpholinoethanesulfonic acid
MIC	minimal inhibitory concentration
NRRL	Northern Regional Research Laboratory
OD_{600}	optical density at 600 nm
PCR	polymerase chain reaction
PDA	potato dextrose agar
T-DNA	transfer DNA
Ti	tumor inducing
USDA	U.S. Department of Agriculture
YEP	yeast extract peptone

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