Molecular Interactions between *Thielaviopsis basicola* and Cotton Governing the Pathogenesis of Black Root Rot

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Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify to the best of my knowledge that any help in preparing this thesis, and all sources used, have been acknowledged and referenced in this thesis.

Rebecca Louise Forbes

Dedication

I would like to dedicate this thesis to my Dad, Max Joseph Forbes.

Thanks for your encouragement, help, love and support throughout the duration of my undergraduate studies. I could not have done it all without you. You have taught me by your unwavering example, that in whatever I do, to try my best and to do it with all my heart as unto the Lord. You are an inspiration to me Dad. Thanks for being the best Dad. Ti amo molto Papa e molto grazie per tutto.

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Abstract

Thielaviopsis basicola, a phytopathogenic filamentous fungus, is the causative agent of black root rot in a variety of host plants, including the economically important crop, cotton. The method of *Agrobacterium tumefaciens* mediated transformation (ATMT) was chosen to investigate the molecular interactions that exist between *T. basicola* and cotton. ATMT has long been used to generate transgenic plants and has more recently become a popular method for random insertional mutagenesis in the transformation of filamentous fungi. Generation of a large number of reduced pathogenicity mutants using this technique will aid to elucidate the identification of key pathogenic genes providing a better understanding of the molecular interactions between *T. basicola* and cotton, governing the pathogenesis of black root rot.

Development of an efficient ATMT protocol, designed specifically for transforming *T. basicola*, required optimisation of the experimental conditions prior to, during and after transformation. Transformation efficiency was found to be dependent upon the duration and temperature of pre-cultivation, co-cultivation and selection. The number of *A. tumefaciens* cells and the status of the *T. basicola* cells were also found to have significant influence on the efficiency of transformation. A consistently high rate of transformation efficiency was achieved by employing the hypervirulent strain AGL1, carrying the binary vector pBHt2, which contains the modified bacterial Hygromycin B phosphotransferase *hph* gene under the control of the *Aspergillus nidulans trpC* promoter. The media used during co-cultivation and the method of selection also played an important role in optimising the ATMT protocol for *T. basicola.* Optimal conditions of transformation led to the production of 300-770 Hygromycin B resistant (Hyg^R) putative transformants per 1 x 10^6 conidia of *T. basicola*.

All 10 Hyg^R putative transformants tested remained mitotically stable, maintaining their Hygromycin B resistance after five generations on non-selective medium. Primary pathogenicity screenings indicated that three of the 10 mitotically stable Hyg^R putative transformants had reduced pathogenicity, showing decreased virulence towards infected cotton seedlings when compared to the WT. Vegetative growth tests of these same 10 Hyg^R putative transformants, displayed varying growth by comparison to the WT; with six showing reduced growth and four growing at a similar rate to the WT. Colony morphology also indicated that at least seven of the Hyg^R putative transformants differed in colour, texture, and number of chlamydospores compared to the WT. Further genetic testing will be required to confirm that single and random insertion of the T-DNA occurs in the *T. basicola* genome.

Southern blot analysis on three of the five *T. basicola* reduced pathogenicity mutants generated by PEG/$CaCl_{2,}$ revealed that in p737 and p888, more than one insertion of pGpdGFP took place at multiple loci in the fungal genome; a common occurrence when using this method of transformation. The reduced pathogenicity mutant p16 instead had a single insert of the plasmid pGpdGFP integrated at a locus in the fungal genome, which suggests that further attempts could be made to recover the tagged pathogenicity gene from this mutant. Phenotypic analyses of all five PEG mutants, as well as 20 Hyg^R putative ATMT transformants, indicated that *T. basicola* most likely has some pathogenicity genes that are similar to those found in other filamentous fungi; including genes involved in the formation of infection structures and hydrophobins, spore development and germination, regulation and biosynthesis of melanin, cuticle and cell wall degrading hydrolytic enzymes, and regulatory proteins, including transcription factors, receptors, G proteins, and enzymes.

List of Abbreviations

µg	microgram
µl	microlitre
µM	micromolar
cm	centimetre
L	litre
ng	nanogram
PCR	polymerase chain reaction
TAIL-PCR	thermal asymmetric interlaced PCR
HygB	Hygromycin B
Hyg^R	Hygromycin resistant
Mef	mefoxin
Amp	ampicillin
dNTP	deoxyribonucleotide triphosphate
LB	Luria Bertani
½ PDA	½ Potato Dextrose Agar
IM	induction medium
ATMT	*Agrobacterium tumefaciens* mediated transformation
PEG	Polyethylene glycol
hph	*hygromycin phosphotransferase*
ER	endoplasmic reticulum
ITC	isothiocyanate
PRPs	Pathogenesis related proteins
HSTs	host selective toxins
REMI	restriction enzyme mediated integration
Ti	tumour inducing
T-DNA	transfer DNA
AS	acetosyringone
ss	single-strand
Km	Kanamycin
mM	millimolar
TAE	Tris-Acetate EDTA
BSA	Bovine Serum Albumen
RE	restrinction enzyme
Vir	virulence
OD	optical density
MIC	minimal inhibitory concentration
TE	Tris-EDTA
PDB	Potato dextrose broth
rpm	revolutions per minute
g	g-force
TES	Tris-EDTA SDS
MAPK	mitogen activated protein kinase

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Chapter 1: Introduction

1.1 General Introduction

Cotton is one of the most important natural fibre crops grown world-wide; constituting about 40% of the world's textile market. Australia is the world's third largest raw cotton exporter; with more than 70% of cotton grown and harvested as an irrigated crop in northern NSW (CRDC 2004).

The history of cotton as a major industry in Australia began in 1788 when Governor Philip first imported cotton seeds from Rio de Janeiro. Forty-three year later, the first reported export comprised three bags of cotton and by 1861 cotton farming had begun to expand rapidly. Cotton exports from Queensland during 1862-1865, netted £30,000-1,300,000. By 1898 there were reports of successful cotton growth in NSW and current estimates now reaching around two million bails harvested per year (Healy 1923; CRDC 2004).

There are a number of native species of cotton, including *Gossypium sturtianum* and *G. australe*, however, most commercially grown and exported cotton is derived from the species *G. barbadense* ("Egyptian" cotton) and especially *G. hursutum* ("Upland" cotton) (Wendel et al., 1992; McFadden, et al., 2004).

The presence of a wide array of diseases in cotton poses a serious threat to crop production. A number of bacteria and nematodes are known pathogens of cotton; including the bacterium, *Xanthononas campestris* pv. *malvacearum*, which causes Angular leaf spot and the nematodes *Meloidogyne spp.*, which cause root-knot (Agrios, 2005; Walker, et al. 1998). At least 20 different fungal genera of cotton pathogens have also been identified. This includes the fungi *Phytopthora capsici* and *Diplodia gossypina,* responsible for leaf spot disease and *Alternaria macrospore* and *Mycosphaerella gossypina*, causing cotton boll rot; both of these diseases primarily affect the growth and survival of the mature cotton plant. A number of fungi are known to cause cotton seedling disease complexes including anthracnose caused by the fungus *Glomerella gossypii,* root, leaf, and stem rot caused by *Rhizoctonia solani,* seed and root rot caused by many *Pythium spp,* root and stem rot as well as vascular and leaf wilt caused by *Fusarium oxysporium,* and black root rot caused by *Thielaviopsis basicola* (Healy, 1923; Lyon & Becerra-LopezLavalle, 2006; Wrather, et al., 2002, Wang & Davis, 1997; McFadden, et al., 2004).

Black root rot, is a seedling disease caused by pathogenic fungi of the species *T. basicola.* Black root rot was first discovered in Britain in 1850 (Berkley & Broom, 1850 cited by Stover, 1950) and was first identified in Australia in 1930, where it was isolated from sweet pea plants (O'Brien & Davis, 1994). Though known to have infected cotton in the USA for many years prior, it was not until the 90's that the first record of *T.basicola* - infected Australian cotton was made (Allen, 1990).

1.2. Geographical Distribution and Plant Host Range of *T. basicola*

T. basicola has a wide geographical distribution spreading virtually throughout the world. This fungus has been found in a large number of American states, especially in the main cotton producing areas of the San Joaquin Valley cotton fields. Though particularly prevalent in USA, *T. basicola* has been found in nearly every country in the world, including China, Belgium, South Africa, UK, Italy, Greece and Australia (Farr, et al., 2005: online; Mathre, et al., 1966; Rothrock, 1997; Stover, 1950; Nehl, et al., 2004; Wrather, et al., 2002: online).

The number of susceptible hosts to *T. basicola* infection is also widespread. The U.S. Systematic Botany & Mycology Laboratory have estimated that there are over 150 genera with more than 307 species of plants known to be hosts to *T. basicola*. This includes a variety of woody and herbaceous plants such as acacia, holly, begonia and cyclamen. Many flowering plants, like pansies, camellias, hibiscus, petunias, tulips, lilacs and lilies are also subject to attack by *T. basicola,* and black root rot in these commercially grown bedding plants has caused significant economic loss. A substantial number of economically important field crops are also susceptible to *T. basicola* infection, including tobacco, legumes (including lupins, faba beans, soybeans, chickpeas and green beans), carrots, lettuce, capsicum and cotton (Farr, et al., 2005: online; O'brien & Davis, 1994; Riggs & Mims, 2000; Moorman:online, Healy, 1923; Allen, 1990; Nehl, et al., 2004; CRDC, 2004; Mondal, et al., 2006).

1.3. Disease Symptoms of Black Root Rot in Cotton

The most obvious disease symptoms of black root rot are the browning and blackening of the roots (due primarily to the production of the highly-pigmented chlamydospores in the root cells) and small lesions, particularly in the main tap roots (Figure 1.1). Early root growth is often stunted by this disease. Due to the decreased ability of the diseased seedling's roots to absorb nutrients from its surrounding environment, stunting and yellowing of the plant's foliage and branch dieback also occur (O'brien & Davis, 1994; Walker, et al., 1998; Mauk & Hine, 1988).

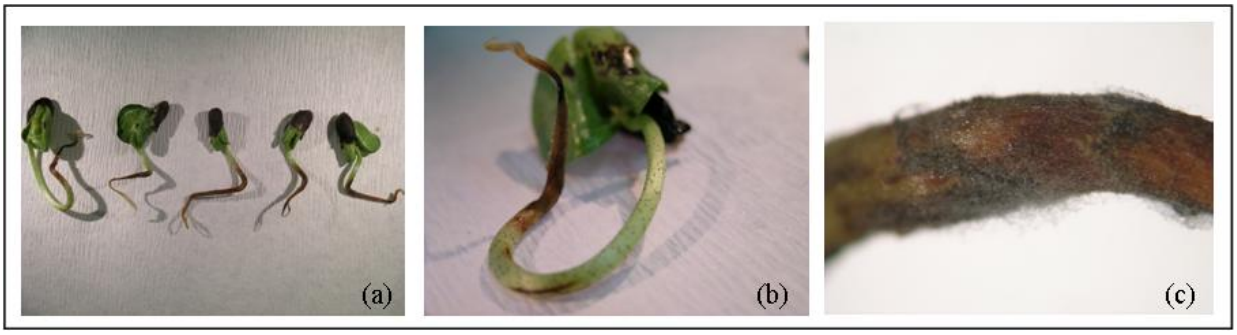

Figure 1.1. Symptoms of Black Root Rot Caused by *T. basicola* on Cotton Roots. (a) cotton seedlings infected with *T. basicola***.** (b) root lesion in main tap root. (c) *T. basicola* hyphae and chlamydospores on infected root.

T. basicola is not a primary cause of seedling death, however infection renders the plant vulnerable to a number of other pathogenic organisms that do cause mortality. The overall effect of *T. basicola* infection therefore results in a serious yield loss of host plants (O'brien & Davis, 1994; Walker, et al., 1998).

Black root rot caused by *T. basicola* is widespread in Australian cotton. In 2004, thirty Australian cotton farms showed 97% distribution of the disease (Nehl, et al., 2004). In 2006-2007 the CRDC reported that every tested cotton field was infected with *T. basicola*, resulting in high mortality rates. Pandemic spread can be primarily attributed to the ease with which the hardy spores of *T. basicola* are dispersed by water, farming machinery, and even footwear. Although a recent disease in Australia, black root rot has rapidly become a serious and challenging problem to Australian cotton production. The economic implications of such severe losses, affecting quality and quantity of yield, are of paramount importance to Australian cotton farmers and industries alike (Nehl, et al., 2004).

Effective control measures against *T. basicola* are required and are of great priori in order to prevent further spread of black root rot. Basic control strategies have included cultural practices like summer field flooding, late crop planting, crop rotations and basic sanitation practices (e.g. cleaning machinery and shoes) (Abawi & Widmer, 2000). Chemicals, e.g. fungicides can have a significant impact on controlling black root rot, however such control measures generally suppress rather than eliminate the fungal pathogen (Atkinson, 1999) Biocontrol schemes have also been implemented, such as planting cover crops like canola and hairy vetch (Abawi & Widmer 2000; Candole & Rothrock, 1996; Rothrock et al., 1994) and the use of microorganisms known to suppress *T. basicola* disease development (Howell, 2003; Shoda, 2000). Work in our group at UNE focuses on knowledge generation towards the development of new research tools to assist in black root rot management.

In the remainder of this introduction, I will introduce the fungus *Thielaviopsis basicola* in more detail giving information on its classification as a fungus, its morphology and spore formation/function. I will then discuss the ecological classification of this fungus and will provide some specifics as to the particular soil environments which assist or suppress *T. basicola* growth. I will give a brief introduction of plant-pathogen interactions and provide details of black root rot pathogenesis. I will conclude with the evident need for more effective control measures of black root rot and a discussion of the molecular techniques available and which need to be developed specifically for *T. basicola* to aid in the prevention of black root rot.

1.4. Taxonomy *T. basicola*

T. basicola (synamorph *Chalara elegans*) is an "imperfect" fungi due to its apparent lack of the sexual or teleomorphic stage. As such, *T. basicola* is classified according to its spore types, as well as other general features of morphology, including the shape and colour of their colonies and spores (Rajan, 2002:146-159). Though the taxonomic history of *T. basicola* has been long and complex, combining classical and modern techniques of classification, it now seems established that this fungus can be classified as Domain Eukarya, Kingdom Fungi, Division Eumycota, Subdivision Ascomycetes, Class Hyphomycetes, Order Moniales/Hyphales, Family Dematiciae, Genus *Thielaviopsis,* Species: *basicola* (Rajan, 2002:146-159; Agrios, 2005).

1.5. Mycology of *T. basicola*

T. basicola possesses typical features exhibited by filamentous fungi, including the characteristic fungal cell wall, vegetative growth structure, general morphology and reproduction. *T. basicola* cell walls are characteristic of many pathogenic fungi, and include the complex carbohydrates pectin, chitin, and cellulose as well as the pigment melanin. One function of its cell wall is to provide protection against toxic external chemicals secreted by the host in response to fungal invasion (Cole, 1991: 935-943).

T. basicola shows a morphology and growth pattern similar to that of other filamentous fungi in which the cells become arranged into long filament-like structures termed hyphae. Growth of these hyphal tubes is achieved by tip extension. The hyphae intertwine to produce a large interconnected mass known as the mycelium. The mycelia develop in an asymmetrically radial fashion, forming what is known as the vegetative body of the fungus (i.e. a fungal colony) (Cole, 1991: 935-943; Schweizer & Oliver, 1999: 165).

T. basicola colony colours are characteristically brown, dark green and/or black; though sometimes patches of white or yellowish colonies have been noted (Figure 1.2).

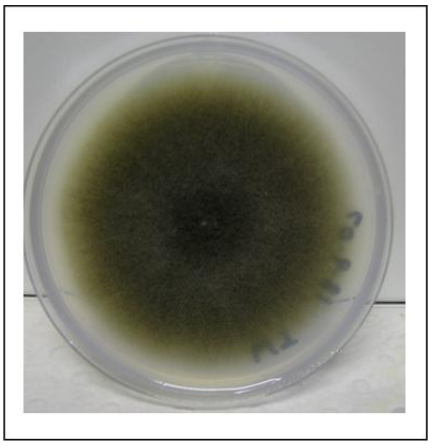

Figure 1.2. Colony Morphology of *T. basicola.*

For multi-cellular filamentous fungi like *T. basicola,* conidiation or conidiogenesis is the primary method of asexual sporulation, which involves the formation of spores from specialized hyphae (Cole, 1990; Agrios, 2005). *T. basicola* produces two distinct types of asexual spores known as endoconidia and chlamydospores, which have their own characteristic morphology, role and genesis (Figure 1.3).

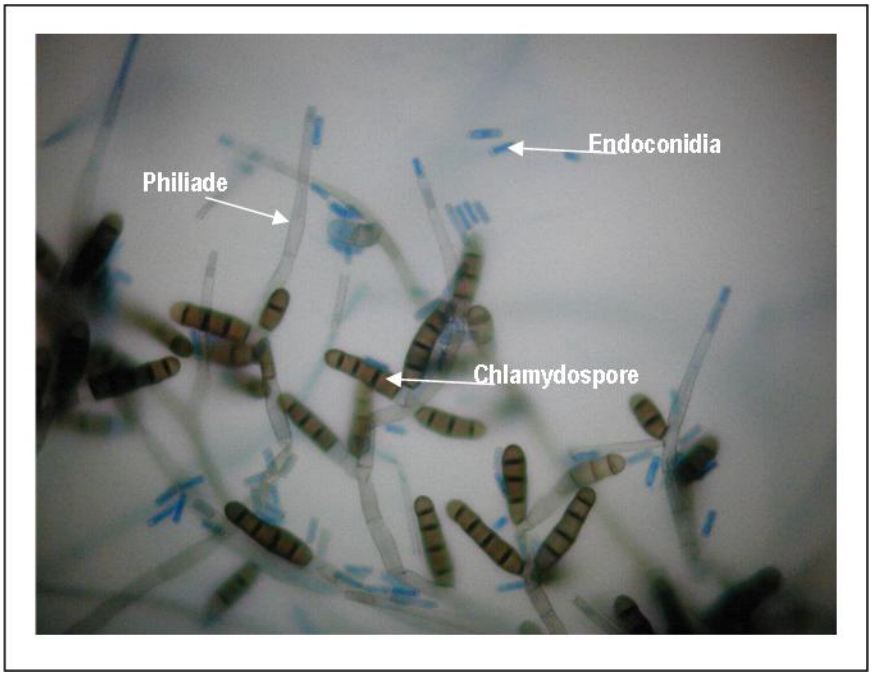

Figure 1.3. *T. basicola* Spore Morphology and Formation. Photo by Al-Jaaidi, 2007. Endoconidia are stained with cotton blue for easy visualisation.

1.5.1 Morphology and Formation of T. basicola Chlamydospores

A single chlamydospore (also called macroconidia or aleuriospore) has a circular structure with a flat top, having an approximate length of 10-16 µm and a width of 5-8 µm (Figure1.3). Chlamydospores are uni-nucleated cells separated by septa. They form short chains, surrounded by a thin membrane and branch from central hyphae. Each chain consists of 3-8 thick-walled highly pigmented apical spores and several thin-walled transparent basal spores. The thickness and characteristic colour of chlamydospores result, at least in part, from the high melanin content found in the spore's cell wall. Apical chlamydospores contain many lipid bodies, which increase in number as the spores mature, but are absent from the basal cells which don't undergo true chlamydospore formation (Riggs & Mims, 2000; Cole, 1991; Stover, 1950; Mauk & Hine, 1988).

A chlamydospore chain begins with the formation of a sporogenous cell, extending laterally from a parental hypha by breaking through the external membrane. After receiving a nucleus, it becomes separated (delimited) from the parental hypha. The sporogenous cell elongates and undergoes mitosis to produce two uni-nucleated cells known as the apical and basal cells. The apical cell continues this process until the entire chlamydospore chain has been produced. The basal cell undergoes one or two cell

divisions, allowing for additional chains to develop from this branch point; this results in the typical chlamydospore "fingers" often observed (Riggs & Mims, 2000; Cole, 1991; Stover, 1950).

Once the chain is complete, the chlamydospores simultaneously develop thickened multi-layered cell walls and the number of lipid bodies increases. The septa dividing each spore consist of a central pore, plugged by a Woronian body. These plugs, together with the bi-layered envelope surrounding the spores, seem responsible for maintaining the integrity of the chlamydospore chain. When mature chlamydospores break apart, the envelope disintegrates and the septal plugs are removed (Riggs & Mims, 2000; Cole, 1991; Stover, 1950).

1.5.2. Morphology and Formation T. basicola Endoconidia

Endoconidia (also called microconidia, philiaspores, or conidia), are thin-walled spores, much less melanised than chlamydospores. Endoconidia are cylindrical, truncated at each end, and measure 8-20 μm in length and 4-6 μm in width (figure 1.3). Conidia emerge and are dispersed as single cells. These spores develop consistently in size and shape except under starvation conditions, when they appear swollen and/or varying in shape and size, and may often form a chain-like appearance, aligning from end to end (Stover, 1950; Hammil, 1974; Cole, 1991; Rigs & Mimms, 2000; Hood & Shew, 1997).

Conidiogenesis begins with the development of a sporogenous cell, which extends apically from the parental hypha and elongates to form a tube-like structure known as the philiade, conidiophore, or endoconidophore (Figure 1.3). During formation, the conidiophore becomes delimited from the parental hypha forming a "neck-like" region at the base of the philiade tube. These specialised spore-producing structures are at least half as wide compared to a chlamydospore chain (i.e. $\leq 4\mu m$) and often extend much further in length. They are uni-nucleate and contain large amounts of rough endoplasmic reticulum (ER), free ribosomes, and lipid bodies (Hammil, 1974; Cole, 1991; Campbell, 1972).

The apex of the philiade elongates and swells as the conidial initial (i.e. endoconidia inside the philiade) forms. The conidial initial acquires a thick outer layer that surrounds the thin inner layer derived from the philiade and continues to expand as it receives large amounts of mitochondria, lipid bodies and rough ER from the philiade. Maturation is completed by transfer of a nucleus. The mature conidial initial becomes delimited and then expelled (or "blown out") from the philiade into the immediate surroundings. Numerous endoconidia will be released from a single philiade (Hammil, 1974; Cole, 1991; Campbell, 1972).

1.5.3. Purpose and Function of T. basicola Spores

Chlamydospores and endoconidia have collectively been termed "resting hyphae" because they are produced during nutritional abundance and are dormant after nutrients are spent. However, subsequent nutrient renewal quickly results in spore germination producing new vegetative bodies (Hood & Shew, 1997).

The primary purpose of these durable chlamydospores, with their thick highly-melanised walls, is long-term survival of *T. basicola*. Melanin in fungal cell walls has a high correlation with pathogenicity towards hosts. This polymeric compound acts as "fungal armour" providing a high level of protection for *T. basicola* against adverse environmental conditions and toxic metabolites released in defence by the host (Gomez & Nosanchuk, 2003). Chlamydospores are generally less abundant than endoconidia, more resilient, and their production is induced upon sudden depletion of nutrients during rapid vegetative growth. Conversely, endoconidia with their less-melanised and thinner walls support earlier stages of *T. basicola* survival. Conidiogenesis begins during early vegetative growth, producing vast numbers of these spores (Stover, 1950). Resistance to heat, drying, and long periods of dormancy (1-2 years) is similar for both spore types, however, beyond this period, majority of endoconidia are not capable of germination whereas chlamydospores are (Stover, 1950).

1.6. Acquisition of Nutrients

Fungi may be classified according to the type of organic matter from which they obtain their carbon compounds. Based upon this nutritional status, *T. basicola* can be classified as an obligate parasite; specifically a necrotrophic hemibiotroph (Hood & Shew, 1997; Mims, et al., 2000).

A fungus is considered a parasite if it obtains its nutrients from living organic materials. A facultative parasite will readily live in the soil, feeding on non-living material (i.e. as a saprophyte) but, upon contact with a living host, it will feed upon the living plant tissue, causing disease. Conversely, an obligate parasite requires a living host in order to survive (Rajan, 2002:1-4; Cole, 1991: 935-943; Kahmann & Basse, 2001; Hood & Shew, 1997; Mims, et al., 2000).

Obligate parasitic fungi are further classified, as biotrophs and hemibiotrophs, according to the duration of parasitic association with their host. To survive, a biotroph depends upon a host for its entire life cycle, whilst a hemi-biotroph requires a living host interaction during a specific part of their life cycle, in which they produce specialised structures not present when separated from the host (e.g. during germination and reproduction) (Rajan, 2002:1-4; Cole, 1991: 935-943; Kahmann & Basse, 2001; Hood & Shew, 1997; Mims, et al., 2000).

Biotrophs and hemi-bitrophs may infect host cells without directly causing cell death; simply using the host to supply it with the necessary nutrients. However, when necrosis and death of the host cells is caused, the fungal parasite is classified as a nectrotroph (Rajan, 2002:1-4; Cole, 1991: 935-943; Kahmann & Basse, 2001; Hood & Shew, 1997; Mims, et al., 2000; Agrios, 2005).

Like other fungi, *T. basicola* acquires carbon nutrients by the external breakdown of macromolecules (e.g. complex polysaccharides, lipids, and proteins) into micromolecules (e.g. mono- and di- saccharides, amino acids, and fatty acids), aided by secreted fungal enzymes, which also play an important role in host penetration and establishment of infection. After external digestion, the fungi ingest the micromolecules by simple diffusion through their cell walls. Once inside the hyphal cell's cytoplasm, nutrients are dispersed to appropriate intracellular locations for immediate use or storage (Rajan, 2002:1-4; Cole, 1991: 935-943).

1.7. Effects of the Soil Environment on Fungal Growth

1.7.1. Receptivity

Being a soil-borne pathogen, the soil environment will influence the radial growth, spore production, and germination of *T. basicola*. Soils can be divided into three types: (1) Suppressive soils, in which *T. basicola* may be present but will not produce significant disease symptoms regardless of which *T. basicola* isolate or plant cultivar is present, (2) Conducive Soils, in which *T. basicola* when present, will produce disease symptoms with susceptible cultivars showing more severe symptoms than resistant cultivars, and (3) Highly Conducive Soils, in which *T. basicola* when present will produce disease symptoms regardless of which isolate or plant cultivar is present. Factors influencing soil receptivity can be broadly divided into biotic, physical and chemical factors (Meyer & Shew, 1991).

1.7.2. Biotic Factors

Common biotic influences on *T. basicola* include (1) microorganisms that are found to co-exist in the soil and (2) the organic content of the soil.

Some microbes will enhance *T. basicola* virulence whilst others are antagonistic. Microplots inoculated with a combination of the root nematode *Meloidogyne incognita* and *T. basicola,* showed significant reduction in cotton seedling growth and survival. Increased yield loss was observed by comparison to uninfected microplots and those separately inoculated with *Meloidogyne incognita* or *T. basicola* (Walker, et al., 1998).

A number of bacteria, such as *Pseudomnas, Agrobacterium, Bacillus, Alcaligenes, Streptomyces,* and *Trichoderma,* act as antagonists to soil-borne pathogenic fungi including *T. basicola.* The suppressive

effects exhibited by these bacteria can be attributed to physical factors such as competition for space and nutrition and infection of the pathogenic fungi by parasitic bacteria, which may weaken or even kill the plant pathogen. Suppressive chemical factors include bacterial extracellular secondary antifungal metabolites (e.g. antibiotics, volatile compounds, and exoenzymes) or plant growth stimulants and activators of natural defence mechanisms (e.g. siderophores and salicylate) (Maurhofer, et al., 1994; Laville, et al., 1992; Haas, et al., 2002; Howell, 2003; Shoda, 2000).

One of the most common bacteria known to suppress fungal plant pathogens is *Pseuodomonas fluorescens* CHA0. This bacterium produces numerous secondary metabolites acting as anti-fungal agents including the volatile compound HCN, the antibiotics pyoluteorin (Plt) and 2,4-diacetylphloroglucinol (Phl), and the fluorescent siderophore pyoveridine (Maurhofer, et al., 1993; Laville, et al., 1992; Haas, et al., 2002). Using a gnotobiotic system that contained tobacco plants in sterile artificial soil inoculated with *T. basicola* and *Pseudomonas fluorescens*, Laville, et al. (1992) showed that *P.fluorescens* mutants were unable to synthesise these secondary metabolites and thus suppress black root rot.

Graham & Timmer (1991) showed that peat-based soil was conducive to *T. basicola* growth, likely due to an increased level of the nutrients consumed by *T. basicola* (including host plant exudates), present in such soil (Meyer et al., 1994; Hood & Shew, 1997). Canola plants/matter showed a suppressive effect on *T. basicola* growth; as this cruciferous plant produces high levels of 2-phenylethyl isothiocynate (ITC), to which *T. basicola* is highly sensitive (Smith & Kirkegard, 2002). Soils containing hairy vetch were also found to show suppression of black root rot disease in cotton; as this legume increases the presence of soluble ammonia in the soil (see chemical factors in section 1.7.4.) (Candole and Rothrock, 1996; Rothrock et al., 1994).

1.7.3. Physical Factors

Several physical properties that may influence *T. basicola* growth include the soil texture, water-holding capacity and temperature. Meyer & Shew (1991*)* reported no major difference between moisture content in suppressive and conducive soils. However, black root rot appears more severe in conducive soils with higher water holding capacity. This is attributed directly to the importance of moisture in the germination of *T. basicola* spores and their penetration of the host cells. Furthermore, poorly drained soils provide adverse growing conditions for the plant, thus favouring *T. basicola,* which tolerates such environments (Rothtrock, 1992).

A temperature range of 16-20°C provides the most advantageous conditions for *T. basicola* infection even though this is not the optimum temperature determined for growth of the fungus. Lower temperatures are ill-suited to cotton growth and development, thereby rendering it more susceptible to infection by this hardy pathogen (Rothrock, 1992; Mauk & Hine, 1988).

Common soil components including sand, silt and clay determine the soil texture. Meyer & Shew (1991) and Rothrock (1992) found that most suppressive and conducive soils contained comparable soil textures and thus any effect exhibited on *T. basicola* virulence is likely due to other factors in the soil that are known to influence soil receptivity.

1.7.4. Chemical Factors

Soil pH, aluminium and ammonium concentrations are abiotic factors, which show consistent influences on the soil receptivity towards *T. basicola.* Though suppressive soils often have a low pH (≤ 5.2), it is now recognized that an acidic soil alone is not sufficient to suppress *T. basicola* growth (Meyer & Shew, 1991; Meyer, et al., 1994; Harrison & Shew, 1999). There exists an inter-relationship between acidity and the concentrations of soluble aluminium present in the soil. A low soil pH increases Al solubility and the presence of Al lowers soil pH (due to release of H^+ upon Al hydrolysis). The suppressive effects on *T. basicola* are directly attributed to the increased Al solubility, which appears to be fungistatic at low levels and fungitoxic at high levels (Meyer, et al., 1994). A similar correlation is seen between soil pH and ammonia; with antagonistic effects on *T. basicola* growth being produced indirectly by low soil pH and directly by free ammonium. Ammonia induces plants to release putrescine, a fungistatic compound (Harrison and Shew, 1999), and also acts directly as a fungitoxic compound by penetrating and disrupting the fungal cell membrane (Candole and Rothrock, 1996).

1.8. Plant-Pathogen Interactions: Complex Associations Mediating Disease Development

1.8.1. Host Plant Susceptibility/Resistance

Host plants can be classified as having low, medium, or high susceptibility/resistance to *T. basicola* (Shew & Meyer, 1991; Trace, n.d; McFadden, et al., 2004). Shew & Meyer (1991) compared severity of black root rot across low, medium, or highly resistant cultivars of tobacco (a susceptible host plant). Regardless of which *T. basicola* isolate was used, in conducive soil, the disease was most severe in the cultivar with lowest level of resistance. Graham & Timmer (1991) compared black root rot severity amongst susceptible host species from the citrus genus; Cleopatra mandarins showed high susceptibility, Ridge Pineapple, sweet orange, sour orange and Volkamer lemon showed moderate susceptibility and rough lemon, trifoliate orange, Carrizo citrange and Swingle citrumelo showed low susceptibility. Native species within the cotton genus, such as *G. australe*, appear more resistant than commercial species, like *G. hursutum* (Wendel, et al., 1992; McFadden, et al., 2004), though, equally high susceptibility was displayed amongst 12 cultivars of the latter (Wang & Davis, 1997).

1.8.2. T. basicola Virulence/Avirulence

The *T. basicola* isolate or strain is no less important in determining the disease severity exhibited by the host plant; different isolates show varying levels of virulence towards a susceptible host (Agrios, 2005). Meyer & Shew (1991) showed that 7 different *T. basicola* isolates caused highly variable disease severity on a given susceptible tobacco cultivar. Pereg-Gerk et al. (2006) showed that cotton seedlings were highly susceptible to black root rot infection by *T. basicola* strains isolated from cotton, lupin, and pansy but showed only low susceptibility when isolates from lettuce and carrot were used. These same five *T. basicola* strains also showed varying levels of virulence towards lupin, lettuce, pansy, and carrot seedlings.

Such phenomena are beginning to be understood by researchers following that plants comprise non-specific and specific immunity, in a manner somewhat analogous to the mammalian immune system. Non-specific and specific immunity have genetic origins and interact to render a plant with varying levels of resistance or susceptibility against a given pathogen (Montesinos, et al., 2002).

1.8.3. Non-Specific Immunity

Non-specific immunity, (also known as partial, race non-specific, polygenic, or horizontal resistance) is exhibited by all plants and is controlled by multiple gene loci. This type of defence is primarily concerned with aspects of the plant's physiological processes, which provide structural and material support. These factors may be constitutively synthesised by the plant or activated in response to infection by the plant pathogen. This type of immunity acting alone does not generally inhibit infection by the pathogen but rather slows down the process of the initial infection and subsequent spread (Agrios, 2005).

In cotton and other plants, root border cells play an important role in the plant's defence against soil-borne parasites including bacteria, nematodes, and fungi. Root border cells are differentiated and form distal from the root tip. Root border cells are a constitutive physical property and though viable, they are detached from the root itself, thereby forming a sheath around the growing root. When suspensions of cotton root border cells and spores from the soil-borne fungus *Pythium dissotocum* were mixed, a rapid (1 min) accumulation of the spores on the root border cells was observed. Within 5-15 min, germination, penetration and extension of fungal hyphae through the cellular cytoplasm occurred. By 2 hours of incubation, the majority of cotton border cells were completely digested and non-viable (Hawes, et al. 1994; 1998; 2000). Thus, root border cells act as "biological goalies," against invading fungal pathogens; upon infection, they are digested and subsequently sloughed off, whilst leaving the majority of the root tips white, free from lesions, and able to continue seedling growth.

Cellular metabolism, including the activity of soluble oxidases and peroxidases, commonly increases in diseased cotton and other plants. Hampton (1963) demonstrated altered metabolism in carrot tissues when

infected with *T. basicola*. Such a response provides the plant with resources required to survive and continue growth when infected by the pathogen as well as the ability to mount other non-specific and specific defensive responses.

Plants synthesise a number of secondary metabolites to aid in their defence against invading pathogens. Phytoanticipins are constitutively expressed secondary metabolites whilst phytoalexins are produced only after pathogen invasion (Idnurm & Howlett, 2001). Glucosinolates are sulphur and nitrogen containing secondary metabolites, produced especially by cruciferous plant roots and used in biological activities to defend against invading pathogens. When hydrolysed, a group of toxic chemicals, collectively known as isothyocynates (ITCs), are produced (Bones & Rossiter, 1996). ITCs have varying levels of toxicity towards different pathogens, including fungi; *T. basicola* is highly sensitive, with greatly suppressed growth in response to this naturally produced plant chemical (Smith & Kirkegaard, 2002).

Pathogenesis related proteins (PRPs) constitute a group of non-specific defence proteins, including glucanases, proteinase inhibitors, and chitinases. Resistance by this diverse group of proteins may act directly against the pathogen itself or by neutralizing/inactivating virulent exo-products released by the invading microorganism. These proteins, whilst normally present in relatively low levels in a host plant, increase dramatically in response to *T. basicola* infection of tobacco roots. In the infected host cells the PRPs increase in concentration and activity, especially in the primary cell walls, papillae and the secondary thickening of xylem vessels. PRPs were also found in high concentrations in the infecting fungal hyphae (Tahiri-Alaoui, et al., 1992; Glick & Pasternak, 2003:556; Montesinos, et al., 2002).

1.8.4. Specific Immunity- Gene-for-Gene Hypothesis

Specific genetic interactions between plant host and pathogen determine the outcome of plant susceptibility/resistance and are governed by the expression of complementing single dominant genes in the plant and pathogen. This gene-for-gene interaction can be interpreted as a receptor-ligand model. Plants possess multiple resistance/susceptibility gene loci to a variety of pathogens that in turn contain the complementary avirulence/virulence gene loci, thus defining a pathogen's plant host range (Heath, 1991; Staskawicz, 2001; Grant, et al., 1995; Sidhu, 1984; Montesinos, et al., 2002; Bonas & Lahaye, 2002; Manning & Ciuffetti, 2005).

In classical gene-for-gene interactions, a pathogen avirulence gene (*Avr*) encodes an 'antigenic' elicitor molecule, recognized by an intra- or extra-cellular 'recognition' receptor molecule, encoded by the plant's complementing resistance (*R*) gene. Upon physical interaction, the Avr molecule acts an "incompatibility agent," causing the plant to mount an array of resistance responses against the pathogen, including the hypersensitive response. In this response, hyper-activation of the plant's non-specific defence mechanisms occurs, leading to localized plant cell death at the site of invasion and thereby marginalizing

the pathogen (Heath, 1991; Staskawicz, 2001; Grant, et al., 1995; Sidhu, 1984; Montesinos, 2002; DeWit, 1997; Manning & Ciuffetti, 2005). This is well illustrated by the salicylate pathway; interaction of the host plant's recognition receptor with the pathogenic avirulence elicitor results in conversion of the plant's salicylate stores (2-O-β-$_D$-Glucoside) into the active form of salicylate. Salicylate molecules then activate the expression of a key transcription factor, NEP1, which controls the expression of a large number of non-specific PRPs (Glick & Pasternak, 2003: 556).

Some pathogenic fungi produce host selective toxins (HSTs), which also follow the gene-for-gene hypothesis; with pathogenic toxin gene (*T*) production and host toxin sensitivity gene (*S*) each conferred by a single dominant gene. Compatibility results as the expression of the pathogen toxin is recognized by the host plant sensitivity receptor, which allows entry of the toxin into the plant cells and likewise results in plant cell death (Manning and Ciuffetti, 2005).

1.8.5. Fungal Pathogenicity Genes

A number of fungal genes are expressed during plant infection, which aid the pathogen in completing its disease cycle in the host plant; however, not all of these genes are essential specifically for pathogenicity. A pathogenicity gene can thus be defined as one which, when disrupted results in reduction or complete loss of pathogenicity. Pathogenicity genes can be divided into three main categories according to their role and time of expression during pathogenesis. A subset of the pathogenicity genes identified so far is presented here from reviews by Idnurm & Howlett, 2001, Kahmann and Basse, 2001, & Tudzynski & Sharon, 2003 and references therein.

1.8.5.1. Initial Host Contact

During the early stages of infection, the host plant's surface influences disease development; for many fungal pathogens, attachment to the host surface is required for subsequent invasion of host tissues. A crucial point in initial host contact is the development of the early infection structures, like the appressorium, which is a penetrating structure that develops from the germ tube. Production and maintenance of the appressorium is under genetic (and environmental) control. In *Magnaporthe grisea, PTH11* and *ACR1,* are pathogenicity genes, which encode proteins that play a role in regulating appressorium development. Mutations in these genes results in a loss of pathogenicity. In *Colletotrichum gloeosporiodes*, the *cap20* gene encodes for the protein CAP20, which is expressed during the formation of the appressorium and is essential for its function. Disruption of this gene produces non-pathogenic mutants. In *M. grisea,* the pathogenicity gene, *MPG1*, encodes the protein hyrdophobin. Hydrophobins are secretory proteins that accumulate at the interface between hydrophobic plant and hydrophilic fungal surfaces. Mutant phenotypes show a reduced ability to form appressorium on hydrophobic surfaces and an overall reduction in pathogenicity.

Melanin plays an important role in successful early penetration by many pathogenic fungi. Melanin confers cell wall rigidity and maintains osmotic potential; both necessary for the high turgour pressure in the appressorium as it punctures the host cell wall. In *C. lagenarium, PKS1, THR1,* and *SCD1* are three structural genes, involved in melanin biosynthesis. Disruption to these genes produces appressoria that lack melanization and a reduction in pathogenicity.

Compatibility is required for host specific interaction between fungal pathogen and host plant. In *Pyrenophora tritici-repentis* the pathogenicity gene, *Ptrl ToxA* encodes for the proteinaceous HST ToxA toxin. Transformation of reduced pathogenicity $ToxA^-$ mutants with this gene resulted in disease development on susceptible plants.

Successful penetration is also dependent upon fungal hydrolytic enzymes, capable of degrading the complex polysaccharides present in the plant cell wall. In *C. lindemuthianum,* the gene *clpg2* encodes an endopolygalactouronase, which breaks down pectin. This enzyme is expressed in germinating endoconidia, appressorium, and penetrating hyphae. In *Fusarium solani* f.sp.*pisi* expression of *pelA* and *pelD* produces the enzyme pectate lyase, which also functions in pectin degradation. Double mutants showed greatly reduced virulence, exhibited most markedly during penetration. Cutinase is another cell wall degrading enzyme expressed by many pathogenic fungi, which functions in the breakdown of cutin in plant cell walls. In *F. solani* f.sp.*pisi,* disruption of the cutinase expressing gene *CutA,* results in non-pathogenic mutants.

1.8.5.2. Biotrophic Phase

During the biotrophic phase the fungus must respond to the host environment. Pathogenic fungi express an array of genes involved in suppressing, avoiding, or overcoming the host plant's defence mechanisms. *C. gloeosporioides* expresses a plant defence-suppressing protein, encoded by the gene *CgDN3.* Disruption of this gene renders the pathogen avirulent. Some pathogenic fungi contain avirulence genes, which prevent incompatibility recognition by the host, allowing the pathogen to avoid detection. Disruption of the avirulence genes *Avr4,9* and *Ecp2* in *C. fulvum*, causes partial reduction in pathogenicity. *M. grisea* overcomes host defence mechanisms by detoxification. This fungus expresses the *ABC1* gene that encodes the ABC (ATP-binding cassette) transporter, which functions as a plant toxin efflux pump. Disruption of this gene renders *M. grisea* non-pathogenic.

1.8.5.3. Necrotrophic Phase

Large amounts of nutrients are required by the fungus for mass spore production and release. During the advanced stages of infection, upregulation of pathogenic genes encoding hydrolytic enzymes and toxins are common. In *C. lindemuthianum,* the *CLPG1* gene encodes an endopolygalacturunase, expressed only

during necrosis, and *Botrytis cineraea* expresses an endopolygalacturonase, encoded by *Bcpg1*. When disrupted this produces mutants with reduced pathogenicity.

There are complex interactions that exist between a pathogen and its host plant, governing disease development. For pathogenesis to occur the plant and pathogen must communicate throughout the disease progression. To control the spread of black root rot, an understanding of the interactions existing between *T. basicola* and its host plants (including cotton) is needed.

1.9. Pathogenesis: Disease Development

1.9.1. T. basicola Infection Stages Leading to the Development of Black Root Rot in Cotton Plants

The pathogenic life cycle of this hemibiotroph constitutes a number of distinct steps, which require intricate host-pathogen interactions throughout the process of disease development. Pereg-Gerk et al. (2006) divided the black root rot disease cycle caused by *T. basicola* into the following steps (1) spore germination, (2) growth towards the host roots (3) initial contact with the root, (4) root penetration (5) biotrophic phase establishment and (6) necrotrophic reproduction.

A spore must first germinate, releasing a germinating hyphae or germ tube into the outer environment. In most cases, a single germ tube extends from any given spore. For endoconidia, the germ tube ruptures through the spore wall, whilst for the chlamydospore chain the germ tube extends from the top of the cell wall, protruding out past the envelope. After initial release of the hyphae from the germinating spore, subsequent steps of the pathway develop similarly for both endoconidia and chlamydospores (Mauk & Hine, 1988). The germ tube grows towards the roots of the host plant, most likely attracted by some host root exudates (Pereg-Gerk, et al., 2006).

Upon contact of the germ tube with an epidermal root hair or the root cell itself, one of two events will occur: (1) inductive contact or (2) non-inductive contact (Hood & Shew, 1996). In inductive contact, which occurs more commonly, the tip of the germ tube swells slightly and ceases growth. In some cases the swollen tip separates from the germ tube by septa production (Mathre, et al., 1966, Hood & Shew, 1996, Mauk &Hine, 1988; Mims, et al., 2000). In non-inductive contact, the hyphae continue growing along the surface of the root hair resulting in vegetative growth without infection. It is possible for non-inductive contact to develop into inductive contact (Mathre, et al., 1966, Hood & Shew, 1996, Mauk & Hine, 1988; Mims, et al., 2000).

Once contact is established, a thin penetration hypha emerges from the tip of the germ tube and penetrates through the cell wall into the cytoplasm of the epidermal root cell. The penetrating hypha swells at its tip, reaching a final diameter 200% greater than that of normal vegetative hyphae. From this globular

infection vesicle emerge multiple infection hyphae, which elongate until coming in contact with the inner cortical root cells (Mathre, et al., 1966, Hood & Shew, 1996, Mauk & Hine, 1988; Mims, et al., 2000).

Infection is established with colonisation of the root's inner tissue by fungal hyphae. Infection hyphae contact and penetrate the cortical cells, producing infection vesicles and establishing multiple infection hyphae in each cortical cell. *T. basicola* becomes well established as it spreads throughout the root tissue from the initial point of infection. The infection hyphae in the cortical cells have morphology somewhat distinct from that of the hyphae, which develop in the epidermal cells. They appear highly branched and have constricted septa separating each cell; this gives rise to a characteristic beaded or lanced shaped appearance. These branched infection hyphae will form within the cortical cells (i.e. intracellular) but are not observed between the cells (i.e. intercellular) (Mathre, et al., 1966, Hood & Shew, 1996, Mauk & Hine, 1988; Mims, et al., 2000).

Numerous endoconidia and chlamydospores are produced in these infected cortical cells and due to host cell necrotrophy, root lesions develop leading to the typical root appearance for which the disease "black root rot" is named. In most cases, each cortical cell becomes highly colonised with multiple hyphae and spores before fungal passage into a neighbouring cell (Mathre, et al., 1966, Hood & Shew, 1996, Mauk &Hine, 1988; Mims, et al., 2000).

This entire process of *T. basicola* pathogenesis is a rapidly occurring event. In cotton roots it was found that chlamydospores began germination by 12 hours post-inoculation, whilst endoconidia had begun by six hours. Penetration of the epidermal root cells took 36-48 hours for the chlamydospores whilst endoconidia had penetrated by 12 hours post-inoculation. By 24-72 hours, *T. basicola* was completely established in the cotton roots, with the numerous spores giving the distinctive black appearance to the roots (Mathre, et al., 1966; Mauk & Hine, 1988).

1.9.2. Host Plant Responses

The root cells of host plants such as cotton, tobacco, and pansy were found to respond to *T. basicola* infection by (1) cytoplasmic streaming, (2) papillae formation and (3) host cell necrosis.

From the moment of inductive contact, cytoplasmic streaming takes place in the epidermal cells at the site of infection; with the accumulation of host cytoplasm carrying organelles including mitochondria and often the host nucleus itself (Mathre, et al., 1966, Hood & Shew, 1996, Mauk & Hine, 1988; Mims, et al., 2000).

Shortly after cytoplasmic streaming, papillae develop in the cytoplasm in the immediate area of invasion. Papillae consist of an electron-transparent membrane surrounding a central core filled with many vesicle-

like structures, containing the carbohydrate callose. The host nucleus is often in close proximity to the papillae (Mathre, et al., 1966, Hood & Shew, 1996, Mauk & Hine, 1988; Mims, et al., 2000). Development of the papillae in host cells seems an essential element of the infection cycle. Mims et al., (2002) discovered that when pansy roots were first washed, no cytoplasmic streaming or papillae formation took place in the epidermal cells. This resulted in the fungal germinating tube passing straight through these outer cells without the formation of penetrating hyphae. Upon contact with the inner cortical cells, which did produce papillae, the typical slender hyphae emerged and the infection process proceeded as usual.

Shortly after infection hyphae emerge, host epidermal cells respond by undergoing necrosis. The plasma membrane detaches from the intracellular face of the cell membrane, the host nucleus breaks down and the vacuolar tonoplast degrades, causing the leakage of cytoplasmic organelles into the vacuolar space. Similar necrosis occurs in the cortical cells after fungal invasion. Whether or not this necrosis results from a hypersensitive response by the plant or is due to fungal toxins released by *T. basicola* is not yet known (Mathre, et al., 1966, Hood & Shew, 1996, Mauk & Hine, 1988; Mims, et al., 2000).

Present control measures to eradicate black root rot in cotton are inadequate. Though the infection process by *T. basicola* has been well characterised at the morphological level, there is a complete lack of knowledge on molecular factors governing its interactions with host plants. Understanding the molecular interactions will provide the ability to develop specific control strategies aimed at disrupting one or more steps of *T. basicola* pathogenesis in cotton.

1.10. Strategies Used for Studying the Molecular Interactions between Plants and Pathogens

Successful strategies for studying the molecular genetics of fungal-host interactions most commonly take the form of generating mutant strains (i.e. mutagenesis) of the fungal pathogen by gene disruption (i.e. insertional mutagenesis). Gene disruptions require the development of suitable and efficient transformation protocols to transfer the foreign DNA into the fungal genome and are broadly divided into targeted (specific) or random (non-specific) mutagenesis (Mullins & Kang, 2001).

Targeted mutagenesis relies upon disrupting or silencing the expression of a specific putative pathogenic gene. For these specific techniques it is essential that the DNA sequence of the pathogenicity gene to be disrupted is known (at least in part). Two common methods of targeted mutagenesis are gene knockouts, in which the putative pathogenic gene is the target and replaced with a null gene, (Watson, et al., 1992:241; Mullins & Kang, 2001; Covert, et al., 2001) and gene silencing, in which the mRNA of the putative pathogenic gene is instead targeted for destruction (Glick & Pasternak, 2003:292-294; Watson, et al., 1992: 228-229; Liu, et al., 2002).

Random mutagenesis relies upon techniques, which randomly disrupt genes within the genome of the fungal pathogen and do not require prior knowledge of the DNA sequences that will be mutated. In such techniques, a DNA fragment is randomly inserted into the fungal genome. Selection of transformants is dependent upon a selectable marker on the DNA fragment, such as the Hygromycin phosphate transferase *hph* gene, under a constitutive fungal promoter. Pathogenicity genes are identified by screening for reduced virulence. Non-specific mutagenesis includes techniques like polyethylene glycol (PEG)/$CaCl_2$ mediated transformation, restriction enzyme mediated (REMI) transformation, and *Agrobacterium tumefaciens* mediated transformation (ATMT) (Mullins & Kang, 2001).

1.11. Conclusion

The fungal pathogen, *T. basicola* is the causative agent of black root rot. This necrotrophic hemibiotroph (Hood & Shew, 1997; Mims, et al., 2000), first isolated over 200 years ago (Berkley & Broom, 1850 cited by Stover, 1950) has been found virtually world-wide and is known to affect a broad host range; including the important natural fibre crop, cotton.

T. basicola in cotton causes root rotting and growth stunting, which leaves the plant weakened, vulnerable and victim to numerous pathogenic microorganisms. Its rapid spread, aided by highly melanised and durable spores, has resulted in large crop losses despite the fact that its appearance in Australian cotton is recent. The detrimental effect of this fungus on cotton is still a major and rapidly growing problem. More research into the molecular interactions that exist between this fungal pathogen and its host may prove of great benefit in providing more effective control measures.

Molecular techniques of fungal mutagenesis have met with varying levels of success, depending on the fungal pathogen being tested. A large number of pathogenic genes, both virulent and avirulent, from a range of pathogenic fungi have been isolated by application of such techniques (Kahmann & Basse, 2001).

To date, little work has been done in elucidating molecular interactions governing pathogenesis of *T. basicola* in cotton or any other plant. There is a need to develop suitable molecular techniques applicable to the analysis of *T. basicola.* From these methods, the isolation of key pathogenic genes involved in host pathogenesis can be achieved and used for developing more effective control strategies to reduce the disease.

1.12. Aims

The aims of the research are to identify and understand the molecular interactions that exist between the pathogen *T. basicola* and its host cotton in order to more effectively control and reduce the symptoms of the disease, black root rot, caused by this fungus.

More specifically, this project aims to elucidate key *T. basicola* pathogenicity genes governing the pathogenesis of black root rot in cotton. The approach taken will be to use a specific method of random mutagenesis in order to generate a wide range of *T. basicola* mutants, which show reduced signs of pathogenicity towards cotton. Identification of such pathogenicity genes will require a range of genetic and microbiological techniques.

In addition, five *T. basicola* reduced pathogenicity mutants, previously generated by an alternative method of random mutagenesis, will be analysed. These mutants will undergo numerous phenotypic tests as well as a genotypic test in order to elucidate information about the targeted pathogenicity genes and to provide confirmation and further insight about the method of random mutagenesis that was used.

The results of this project will thus be presented in two subsequent chapters, Chapter 2: 'Development of an *Agrobacterium tumefaciens* Mediated Transformation Protocol for *T. basicola'* and Chapter 3: 'Analyses of *T. basicola* Reduced Pathogenicity Mutants Generated by PEG/$CaCl_2$ Mediated Transformation'.

Chapter 2: *Agrobacterium tumefaciens* Mediated Transformation Protocol for *Thielaviopsis basicola*.

2.1. Introduction

Since the first achievement in 1979 (Case et al.), a number of filamentous fungi have been successfully transformed using a variety of mutagenesis techniques. Efficient introduction of foreign DNA, specifically into the host genome at random locations (i.e. random mutagenesis), has been commonly performed using polyethylene glycol (PEG)/$CaCl_2$ mediated transformation, restriction enzyme mediated integration (REMI), or *Agrobacterium tumefaciens* mediated transformation (ATMT).

Polyethylene Glycol/$CaCl_2$ -Mediated Transformation

For PEG/$CaCl_2$ –mediated transformation, fungal protoplasts (i.e. cell wall-free cells) are induced to take up a recombinant integrative shuttle vector by incubation in the presence of $CaCl_2$ and high concentrations of PEG. Transformants with randomly integrated foreign DNA in their genomes are selected on antibiotics. Though PEG/$CaCl_2$ mediated transformation has proven an efficient method of transforming a number of filamentous fungi, including *T. basicola*, there are many disadvantages. This includes the time-consuming and laborious preparation of protoplasts, as well as their regeneration on osmotically neutral medium. This technique has also proven difficult to optimise and often shows unstable insertions, (either from loss of the plasmid from the genomic DNA or lack of insertion in the first place) resulting in abortive transformants. Furthermore, several copies of the plasmid can integrate making recovery and genetic analysis difficult. Multiple insertions at more than one location in the genome are also a common occurrence (Amey, et al., 2002; Al-Jaaidi, 2007; Ruiz-Diez, 2002; Shi, et al., 1995; Amey, et al., 2001; Meyer, et al, 2003).

Restriction Enzyme Mediated Integration

In this method, fungal protoplasts are induced to take up a linearised recombinant vector (with the same features as PEG/$CaCl_2$ vector but also containing at least one unique restriction enzyme site). The protoplasts are incubated with PEG/$CaCl_2$ and the same RE used to linearise the vector. Upon entry into the nucleus of the fungal cell, the enzyme digests the fungal genomic DNA, creating a number of exposed ends that are compatible to that of the linearised plasmid. Plasmid and chromosomal complementing ends fuse together, resulting in the incorporation of the recombinant DNA into any of a large number of possible locations in the host genome. Like PEG/$CaCl_2$, efficient transformation of filamentous fungi has been achieved with REMI, however several major drawbacks have been noted. In addition to tedious protoplasts preparation, it is common that a number of mutations are generated resulting from genomic rearrangements that don't involve the inserted plasmid but are due to incorrect rejoining of chromosomal DNA. In many cases non-random insertion of the plasmid was actually observed to occur. Furthermore,

strain specific variation in the transformation efficiency commonly occurs, resulting from differing host DNA repair systems (Maier & Schafer, 1999; Kahmann & Basse, 1999; Mullins & Kang, 2001; Sweigard, et al., 1998; Shi, et al., 1994; Sexton & Howlett, 2001; Leclerque et al., 2003).

Agrobacterium tumefaciens-Mediated Transformation

Agrobacterium tumefaciens is a gram negative soil bacterium, which causes crown gall tumours to form at the wound sites of infected plants. *A. tumefaciens* contains a large plasmid known as the Ti or tumour inducing plasmid (Figure 2.1) that contains (1) the *vir* genes, which are clustered into around 10 operons, (2) an opine catabolism gene, (3) the T- (Transfer) DNA, which contains genes encoding enzymes involved in the synthesis of auxins, cytokinins and opines The T-region is flanked by left and right 25bp imperfect repeats known as the left and right border (LB and RB) sequences (Wei, et al., 2000; Bundock et al., 2002; Mullins & Kang, 2001; Glick & Pasternak, 2003:514-520; Madigan, et al., 2003: 683-685),

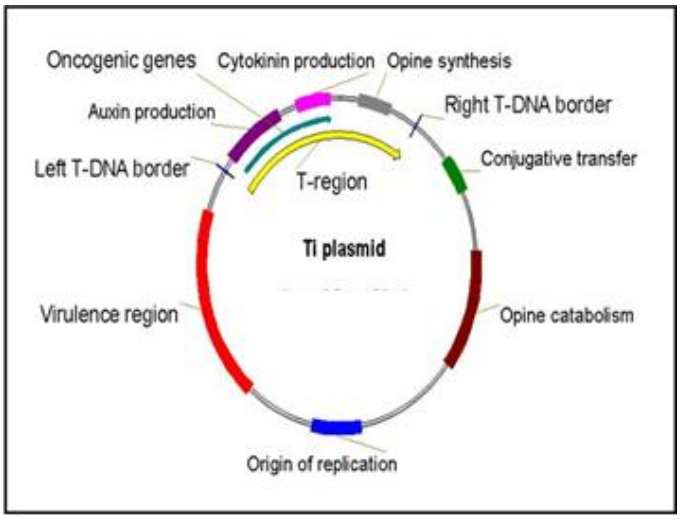

Figure 2.1. Tumour Inducing (Ti) Plasmid.

A wounded plant releases phenolic compounds (such as acetosyringone, AS), which bind to and trigger the Vir A receptors located on the bacterial cell membrane. The Vir A receptors activate the intracellular Vir G transcription factor, which regulates the expression of the *vir* genes. Expression of Vir D proteins causes recognition and nicking of the left and right border sequences, resulting in single-strand (ss) displacement of the T-DNA. The released ss-T-DNA, bound at its 5' end by Vir D proteins, is transported from the bacterial cell into the host cell through a pilus-like structure made from Vir B proteins. Also accompanying this convoy, are ss-DNA binding Vir E proteins that protect the travelling T-DNA from degradation by host nucleases. The VirD proteins display nuclear localisation signals that allow this foreign DNA to enter the host nucleus (Figure 2.2). The T-DNA is integrated into the host's chromosomal DNA by illegitimate recombination, which commonly results in deletions and/or truncation of the border sequences. Integration is usually random and occurs as single or low copy number inserts. The net effects of this natural transformation system is excessive host cell proliferation (i.e. crown gall formation) and

opine synthesis, which can then be utilized as unique substrates by the infecting *Agrobacterium* (Wei, et al., 2000; Bundock, et al., 2002; Mullins & Kang, 2001; Glick & Pasternak, 2003:514-520; Madigan, et al., 2003: 683-685).

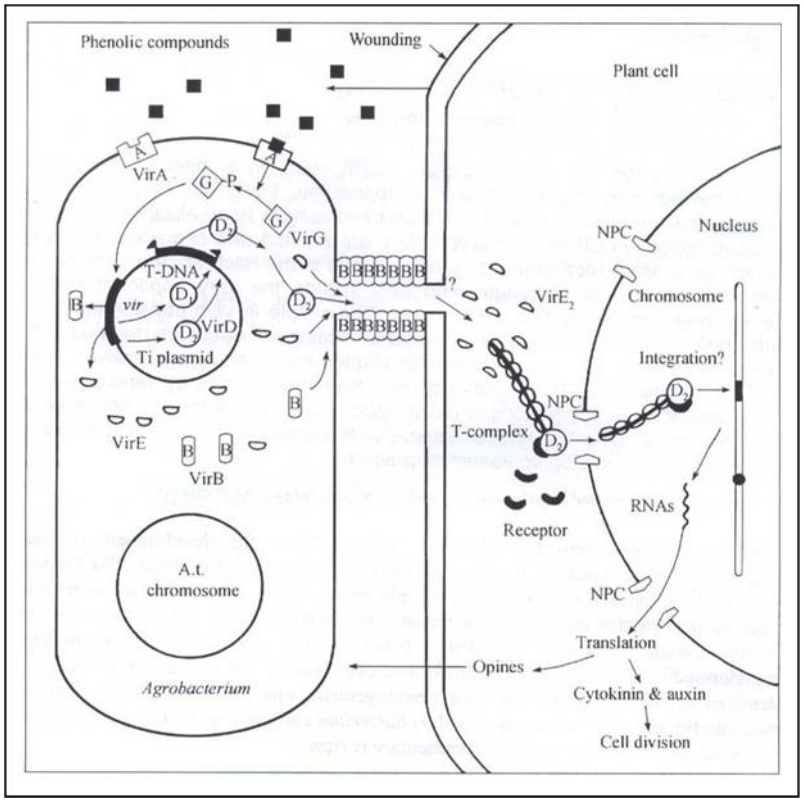

Figure 2.2. *A. tumefaciens*-mediated Transformation. *Figure from Wei, et al., 2000.*

The only part of the T-DNA required for its transfer are the 25bp left and right border flanking repeats. As such, the virulence-related genes within the T-DNA can be replaced with any given sequence, provided the borders remain intact. The ATMT system has become an invaluable tool for transformation of plants and recently has been found effective in the transformation of single-celled and filamentous fungi. For plant and fungal random mutagenesis, the binary vector system is most commonly employed and requires two plasmids for successful T-DNA transfer; the binary vector and the helper plasmid (Figure 2.3.). The binary vector is a modified Ti-plasmid that consists of (1) *A. tumafaciens* ori, (2) left and right border sequences flanking a fungal selectable marker, and (3) an *E.coli* selectable marker and ori, which may or may not be located inside the border repeats. The helper or disarmed plasmid is a modified Ti plasmid that lacks the left and/or right border sequences but contains an *A. tumafaciens* ori and the *vir* genes. Co-cultivation of an appropriate *A. tumefaciens* strain and binary vector system, together with the plant/ fungus of interest, in the presence of AS, results in random DNA integration of the T-DNA into the host genome (Glick & Pasternak, 2003:514-520; Bundock, et al.,, 2002; de Groot, et al., 1998; Covert et al., 2000; Hoekema, et al., 1983).

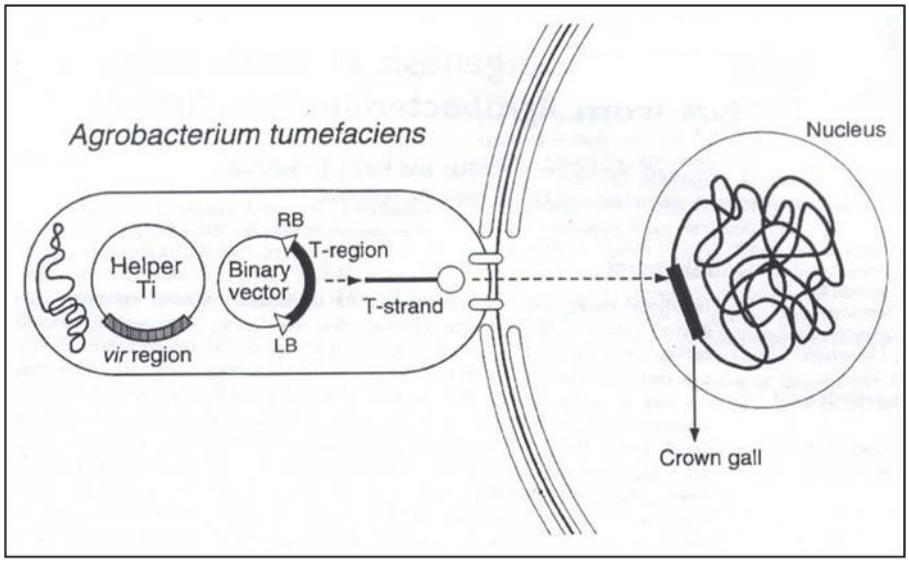

Figure 2.3. *A. tumefaciens* Binary Vector System. *Figure adapted from Bundock ,et al., 2002*

ATMT is an excellent method for fungal mutagenesis, showing thus far a high rate of success with many filamentous fungi, including *Fusarium* spp., *Verticillium dahliae, Magnaporthe grisea, Hebeloma cylindrosporum, Beauveria bassiana* and *Leptosphaeira maculans.* Efficient transformation has been achieved with endoconidia, hyphae, and mycelia.

A major advantage of this method is that the time consuming and often unreliable preparation of protoplasts is not required. In addition, transformation generally results in the stable integration of single or low copy number inserts. Furthermore, random integration usually occurs with minimal chromosomal rearrangements. Since the flanking left and right border sequences of the T-DNA are known, this eases later recovery and analysis of the tagged host genomic sequences. ATMT is an overall low cost, easy to operate transformation system (Glick & Pasternak, 2003:514-520; Bundock, et al., 2002; de Groot, et al., 1998; Covert, et al., 2000; Hoekema, et al., 1983; Leclerque, et al., 2003; Mullins, et al., 2000; Amey, et al., 2002; Gardiner & Howlett, 2004).

2.1.1. Objectives

The objectives of this part of the project were to develop an efficient method of random mutagenesis for *T. basicola.* The method developed is based upon the technique of *Agrobacterium tumefaciens*-mediated transformation and has been refined specifically for efficient transformation of *T. basicola*. ATMT has been developed for use in generating a large number of stable *T. basicola* mutants, especially those that show significant reduction of pathogenicity towards cotton. Isolation and identification of those tagged pathogenicity genes can be performed using methods such as TAIL-PCR and plasmid rescue.

2.1.2. Research Strategy

The development of an ATMT protocol for *T. basicola* involved the optimisation of five major experimental conditions: (1) establishing the optimal growth and induction conditions of *A. tumefaciens*, which includes the temperature and duration of bacterial growth prior to transformation, as well as the addition of the inducer acestosyringone, (2) optimising the co-cultivation conditions for *A. tumefaciens* and *T. basicola*, which includes the temperature, duration, bacterial to fungal cell ratios and media used, (3) finding the most efficient *A. tumefaciens* strain and binary vector for *T. basicola* transformation, which included trying the strains AGL1 or LBA4404 in combination with the binary vectors pBHt2, pPK2, or pCAMgfp, (4) finding the most suitable *T. basicola* cell status, which included testing germinating and non-germinating endoconidia, different ages of endoconidia and mycelia and (5) optimising the conditions for selection of *T. basicola* transformants, which included testing methods of selection (filter transfer vs top agar), finding the antibiotic concentration required for distinguishing transformants from the WT, temperature and duration of growth and optimising the method of final selection and isolation.

To confirm successful transformation, Hygromycin B resistant (Hyg^R) putative transformants were subjected to genetic analysis by Southern blot and PCR. To assess the stability of the insert into the fungal genome, mitotic stability tests were performed on a subset of Hyg^R putative transformants.

As the aim of the project is to target pathogenicity mutants, further characterisation of *T. basicola* transformants was undertaken by pathogenicity testing. General characterisation tests were also conducted on these Hyg^R putative transformants, which included vegetative growth tests and colony morphology.

Green fluorescence of *T. basicola* transformed with pCAMgfp, was also tested to examine the possibility of using this tool in studying *T. basicola* – cotton interactions.

As time ran out, recovery of the tagged genes (using TAIL-PCR and plasmid rescue) from reduced pathogenicity transformants was not performed.

2.2. Methods

2.2.1. Microbial Species and Strains

Table 2.1 Species and Strains Used in this Work

Strains and Plasmids	Features	Source/Reference
E.coli		
DH5α	*Ø* 80*lacZ*ΔM15, *recA*1, *endA*I, *gyrA*96, *thi*-1, *hsdR*17(r_K-,mK+), *supE*44, *relA1*, *deoR*, Δ(*lacZYA-argF*) U169, *phoA*	Promega
JM109	*endAI, recAi, gyr*A96, *thi, hsd*R17 (r_K-,mK+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F, *tra*D36, *pro*AB, *lacI*^{q}ZΔM15]	Promega
A. tumefaciens		
AGL1	Hypervirulent strain, derivative of AGL0, pTiBo542ΔT disarmed Ti plasmid , *recA::bla, Mop+CbR, non-oncogenic*	ATCC
LBA4404	Common strain , pAL4404 disarmed Ti plasmid	Invitrogen
T. basicola		
BRIP 40192	Host genus G. *hirsutum* obtained from DPI, QLD	O'Brien and Davis, 1994
Gossypium hirsutum	Cotton Cultivar SICOT 189 BR	NSW DPI Narrabri

2.2.2. Growth and Maintenance of Bacterial Cultures

For short term storage, bacterial cultures were streaked on LB (1.6% agar) medium (Appendix 1) and grown O/N at 37^{o}C for *E.coli* and 48hrs at 28^{o}C for *A. tumefaciens*. Cultures were stored at 4^{o}C for up to 2-4 weeks. For long term storage, a single colony was inoculated into 5ml LB liquid medium (Appendix 1) and grown O/N at 180rpm, 37^{o}C for *E.coli* and O/N (AGL1) or 2 days (LBA4404) at 250rpm, 28^{o}C for *A. tumefaciens*. Subsequently, 500µl of this culture was transferred into a sterile screw cap tube containing an equal volume of sterile 100% glycerol. This suspension was vortexed and stored at -70^{o}C.

For recombinant *E.coli* and *A. tumefaciens* strains the final antibiotic concentration used in the selective medium was 100µg/ml for Ampicillin (Amp) and 50µg/ml for Kanamycin (Km) (Appendix 1).

2.2.3. Preparation of $CaCl_2$ Competent *E.Coli* DH5α Cells

A single colony was inoculated into 5ml of YT liquid medium (Appendix 1) and grown O/N at 37^{o}C and 180rpm. Two ml of this O/N culture was used to inoculate 100ml YT liquid medium supplemented with 1M KCl (1ml) and 1M $MgSO_4$ (2ml). This culture was grown at 37^{o}C and 180rpm until it reached OD_{600} of 0.45-0.55 and then cooled on ice for 20min. The cells were then centrifuged at 4,200rpm (4^{o}C) for 5min. The resulting pellet was resuspended in 15ml chilled 50mM $CaCl_2$, incubated on ice for 15 min,

centrifuged again at 4,200 rpm (4°C) for 5min and resuspended in 2ml 50mM $CaCl_2$/15% glycerol. Competent cells were stored in 100µl aliquots in sterile eppendorf tubes at -70°C.

2.2.4. Transformation of *E.coli* with a Plasmid Solution by Heat Shock

An aliquot of competent cells was thawed on ice and mixed with 1µl of plasmid DNA (a ratio of 1:10 plasmid DNA to competent cells should not be exceeded). Following incubation on ice for 20min, cells were subjected to heat shock in a 42°C water bath for 2 min followed by 2 min on ice. After adding 300-450µl LB liquid medium (Appendix 1) to the cells, they were incubated at 37°C for 30min (if using Amp) or 1hr (if using Km). Subsequently, 50-100µl of recovered culture was spread on each of two YT (1.5% agar) selective medium (Appendix 1). When low frequency of transformation was expected, the culture was pelleted at full speed in a standard tabletop microcentrifuge for 5min, resuspended in 100µl of supernatant, and spread on YT (1.5% agar) selective medium. Cultures were incubated O/N at 37°C and successful transformation confirmed by the presence of transformed colonies growing on the selective medium, then by plasmid mini extraction (section 2.2.8.1.) and restriction enzyme analysis (section 2.2.8.6.).

2.2.5. Preparation of *A. tumefaciens* Electroporation Competent Cells

A single colony of *A. tumefaciens* AGL1 was used to inoculate 5ml LB liquid medium (Appendix 1) and grown for 24 hrs. Two ml of the culture were used to inoculate 200ml of pre-warmed (at 28°C) liquid LB. The culture was then grown at 28°C, 250rpm, until reaching an OD_{600} of 0.5-0.7 (not above 0.8), cooled on ice for 15min and centrifuged at 4,200rpm (4°C) for 5min. The pellet was resuspended in 3ml of chilled sterile dH_2O and centrifuged at 4,200rpm (4°C) for 15min twice. The pellet was resuspended in 2ml chilled 10% sterile glycerol, centrifuged at 4,200rpm (4°C) for 10min, and resuspended in a final 1.5ml chilled 10% sterile glycerol. Aliquots of 100µl competent cells were placed in pre-chilled sterile eppendorfs and stored at -70°C.

A. tumefaciens LBA4404 was purchased from Invitrogen as ready-made electroporation competent cells and thus required no prior preparation for subsequent transformation with plasmid DNA.

2.2.6. Transformation of *A. tumefaciens* with plasmid DNA by Electroporation

To transform AGL1, an aliquot of 100µl competent cells was thawed on ice and 40µl of the cells transferred into a pre-chilled eppendorf. Three ng of plasmid DNA was then added to the cells. After gently mixing, the sample was transferred into a pre-chilled 0.1cm BioRad cuvette, inserted into the pre-chilled slider of the BioRad Gene Pulser, and transformed at 2.5kV with 400 pulse (field strength was 12.5kV/cm). To recover the cells, 800µl of SOC (Appendix 1) was immediately added to the cuvette and mixed with the cells by pipette gently up and down. The cells were then transferred into a sterile chilled 10ml Falcon tube and incubated at 28°C, 250 rpm for 3 hrs. After incubation, 50-100 µl of recovered cells

were then plated on LB (1.6% agar) selective medium. When low frequency of transformation was expected, cultures were prepared as in section 2.2.4. Cultures were incubated for 48hrs at 28^{o}C and successful transformants (determined as in section 2.2.4.) were stored at 4^{o}C.

LBA4404 cells were transformed following the same protocol as AGL1 transformation, with the following alterations. A 40µl aliquot of commercially-competent cells was thawed on ice and 18µl of the cells then transferred into a pre-chilled eppendorf. One to two µl of concentrated (500ng-1µg) appropriate plasmid DNA was then added. After electroshock, cells were immediately recovered in 2ml YM liquid medium (Appendix 1) and plated on YM (1.5% agar) selective medium.

2.2.7. Growth and Maintenance of Fungal Cultures

To recover fungal colonies from water or glycerol storage, (see below) a single agar block was taken, using sterile tweezers, and placed mycelial side down onto fresh ½ PDA (1.2% agar) medium (Appendix 1). Cultures were incubated at 25^{o}C for ~ 10 days before use.

To prepare streak plates, a sterile inoculation loop was scraped across the surface of a fungal culture plate to obtain a mixture of mycelia and spores, and streaked in a star shape across the surface of a fresh ½ PDA (2.2% agar) medium plate, supplemented with HygB (10-25µg/ml) when growing transformants. Cultures were incubated at 25^{o}C for 4 days with 12hr light/dark cycling and then stored at 4^{o}C for 1-2 months.

To prepare stab plates, a sterile inoculation needle was scraped across the surface of a fungal culture plate and the needle tip gently stabbed into the centre of a fresh ½ PDA (1.2% agar) medium plate, supplemented with HygB (10-25µg/ml) when growing transformants. Care was taken not to press the needle all the way to the bottom of the plate. Cultures were incubated at 25^{o}C for ~ 2-4 weeks prior to being stored at 4^{o}C for 1-2 months.

For long term storage, several small agar blocks were excised from a fungal streak or stab plate (see above) using a sterile sharp flat-edged excision instrument. For dH_2O storage, 5-6 agar blocks were placed into 7-10ml of sterile dH_2O and stored at room temperature and can be kept for several years. For glycerol storage, 2-3 agar blocks were placed into a 2ml screw cap tube containing 1ml of 10% glycerol (in dH_2O) and stored at -70^{o}C and can be kept indefinitely.

2.2.8. General DNA Manipulations

Table 2.2. Plasmids Used in This Work

Plasmid	Details	Reference/Source
pBHt2*	8.4kb binary vector derived from pCAMB1300; 1.4kb *Hpa*I digested fragment containing the modified fungal $HygB^R$ selectable marker *hph* gene from pCB1004, under the control of the *Aspergillus nidulans* trpC promoter and Cauliflower mosaic virus 35SCaMV35S terminator; pBR322 ori and bom; bacterial Km^R selectable marker *aadA* gene	Mullins, et al., 2000
pPK2*	10.4kb binary vector derived from pPZP201 (Hajdukiewicz, Svab, & Maliga 1994); fungal selectable marker *hph* gene with *Aspergillus nidulans* gpd promoter and trpC terminator; bacterial Km^R selectable marker *aadA* gene	Covert, et al., 2000
pCAMgfp*	9.7kb binary vector modified from pCAMBIA1300 backbone; 3kb *Eco*RI/*Xho*I cassette with fungal $HygB^R$ fungal selectable marker *hph* gene under the control of the *Aspergillus nidulans* TrpC promoter; green fluorescenct protein *SGFP* gene under the control of the *Pyrenophora tritici-repentis* TOXA gene promoter; bacterial Km^R selectable marker *aadA* gene; pBR322 ori	Sesma, 2005
pBR322*	Bacterial Amp^R and Tet^R selectable marker genes;pBR322 ori	New England BioLabs Catalogue 2002-2003

See Appendix 4 for Plasmid Figures

2.2.8.1. Plasmid Mini Preparations

A single transformed colony was used to inoculate 5ml of LB selective liquid. For *E.coli*, the culture was grown O/N at 37°C, 180rpm and for *A. tumefaciens*, O/N at 28°C, 250rpm. Plasmid DNA was isolated from 2ml of culture using the Roche HighPure Plasmid Isolation Kit (Roche Molecular Sciences), following the manufacturers protocol. Plasmid DNA was usually eluted in 80 µl final volume and yielded 50-150ng/µl.

2.2.8.2. Plasmid Midi Preparations

A single transformed colony was used to inoculate 7ml LB selective liquid and incubated for 8 hrs at 37°C, 180rpm. Two hundred µl were used to inoculate 100ml LB selective liquid (Appendix 1) in a 1L flask and grown O/N at 37°C, 180rpm. Plasmid DNA was isolated from the cells using the QIAGEN Plasmid Midi Kit, following the manufacturer's protocol. Plasmid DNA was usually eluted in 80 µl final volume and yielded ~1µg/µl.

2.2.8.3. Estimating plasmid DNA Concentrations

The plasmid was first linearised (section 2.2.8.6.) and then run on a 1% mini gel (section 2.2.8.5.) along with λ *Hind*III, 1kb or 100bp ladders of known DNA concentrations. DNA concentration was estimated by comparing the intensity of the plasmid DNA band with the band in the ladder having the closest size.

2.2.8.4. Ethanol Precipitation

To concentrate a sample of DNA, 0.1 volumes of 3M sodium acetate (pH5.5) was added to the sample and mixed gently by inverting the tube several times. Then 2.5 volumes of chilled (at -20^{o}) absolute ethanol were added, inverted 3-4 times to mix, and incubated at -70^{o}C for 30min. The sample was then spun at top speed in a benchtop microcentrifuge for 10min at 4^{o}C. After decanting the ethanol from the tube, the pellet was washed in 1ml of chilled 70% ethanol, and spun as before, but for 2min. After carefully decanting the ethanol (as the pellet may be loose), the open tubes were dabbed onto tissue paper and then incubated at 37^{o}C for 10-15min. The dried pellet was resuspended in a desired volume of 10mM Tris · Cl pH 8.5 and stored at -20^{o}C.

2.2.8.5. Agarose Gel Electrophoresis

Agarose powder was added to 1x TAE buffer (Appendix 1) in a conical flask and briefly swirled before heating on low for 6-8min or until all agarose was dissolved. The solution was cooled before being poured into an appropriate gel mould and allowed to set for 10-20min. Gels not used immediately, were stored in 1xTAE at 4^{o}C. To prepare DNA samples for separation by electrophoresis, 6x loading buffer (Appendix 1) was first added. Samples were loaded into the gel wells and run alongside a DNA standard ladder. Samples were run at an appropriate voltage until the bromophenol blue dye reached near the end of the gel. To visualise the DNA bands the gel was stained in ethidium bromide (0.5µg/ml) for 10-30 min and then viewed under long-wave UV light in a transilluminator.

Three types of gels were used, mini, midi, and maxi gels. For most analytical purposes, DNA samples were separated using 1% agarose mini gels (3cm x 5cm, 300mg agarose/30ml 1xTAE buffer) and run at constant 90-100V for 30-45min (or until the blue dye reached near the end of the gel). Gels were stained for 10-15mins and then visualised under UV light. For gel extraction/purification, DNA samples were separated using 0.8% agarose midi gels (6.2cm x 10cm, 320mg agarose /40ml 1x TAE buffer) and run at a constant 80V for 60-90min (or until the blue dye reached near the end of the gel). Gels were stained for 6-8 mins (to minimise mutagenic effects to DNA samples) and then visualised under UV light (UV exposure was also minimised to prevent DNA damage). For Southern blot analysis, DNA samples were separated using 0.8% agarose maxi gels (15.7cm x 20.4cm, 3.2g agarose/400ml 1x TAE buffer) and run at a ~40V for 16-18 hrs. Gels were stained for 30-60min and then visualised under UV light.

2.2.8.6. Restriction Enzyme Digestions

The following reagents were added to a sterile 1.5ml microcentrifuge in this order:

1. Sterile dH_2O
2. DNA solution to be digested
3. 10x Restriction Enzyme Buffer
4. 10x BSA (when required)
5. Restriction Enzyme (RE)

The volume of water added depended upon the amount required to bring the sample to the final volume, which was usually 10-30µl. The volume of DNA added depended upon the concentration of DNA to be digested. The volume of RE added never exceeded 10% of the total volume of the restriction solution and as a general rule, 1U = the amount of enzyme activity required to completely digest 1µg substrate DNA in a 50µl total reaction in 60min .

The restriction solution was incubated at the appropriate temperature for the particular RE being used (as instructed by the manufacturer) and allowed to digest for a given period of time ranging from 3-16hrs. Complete digestion was confirmed by running an aliquot of the digestion reaction on a 1% mini-gel along with undigested control DNA. When required, enzyme activity was terminated by heat inactivation following the manufacturer's recommendations.

Double digestions were performed with the same reagents as for a single digest, except that two different restriction enzymes were added. Manufacturer's recommendations of enzyme compatibility and reaction conditions were followed.

2.2.9. Development of the ATMT Protocol

2.2.9.1 .Preparation of A. tumefaciens for ATMT: Pre-Cultivation and Induction Conditions

For AGL1, a single transformed colony from a 1-2 day old streak culture (grown on LB (1.6% agar) + Km medium) was used to inoculate 5ml LB + Km liquid broth in a McCartney bottle and grown for 16-17 hrs at 28°C or 29°C, 250rpm. To induce *vir* gene expression, 20ml of IM supplemented with 200µM AS (see Appendix 1) in a 50ml flask, was inoculated with the culture to achieve a final OD_{660nm} of 0.15.

For LBA4404, a single transformed colony from a 2 day old streak culture (grown on LB (1.6% agar) + Km medium) was used to inoculate 7ml of LB + Km liquid broth in a 50ml conical flask and grown for 2 nights and 1 day at 28°C, 250rpm. To induce *vir* gene expression, 19ml of liquid IM supplemented with 200 µM AS in a 100ml conical flask, was inoculated with 1ml of the culture.

For both strains, induction cultures were incubated at 28°C or 29°C, 250rpm for 4-8hrs. For AGL1, a final OD_{660nm} of 0.6-0.8 was achieved. For LBA4404, OD_{660nm} was not tested due to the clumping nature of this strain.

2.2.9.2. Preparation of T. basicola for ATMT- Cell Status

Non-Germinating Endoconidia

The mycelial mass from 15 four-day old ½ PDA (2.2% agar) streak cultures (section 2.2.7.) was scraped into a McCartney bottle containing 6ml sterile dH_2O and vortexed vigorously for 1 min. Endoconidia were separated from chlamydospores and mycelia by filtering the mycelial suspension through a single layer of sterile (soaked in 100% ethanol and dried for 15min prior to use) mira cloth into a sterile 50ml conical flask. Endoconidial concentration was estimated using an Improved Neubauer Counting Chamber viewed under the compound microscope at x 40 objective. The spore suspension was diluted in liquid IM to obtain a final concentration of 1.0×10^6 endoconidia/ml. Spore suspensions were prepared 1hr prior to completion of *Agrobacterium* induction. When testing the effect of spore age on transformation efficiency, endoconidial suspensions were prepared as above, except that mycelial mass was instead taken from 7 or 14 day old streak cultures.

Mycelia

To test the effect of mycelia on transformation efficiency, either the mycelia was scraped from a 4 day old streak culture plate (section 2.2.7.) or a 100µl aliquot of induced AGL1 [pBHt2] was added directly onto a 4 day old streak culture plate, growing on IMAS (1.5% agar) medium.

Germinating Endoconidia

When testing the effect of endoconidia germination on transformation efficiency, endoconidia were separated from the 4 day old total mycelia as for preparation of non-germinating endoconidia (above), except that they were filtered directly into a 250ml flask containing 50ml of PDB. The flask was incubated at 25°C, 120rpm for 2 hrs, after which, the progress of endoconidia germination was checked using the Improved Neubeur Counting Chamber. The percent of germinating conidia from total conidia was calculated and incubation was allowed to proceed until 50-70% of the spores had germinated (~3.5 hours).

The germinating endoconidia were transferred to 2 x 50ml polypropylene tubes (25ml/ tube) and centrifuged at 3,500rpm, 4°C, for 20min. The supernatant was carefully removed and the pellet resuspended in 25ml sterile dH_2O, followed by a second centrifugation as above. The dH_2O wash was repeated 2-3 more times, each pellet resuspended in 1ml IM (Appendix 1) and then combined (i.e. 2ml

total) into a new 50ml tube. A final concentration of $1.0x10^6$ germinating endoconidia/ml was achieved as for non-germinating endocondia described above.

2.2.9.3. Determining the Minimal Inhibitory Concentration of HygB for T. basicola

To determine the minimal inhibitory concentration (MIC) of HygB for *T. basicola,* 100µl aliquots of a $1.0x10^6$ endoconidial suspension (section 2.2.9.2) were spread onto IMAS (1.5% agar) medium (Appendix 1) and incubated at 25°C for 2 days. Plates were overlayed with 20 ml ½ PDA (2.2% agar) medium, supplemented with 5, 7, 10, 15, or, 20µg/ml of Hygromycin B. Cultures were incubated at 25°C for 12 days before recording the presence of fungal growth on top of the agar overlay.

2.2.9.4. Co-Cultivation of A. tumefaciens with T. basicola

One hundred µl aliquots of *T. basicola* spore suspension (1.0×10^6) were mixed with an equal volume of induced *A. tumefaciens* culture in a sterile microcentrifuge tube. To test the effect of bacterial to fungal cell ratios on transformation efficiency, 2ml aliquots of induced *Agrobacterium* pre-cultivation cultures were centrifuged for 5min at top speed in a table top microcentrifuge and the pellet then resuspended in 1-2ml of fresh IM, supplemented with 200µM AS. Dilutions were prepared in IM to achieve the ratios of 1 endoconidium: 10, 100, 250, 500, 1000, and 2000 bacterial cells. In all other experiments, the *Agrobacterium* aliquot was taken directly from the pre-cultivation culture without further dilutions.

A 200µl aliquot of the *T. basicola* and *A. tumefaciens* mixture was then spread, using a sterile glass spreader, onto the surface of IMAS (1.5% agar or agarose) medium, adjusted to pH 4.8 or 6 (Appendix 1). When filters were used for co-cultivation, the 200µl aliquot was spread onto a sterile black filter (Schleicher&Schuell Ø 90mm) or white filters (Whatman Ø 90mm) that overlayed the IMAS (1.5% agar) medium. Co-cultivation plates were incubated at 25°C or 28°C for 2, 3, or 7 days.

2.2.9.5. Selection for T. basicola Transformants

Selective Top Agar

Twenty- to twenty five ml ½ PDA (2.2% or 1.2% agar) medium, supplemented with 22.5 µg/ml or 112 µg/ml HygB to positively select for *T. basicola* transformants and 675µg Mefoxin (Appendix1) to negatively select against *A. tumefaciens* was poured over the IMAS plate containing the co-cultivants. Since the antibiotics was expected to diffuse from the top agar to the bottom agar, when calculating the amount of each antibiotic added to the medium, the volume of the bottom agar had to be taken into account to ensure that the desired final concentration was maintained (i.e. 10 or 25 µg/ml HygB and 300 µg/ml Mef). The top agar was poured onto the plate using a sterile measuring cylinder, positioned at the very edge of the plate. Pouring was done very slowly so as to minimise the spreading of spores.

Filter Transfer

Where filter paper was used, the entire filter, containing the co-cultivants was transferred using sterile tweezers, onto M-100 (1.5% agar) or ½ PDA (2.2% agar) medium, supplemented with 10 or 25µg/ml HygB and 300µg Mefoxin. The filters were placed onto the selective plate spread side up. As a control, *T. basicola* was also tested for its ability to grow on M-100 minimal medium under non-selective conditions by stabbing the WT into non-selective M-100 (1.5% agar) and ½ PDA (2.2% agar), medium incubated at 25^{o}C for 10 days.

Selection plates from both methods were incubated at 25^{o}C for up to 24 days. Observation and recordings of colony growth were made every 3-4 days.

2.2.9.6. Further Selection and Isolation

To confirm HygB resistance and isolate individual colonies of Hyg^{R} putative transformants for further manipulations, fungal colonies successfully grown on initial selective medium (either filter transfer or top agar) were transferred onto ½ PDA (1.2% agar) medium with 0µg, 10µg, or 25µg/ml Hyg B and incubated at 25^{o}C for 2 days-4 weeks (depending upon the HygB concentration used). The flowchart in Figure 2.4, gives an overview of the selection and isolation procedure and indicates the antibiotic concentrations used as well as the types of transfer performed at each step. The methods of transfer tested are briefly described below.

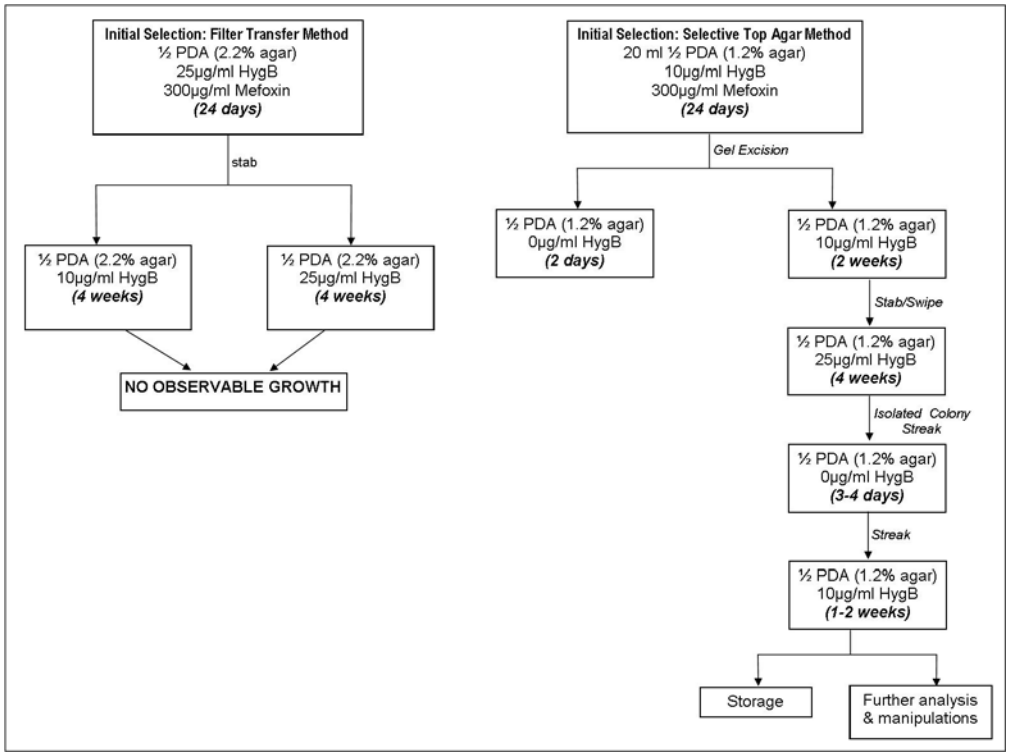

Figure 2.4. Further Antibiotic Selection and Colony Isolation Flow Chart.

Gel Excision Transfer

This type of transfer was performed when transferring colonies from top agar selective plates. Small pieces of fungal colonies were excised from the agar using a sterile blade. Only those colonies that appeared close to or on top of the agar overlay were excised. The agar pieces were placed onto ½ PDA selective medium plate, divided into 12 squares, and spread out as much as possible within the designated square.

Stab or Swipe Transfer

This method was used to transfer the fungi that grew on the filter paper (i.e. filter transfer method) or from the small gel pieces (i.e. selective top agar method). When swiping, a sterile inoculation loop was used to obtain a loop full of mycelia, which was swiped three times within a designated square on the ½ PDA selective medium plate. If stabbing, a sterile needle was used and a needle tip full of mycelia was inserted once into the centre of each square. Fungal cultures were transferred from gel pieces, only after the mycelia had begun to spread from the agar pieces onto the surrounding medium within the square.

Isolated Colony Streak Transfer

This method was used to produce a single isolated colony of each Hyg^R putative transformant and was performed using the standard microbiological technique of streak diluting to achieve individual colonies on ½ PDA.

Streak Transfer

This transfer was performed on the isolated colonies. A sterile inoculation loop was used and loop full of mycelia was streaked in a star shape on ½ PDA medium (55x14mm plates).

For all ATMT experiments, a minimum of 3 replicates was prepared for co-cultivation and initial selection plates. Positive and negative controls of both *A. tumefaciens* and *T. basicola* were always included. Two to three replicates of every Hyg^R putative transformant was prepared and underwent further confirmation of HygB resistance and isolation before any manipulations (e.g. Southern blot analysis, pathogenicity screening, mitotic stability, and general characterisation tests) and long term storage.

2.2.10. Confirmation of T-DNA Integration: Southern Blot Analysis

2.2.10.1. Preparation of the hph and Km DIG-Labelled Probes

Preparation of hph Probe Template DNA

The hph probe (Figure 2.5a) was derived from the plasmid pBHt2 and consisted of a 1.8kb fragment constituting the *hph* gene and left border of the T-DNA. Template DNA was generated by PCR amplification using the primers HYKasF1 and LBKasR2 (GeneWorks; Table2.3), which have complementary sequences within the T-DNA of the plasmid. A master mix was first prepared by adding the following reagents to a sterile 1.5ml microcentrifuge in this order:

Reagent	Volume
x10 Taq polymerase Buffer*	2.5µl
10mM dNTPs mix (Fischer Biotech; 2.5mM of each dATP, dCTP, dGTP, and dTTP).	0.75µl
20pm/µl forward primer (GeneWorks)	0.5µl
20pm/µl reverse primer (GeneWorks)	0.5µl
x10 BSA (Sigma)	2.5µl
Sterile miliQ	16.25µl
Taq polymerase	1µl
Total	24µl

(* homemade prepared by Godwin, S., 2006; Appendix 1).

After preparation of the master mix, 24µl aliquots were transferred into sterile 0.5ml microcentrifuge tubes and 1ul of 0.5-10ng template DNA added to each tube.

PCR was performed as follows: initial denaturation at 94^{o}C for 1min, followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 68^{o}C for 30 sec, and elongation at 72^{o}C for 90sec. After the 30 cycles were complete, a final elongation step was performed at 72^{o}C for 5min followed by incubation at 4^{o}C until samples were removed (max up to 16hrs at 4^{o}C).

A gel check was performed to ensure both the presence of a single PCR product and its position at the expected size. Template DNA was cleaned using the QIAquick PCR Purification Kit using the microcentrifuge (according to manufacturer's instructions) and eluted in 50µl total of 10mM Tris-Hcl (pH8.5).

Table2.3 : Forward and Reverse Primer Set Used in PCR Amplification for DIG-Labelled hph Probe Template

Primer	***Sequence***
HYKasF1 (Forward)	5'-aatcggccaaggcgccgggagaggcgg-3'
LBKasR2 (Reverse)	5'-ggctctcccgctggcgccgtcccgg-3'

Preparation of Km Probe Template DNA

The Km probe was derived from the plasmid pBHt2 and consisted of a 2.9kb fragment that contained the Km^R gene (Figure 2.5b). Template DNA was generated by double digestion of ~12µg of pBHt2 with the restriction enzymes *Sac*II (New England Biolabs, 20U/µl) and *Nhe*I (New England Biolabs, 10U/µl) in 50µl total reaction volume. After O/N digestion, gel analysis was performed to ensure complete digestion of the plasmid and the presence of the two expected bands for pBHt2 when digested with *Sac*II and *Nhe*I (i.e. 5.5kb and 2.9kb).

After heating the reaction sample at 65°C for 10min to inactivate the REs, the entire sample was run on a preparative gel at 80V for 1hr to separate the two fragments. The 2.9kb fragment was excised from the gel and cleaned using the QIAGEN gel extraction/purification microcentrifuge protocol (following the manufacturer's instructions). The cleaned template DNA was eluted in a total volume of 60µl Tris·Cl pH 8.5. To concentrate the DNA, ethanol precipitation was carried on the cleaned 2.9kb Km fragment and eluted in 30µl total volume.

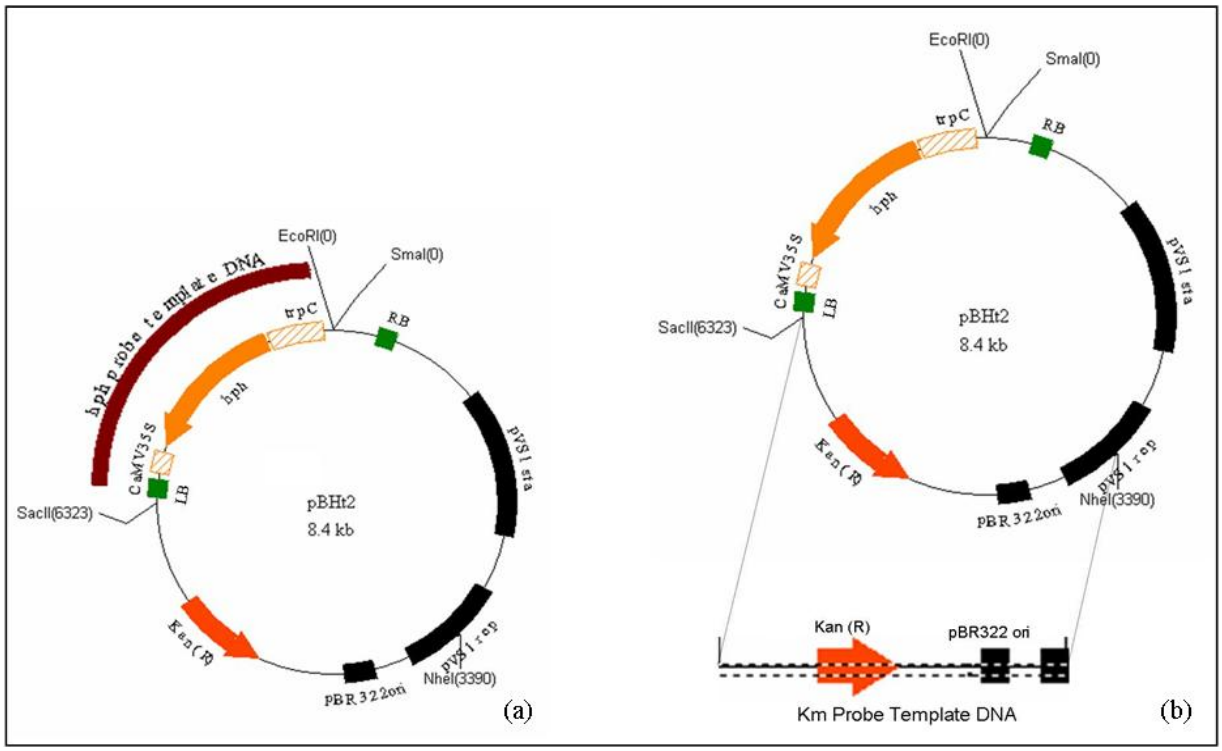

Figure 2.5. Generation of the hph and Km DIG-Labelled Probe Template DNA. (a) Generation of the 1.8kb hph probe template DNA from the plasmid pBHt2 by PCR amplification using the forward primer HYKasF1 and the reverse primer LBEcoR1. (b) Generation of the 2.9kb Km probe template DNA from the plasmid pBHt2 by double digestion with the restriction enzymes *Sac*II and *Nhe*I.

DIG-Labelling Reaction

Just prior to labelling, 1µg of DNA template was transferred to a sterile 1.5ml eppendorf and placed in a boiling water bath for 10min in order to denature the DNA into single strands. The denatured sample was then immediately chilled on ice for 30-60 sec.

Large scale labelling reactions of 100µl total volume were prepared for both the hph and Km probes with reagents added in the following order:

10x Hexanucleotide mix (Roche)

10x DIG DNA Labelling Mix (Roche)

bring to 95µl with sterile miliQ water

5 µl Klenow enzyme 1U/µl (Pharmacia)

After adding all reagents, the reaction sample was gently mixed by tapping/flicking the tube, and briefly zip spun to ensure that no solution remained on the tube walls. The labelling reaction was then incubated at 37^{o}C for 16-20hrs.

To terminate the DIG-labelling reaction, 0.1 volume of 0.25M EDTA was added to the tube and gently mixed by pipetting. Labelled DNA was precipitated from solution by the addition of 0.1 volume of 4M LiCl and 3 volumes of chilled (at -20^{o}C) absolute ethanol and mixed well by gently inverting the tube 5-6 times, followed by a brief zip spin. Samples were then incubated at -70^{o}C for 30min.

DNA was pelleted by centrifuging the solution at 13,000g for 15min in a table top microcentrifuge at 4^{o}C. Ethanol was then decanted and the pellet was washed in 100µl of chilled (at -20^{o}C) 70% ethanol and centrifuge as before but for 5 min. The ethanol was decanted and the DNA pellet was dried by incubation the open tube at 37^{o}C for ~15 min. The dried DNA was resuspended in 50µl 1x TE and stored at -20^{o}C until needed.

Probe Check - NBT/BCIP Colour Detection

To confirm successful DIG labelling and estimate the concentration of labelled probe in a reaction mix, a probe check was performed. Ten-fold serial dilutions were prepared for labelled control DNA (Roche DIG Nucleic Acid Detection Kit Vial 1) diluted in DNA buffer (Roche DIG Nucleic Acid Detection Kit Vial 2) and for experimental DNA diluted in 10x TE buffer as described in Table 2.4.

Table 2.4. Serial Dilutions for DIG Labelled Probe Check

	Labelled Control DNA Stock	Stepwise Dilution	Final Concentration	Total Dilution
A	20ng/µl	2 µl/ 38µl	1 ng/µl	1:20
B	1 ng/µl	5 µl/45 µl	100 pg/µl	1:200
C	100 pg/µl	5 µl/45 µl	10 pg/µl	1:2,000
D	10 pg/µl	5 µl/45 µl	1 pg/µl	1:20,000
E	1 pg/µl	5 µl/45 µl	0.1 pg/µl	1:200,000
F	0.1 pg/µl	5 µl/45 µl	0.01 pg/µl	1:2,000,000

One µl of control and experimental dilutions (B-F) were applied onto the surface of a positively charged nylon membrane. DNA was fixed to the membrane by cross-linking with UV light for 3min on a UV transilluminator. The membrane was washed in Buffer 1 (Appendix 1) for several minutes, incubated for 30min in Buffer 2 (Appendix 1), and then incubated for 30min in diluted Anti-DIG-alkaline phosphatase (Roche DIG Nucleic Acid Detection Kit Vial 3) solution (Appendix 1).

The membrane was washed for 15 min twice in Buffer 1 and then once for 2min in Buffer 3 (Appendix 1). For colour detection to occur, the membrane was incubated in freshly prepared NBT/BCIP (Roche DIG Nucleic Acid Detection Kit Vial 4) solution (Appendix 1). Detection occurred in the dark and was allowed to proceed until the desired intensity was achieved (up to 16 hrs). The reaction was terminated by washing the membrane in sterile miliQ water and stored in 1x TE to preserve the colour.

2.2.10.2. DNA Preparations

Fungal Genomic DNA Extraction

A 3-4 day old fungal culture plate was scraped into 200ml PDB in a 1L flask and incubated for 3-5 days at 25^{o}C and 120rpm. Mycelia were harvested by pouring the contents of the flask into a funnel lined with mira cloth and then washing the mycelia, which remained in the cloth, with ~1L of sterile pre-chilled miliQ water.

The mycelia were transferred onto paper towelling and blotted dry, then wrapped in foil and freeze-dried O/N. One hundred mg of the fresh dried mycelia was transferred into a sterile 2ml microcentrifuge tube and a 20mm stainless steel ball was added. Tubes were placed in the QUIGEN Tissue Lyser for 1 min to grind the samples to a fine mycelial powder. To lyse the cells, 1ml 10x TES (Appendix 1) was added to the tube, the content gently mixed by inverting several times, and then incubated at 65^{o}C for 1hr, gently mixing the sample every 15min.

After incubation, the sample was spun in a bench top microcentrifuge at full speed for 5min and the supernatant transferred to a sterile 2ml microcentrifuge tube. To precipitate proteins and other cellular contaminants, 200µl chilled (at -20^{o}C) 5M potassium acetate (Appendix 1) was added to the supernatant, mixed gently by inversion and incubated on ice for 1hr. The sample was centrifuged at full speed for 15min in a table top microcentrifuge and the resulting supernatant (containing the genomic DNA) transferred to a sterile 2ml microcentrifuge tube. An equal volume of room temperature isopropanol was added to the supernatant, gently mixed by inverting, and centrifuged at full speed for 2 min. To wash the DNA pellet, the isopropanol was drained, the tube was blot on paper towelling, and 1ml of chilled (-20^{o}C) 70% ethanol was added to the DNA pellet. After briefly mixing, the tube was centrifuged for 1min at full speed in a bench top microcentrifuge and all ethanol drained from the tube. After repeating the ethanol wash several times, all traces of ethanol was removed by briefly zip spinning the tube and pipetting off any remaining ethanol. The pellet was dried by leaving the tube lid open and incubating at 37^{o}C for 10-30min (depending upon the size of the DNA pellet). To dissolve the DNA pellet 100-200µl of 1x TE buffer (Appendix 1) was added and then mixed by flicking the tube several times, before leaving on the bench O/N.

If the pellet was still not dissolved the following morning, another 100-200µl of 1x TE was added to the tube and again mixed by flicking and incubating on the bench for several hrs. DNA concentration was estimated by running 1-2µl of the genomic DNA on a 0.8% midi gel and if required, DNA samples were further concentrated by ethanol precipitation and eluted in a total of 50-100µl of Tris·Cl pH 8.5. Dissolved DNA samples were stored at -20^{o}C.

NOTE: prior to running a gel of fungal genomic DNA, RNAseA (10mg/ml; constituting no more than 10% of the reaction total volume) was added to each DNA sample in order to degrade any contaminating RNA present in the genomic DNA. For gel analysis, ~5µl total reactions were prepared and for Southern analysis, ~30µl. After addition of RNAseA, the tubes were incubated at 37^{o}C for 30-60min before running the gel.

Digestion of Genomic and Plasmid DNA

A 1-1.5µg of fungal genomic DNA was digested with (1) *Nhe*I (10U/µl New England Biolabs) (following manufacturer's guidelines) in 25µl total reaction and incubated O/N at 37^{o}C.

Control digestions of pBHt2 and pPK2 were prepared with *Nhe*I (10U/µl New England Biolabs) and *Kpn*I (10U/µl Pharmacia) respectively. For pPK2, 17.5ng of plasmid DNA was digested in a 10µl total reaction, incubated at 37^{o}C for 3hrs, and complete digestion confirmed by running sample on a 0.8% mini gel. For pBHt2, 245ng of plasmid DNA was digested and confirmed as for pPK2, following which, the linearised plasmid solution was diluted to a final concentration of 1.5ng/µl.

Two µl aliquots of each genomic DNA digest were run on a midi gel to confirm complete digestion. The remaining genomic DNA, along with 4µl of control pBHt2 and pPK2 plasmid digests, and 5 µl of diluted (1ng/µl) control hph and Km templates (each constituting ~5ng of plasmid DNA), were run on a maxi gel at 40v for 16-18 hrs. The gel was stained for 30-60min and then visualized and photographed under the uv transilluminator with a ruler alongside the gel, with 0cm aligned with the bottom of the wells. The gel was then trimmed at the top to remove the wells, at the left and right sides to remove the λ-*Hind*III and 1kb ladder respectively, and at the bottom to remove the digested low molecular weight RNA.

2.2.10.3. Southern Blot Procedure

All washes were performed in enough solution to cover the gel or membrane and incubated with gentle shaking on an environmental shaker.

DNA Denaturation and Neutralization

To denature and neutralize the DNA in situ, the gel was washed in 0.25M HCl acid, then in 0.5M NaOH/1M NaCl, and 0.5M Tris-HCl/1.5M NaCl pH7.4 (Appendix 1). Each of these three washes was performed twice for 15 min each and the gel rinsed in between each wash in sterile dH_2O for 2min.

Transfer to Nylon Membrane

A thick sheet of glass was placed atop a pyrex dish and 3 pieces of 3MM filter paper (Whatman) were cut to a size ~12cm longer than the glass plate and ~2cm wider than the gel. The filter paper was placed on top of the glass plate with the ends overhanging into the pyrex dish containing 20x SSC (Appendix 1) and left for ~30min to soak up the SSC by capillary action. The gel was then placed on top of the moist filter papers. A positively charged nylon membrane (Roche) was cut to the same size as the gel and briefly soaked in sterile dH_2O, followed by soaking in 20x SSC, and then placed on top of the gel. Air bubbles were removed from the filter papers and nylon membrane.

To ensure the 20xSSC was soaked only via the gel, parafilm strips were placed on top of the filter paper that surrounded the gel. To prevent drying out of the gel, cling wrap was positioned on the sides of the gel and over the glass pyrex dish. Three pieces of 3MM filter paper, slightly larger than the size of the gel were then placed atop the membrane, followed by a 3-4cm wad of filter paper. A glass plate was positioned atop of the setup and weighed down by a 1kg bottle. DNA transfer was left to proceed over night. The DNA was then cross-linked onto the nylon membrane by placing the membrane DNA side down on a UV transilluminator for 3-5 min and the membrane then dried at 37^{o}C for ~30min.

Hybridization with DIG-Labelled Probe

The membrane was sealed inside plastic sheeting with 20ml of hybridization solution (Appendix 1) and incubated at 37^{o}C for 6hrs. The pre-hybridization solution was removed and replaced with 10ml of

freshly prepared probe/hybridisation solution (Appendix 1), which contained the DIG-labelled probe. The membrane was then incubated at 37^{o}C O/N.

To remove excess unbound probe, the membrane was washed twice in pre-heated (at 65^{o}C) 2x SSC, 0.1% SDS for 5 min each, twice in 0.1X SSC, 0.1% SDS for 15 min each, and then once in washing buffer (Appendix 1) for 1min.

Detection by Chemiluminescence

The membrane was incubated at room temp for 30min in Buffer 2 (Appendix 1), and then 30min in Anti-DIG-AP conjugate solution (Appendix 1). Excess antibody solution was removed by washing the membrane in washing buffer for 15 min twice.

The membrane was equilibrated for 5min in Buffer 3 (Appendix 1), transferred into fresh plastic and the CSPD substrate solution (Appendix 1) was added. After gently massaging the solution over the entire membrane 6-8 times, excess substrate and air bubbles were removed and the plastic pocket was sealed. The membrane was placed DNA side up into a pre-warmed (at 37^{o}C) cassette, incubated at 37^{o}C for 15min, and then a piece of Kodak X-ray film was placed emulsion side down onto the sealed membrane. Two exposures were taken at 30min and 2 days and each film was placed in developer for 2min, rinsed in water for 1min, placed in fixer for 5 min, and again rinsed in water for 5min.

Stripping and Re-Probing the Membrane

The membrane was removed from the plastic bag and washed twice in pre-heated (at 56^{o}C) 0.2M NaOH, 0.1% SDS for 15 min at 37^{o}C. Three washes in 2x SSC for 5min each were then performed before storing the membrane in a fresh plastic bag containing 10ml 2x SSC at 4^{o}C. It was important not to let the membrane dry out at any stage of this stripping procedure. The membrane could be re-used by following the methods of hybridisation and detection described above.

2.2.11. Mitotic Stability

Ten putative transformants were randomly selected to examine the stability of the T-DNA insert. Putative transformant (10/ plate plus a WT) were subcultured every 3 days on unamended ½ PDA (1.2% agar) medium and then replicated on ½ PDA (1.2% agar) medium supplemented with 10µg/ml HygB. The latter was used to determine at what generation the putative transformant lost its resistance to HygB. After 5 generations without selective pressure, a final transfer was performed onto one ½ PDA (1.2% agar) medium supplemented with 10µg HygB and a second supplemented with 25µg HygB; these plates were grown for 14-28 days before final recording. Putative transformants were classified as mitotically stable if they remained resistant to HygB after being subcultured for 5 generations on unamended medium. Two replicates of each isolate were used.

2.2.12. Confirmation of T-DNA Insertion by PCR

Confirmation of T-DNA insertion was also performed by PCR. The same set up and reaction conditions were used as for preparing the hph probe template DNA (section 2.2. 11.1) except that fungal genomic DNA was used rather than pBHt2 plasmid DNA.

2.2.13. Pathogenicity Test- Screening for Putative Transformants with Reduced Pathogenicity

Cotton Seed Sterilisation

Cotton seeds were first soaked in sterile dH_2O for 5min, drained and then soaked in surface sterilisation solution (Appendix 1) for 5min. The seeds were rinsed in sterile dH_2O for 1min with regular shaking. The dH_2O wash was repeated 5 times before transferring the seeds to YMA (1.5% agar) medium (Appendix 1) using sterile forceps. Five to six seeds were placed on each YMA medium plate and then grown at 25^{o}C for 2 days.

Root Dip Assay

Ten Hyg^R putative transformants (the same 10 tested for mitotic stability) were assessed for reduced pathogenicity. For primary screening, the mycelial growth from a 4-day old streak culture, grown on ½ PDA (2.2% agar) medium (Appendix 1) was scraped into 5ml sterile dH_2O and vortexed for ~30sec. Two-day old sterile cotton seedlings were dipped in the fungal suspension, (containing a mixture of mycelia, endoconidia, and chlamydspores) for ~1min with occasional shaking. Control seedlings were dipped in sterile dH_2O (i.e. negative control) or WT fungal suspension (i.e. positive control). Each seedling was transferred to a separate water agar (1.2% agar) medium plate (55x14mm) (Appendix 1) and grown at 25^{o}C. Seven days after inoculation, the total root length and length of root lesions was measured (in cm) using a ruler. Roots were also examined under the stereomicroscope for the presence of chlamydospores. Five replicates were prepared for each isolate.

2.2.14. General Phenotypic Characterisation Tests

Stab cultures of the same 20 Hyg^R putative transformants (which included the 10 that underwent mitotic stability), plus the WT were prepared as described in section 2.2.7. After 3 days, standard vegetative growth was assessed by measuring the colony diameter (in cm) using a ruler. Colony morphology (i.e. texture, colour, and patterning) and presence of chlamydospores was assessed by naked eye and stereomicroscope analysis. Experiments were repeated five times with two replicates of each isolate per experiment.

2.2.15. HygB Resistant Growth Tests

Ten putative transformants (the same 10 tested for mitotic stability) were grown as for the phenotypic characterisation tests (2.2.14), except that the medium was supplemented with 10µg/ml and 25µg/ml HygB. Experiments were repeated six times with two replicates of each isolate per experiment.

2.2.16. Green Fluorescence Microscopy

Liquid cultures of ten putative transformants containing the T-DNA of pCAMgfp, and the WT were prepared for fluorescence microscopy by scraping a 3-4 day old ½ PDA (1.2% agar) culture plates into 10ml PDB in a 50ml flask and incubated at 25^{o}C for ~5hrs. Glass slides were prepared with 1-2 drops of liquid culture and viewed under the Olympius BHF2 Fluorescent microscope, fitted with a 550nm barrier filter and 490nm blue excitation filter, at 10-40x objectives. Pictures were taken with Nikon Coolpix 5400 camera.

2.2.17. Plasmid Construction

The construction of two plasmids, pMAX and pAIM3 was performed in order to attempt plasmid rescue of T-DNA tagged fungal genomic sequences.

2.2.17.1. pMAX

To construct the vector pMAX, a 2kb *Eco*RI/*Bsa*AI fragment, containing the pBR322 Amp^{R} gene and ori (Figure 2.6c), was cloned into the multiple cloning site (MCS) of the 8.4kb binary vector pBHt2 digested with *Eco*RI and *Sma*I. Since the RE sites in pBHt2 are located in close proximity, two separate double digests of pBHt2 with (1) *Eco*RI and *Nhe*I and (2) *Sma*I and *Nhe*I first had to be performed. Both double digests produce a 5kb and 3.4kb fragment: the *Eco*RI/*Nhe*I 5kb fragment (Figure 2.6a) and *Sma*I/*Nhe*I 3.4kb fragment (Figure 2.6b) are the desired products. Thus a tri-ligation of the 5kb, 3.4kb and 2kb fragments was required to generate this 10.4kb pMAX plasmid (Figure 2.6d).

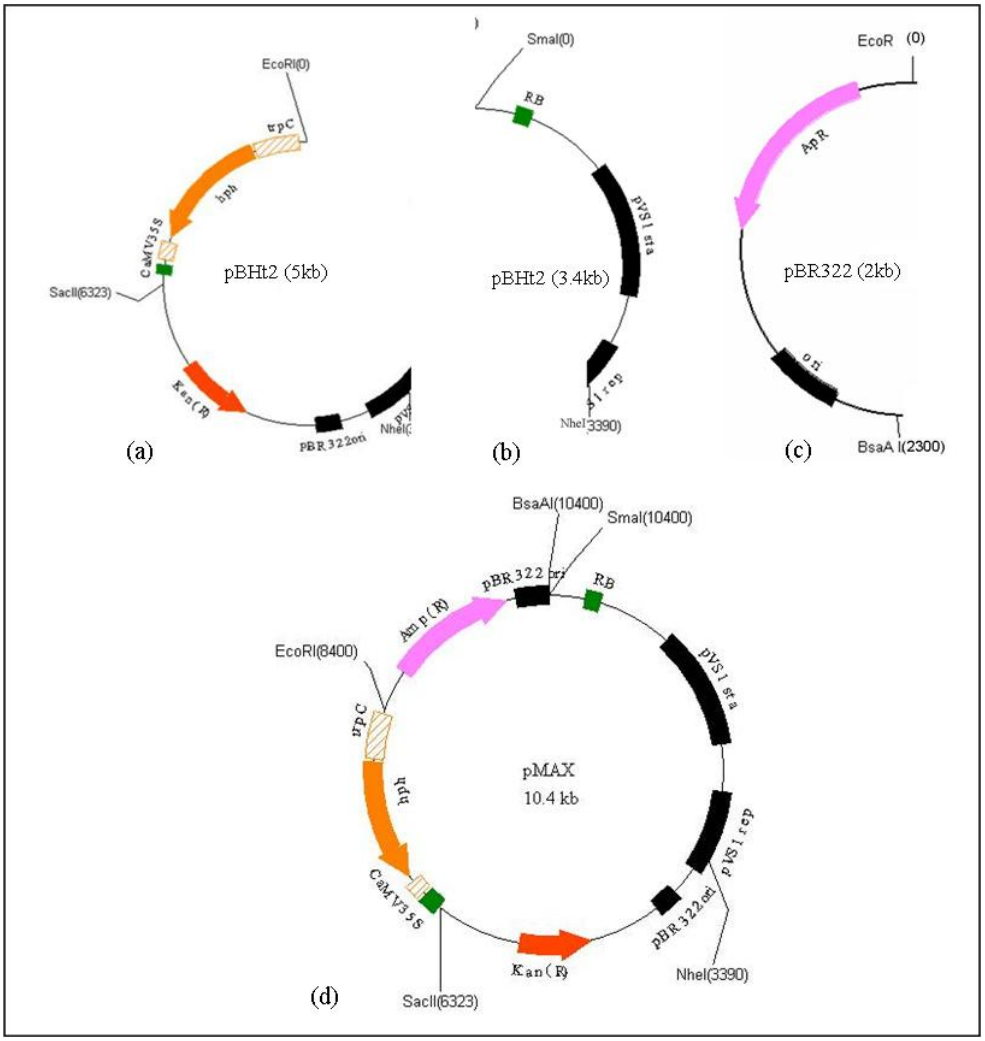

Figure 2.6. Construction of the Binary Vector pMAX. (a) The 5kb fragment generated by double digestion of the binary vector pBHt2 with the restriction enzymes *Eco*RI and *Nhe*I. (b) The 3.4kb fragment generated by double digestion of the binary vector pBHt2 with the restriction enzymes *Sma*I and *Nhe*I. (c) The 2kb fragment generated by double digestion of the plasmid pBR322 with the restriction enzymes *Eco*RI and *Bsa*AI. (d) The 10.4kb binary vector pMAX constructed by tri-ligation of the 5kb (a), 3.4kb (b) and 2kb (c) fragments.

To generate the 5kb pBHt2 fragment, 1.5µg pBHt2 was double digested with *Eco*RI (Promega 12U/µl) and *Nhe*I (New England Biolabs 10U/µl) in a 50µl total volume. For generation of the 3.4kb fragment, 1.5µg of pBHt2 was double digested with *Sma*I (New England Biolabs 5U/µl) and *Nhe*I (New England Biolabs 10U/µl) in 50µl total volume. After confirming complete digestion, the remainder of each digest was run on a midi gel at 80V for 60 min and then stained in ethidium bromide for 10min. The appropriate fragments were excised from the gel and cleaned using the QIAQuick Gel Extraction Purification Kit, following the manufacturer's instructions and DNA was eluted in 30µl total volume.

To generate the 2kb fragment, 60ng of pBR322 were double digested with the restriction enzymes *EcoRI* (Promega 12U/µl) and *BSa*AI (New England Biolabs 5U/µl) in 20µl total volume. After confirmation of complete digestion, the sample was cleaned using the QIAQuick PCR Purification Kit, following the manufactuer's instructions and DNA was eluted in 30µl total volume.

Tri-ligation, was performed using ~50ng of the 5kb fragment, 100ng of the 3.4kb fragment, and 200ng of the 2kb fragment and ligating using T4 DNA ligase (Promega 3U/µl) in 15-25µl total volume. The ligation solution was incubated for 16 hrs at 16^{o}C, after which T4 ligase was heat inactivated at 65^{o}C for 10min.

To confirm successful construction and functionality of pMAX, two strains of competent *E.coli* cells, DH5α (prepared as described in section 2.2.3) and JM109 (Invitrogen) were transformed. Transformation of DH5α was performed as described in section 2.2.4. with the following modifications; 10µl of ligation solution was used (a ratio of 1:10 ligation solution to competent cells) and transformed cells were recovered in 600-800µl LB liquid. Transformation of JM109 was performed as for DH5α, except that 10% of all volumes were used. One hundred µl aliquots of transformed cells were plated onto LB (1.6% agar) medium supplemented with (1) Amp, (2) Km, or (3) Amp+ Km. Medium plates were incubated O/N at 37^{o}C.

2.2.17.2. pAIM3

Construction of pAIM3 was performed as described by Leclerque et al., 2004.

For amplification of the ~250bp linker fragment from pBHt2, the same reagent setup was used as in section 2.2.11.1. except that the primers LBEcoF1 and LBKasR1 (GeneWorks) were used (Table 2.5.)

PCR was performed as follows: Initial denaturation at 94^{o}C for 1min, followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 54^{o}C for 30 sec and elongation at 72^{o}C for 60 sec. After the 30 cycles were complete, a final elongation step was performed at 72^{o}C for 5min followed by incubation 4^{o}C until samples were removed (max up to 16hrs at 4^{o}C).

Table 2.5. Forward and Reverse Primers used to Generate the 250bp Linker Fragment for pAIM3 Construction

LBEcoF1	CTC GGC ACG AAT TCA CCA CTC
LBKasR1	CAT AGT ATC GAC GGC GCC GAT

For amplification of the 1.8kb fragment containing the *hph* gene from pBHt2, the same reagent setup and PCR conditions was used as in section 2.2.11.1.

The construction of pAIM3 was stopped at this stage due to time restriction.

2.3. Results

For a comprehensive list of all parameters tested in establishing the ATMT protocol see Appendix 2 and for a detailed list of the published conditions of ATMT used by various other researchers in transforming a variety of fungi see Appendix 3.

2.3.1. Optimal Conditions for *A. tumefaciens* Pre-Cultivation Growth and Induction

It was found that for both pre-cultivation growth and induction, AGL1 and LBA4404 were best grown at 28^{o}C and 250rpm. Induction was achieved by growing the cells in 200µM AS for 7hrs. For AGL1, this corresponded to an OD_{660nm} 0.6-0.8 and ~10^{7}-10^{9} viable colony forming units. The OD_{660nm} was not assessed for LBA4404 since the clumping nature of this strain during liquid growth would give incorrect readings.

2.3.2. Co-Cultivation of *A. tumefaciens* and *T. basicola*

Note: Since these were found to be optimal conditions, the experiments presented in Tables 2.6 to 2.10 utilise *A. tumefaciens* pre-induced in the presence of 200µM AS for 6-8hrs at 28^{o}C and 250rpm. One hundred µl aliquot of bacterial cells and an equal volume of 4-day old endocondia (10^{6}/ml) were co-cultivated on IMAS (1.5% agar) medium. HygromycinB resistant (Hyg^{R}) putative transformants were selected with 20ml of ½ PDA 1.2% top agar amended with final concentrations of 10µg Hygromycin B (HygB) /300µg Mefoxin (Mef) and incubated at 25^{o}C for up to 24 days.

2.3.2.1. Effect of Co-Cultivation Temperature on Transformation Efficiency

Table 2.6 indicates that a higher number of *T. basicola* Hyg^{R} putative transformants were produced by both bacterial strains when co-cultivation with *T. basicola* endoconidia was carried out at 25^{o}C compared to 28^{o}C.

Table 2.6. Effect of Co-Cultivation Temperature on Transformation Efficiency.

Strains	25^{o}C	28^{o}C
AGL1 [pBHt2]	80	40
LBA4404 [pBHt2]	70	40

AGL1 [pBHt2] and LBA4404 [pBHt2] were co-cultivated with *T. basicola* at 25^{o}C or 28^{o}C for 2 days. Values represent the number of HygB resistant colonies produced/ 10^{6} endoconidia. For AGL1, values are the mean of 4 replicates and for LBA4404 values are the mean from 6 replicates.

2.3.2.2. Effect of Co-Cultivation Duration on Transformation Efficiency

Table 2.7 shows that both *A. tumefaciens* strains carrying the plasmid pBHt2, generated Hyg^R putative transformants within 2 days of co-cultivation with *T. basicola* endoconidia. Regardless of the strain, the highest transformation efficiency was seen after 3 days compared to 2 or 7 days. For AGL1 [pBHt2], after 7 days co-cultivation, the efficiency of transformation decreased by 62%.

Table 2.7. Effect of Co-Cultivation Period on Transformation Efficiency.

A. tumefaciens Strains	2 Days	3 Days	7 Days
AGL1[pBHt2]	130	160	60
LBA4404[pBHt2]	70	90	*n.t.

AGL1 [pBHt2] or LBA4404 [pBHt2] were co-cultivated with *T. basicola* at 25°C for 2 , 3, or 7 days. Values represent the number of Hyg^R putative transformants produced/ 10^6 endoconidia and are the mean of 6 replicates. *LBA4404[pBHt2] was not tested for 7 days growth.

2.3.2.3. Cell Ratios: The Effect of Bacterial Cell to Endoconidium on Transformation Efficiency

To test the effect of bacterial cell concentration on transformation efficiency, ratios ranging from 10-2,000 bacterial cells per endoconidium were tested. Table 2.8 shows that as the number of endoconidium:bacterial cells increased from 1:10 to 1:250, so did the transformation efficiency. The maximum number of Hyg^R putative transformants, (180 Hyg^R putative transformants/10^6 endoconidia transformed) was achieved at ratio of 1:250, after which, the efficiency began to decline. A higher number of Hyg^R putative transformants were obtained at ratios below the optimum compared to ratios above the optimum, e.g. a 1:10 ratio produced 80 Hyg^R putative transformants / 10^6 endoconidia, while the 1:2,000 ratio produced 10 Hyg^R putative transformant/10^6 endoconidia.

Table 2.8. Effect of *A. tumefaciens* and *T. basicola* Ratios on Transformation Efficiency.

A. tumefaciens Strain	1:10	1:100	1:250	1:500	1:1000	1:2000
AGL1[pBHt2]	80	80	180	50	50	10

Ratios of *T. basicola* conidia to AGL1[pBHt2] cells from one conidium to 10, 100, 250, 500, 1,000, and 2,000 bacterial cells was tested. Co-cultivation was carried out at 25°C for 3 days. Values represent the number of Hyg^R colonies produced/ 10^6 endoconidia and are the mean of 4 replicates.

2.3.2.4. Co-Cultivation Media - the Effect of pH and Glucose on Transformation Efficiency

To test the effect of suppressed bacterial growth and enhanced fungal growth during co-cultivation the pH of the IMAS (1.5% agar) co-cultivation medium was lowered from pH 6 to pH 4.8. After co-cultivation on this medium, *T. basicola* growth appeared as small dense patches, thick with chlamydospores and *A. tumefaciens* growth was not observed. After addition of the selective top agar, Hyg^R putative transformants were never observed. Addition of agarose (1.5%) instead of agar to the IMAS medium was also tested. However no Hyg^R putative transformants were produced on this medium.

In early experiments, glucose was added to the IMAS media (liquid and solid) before autoclaving (Covert, et al., 2000). This often resulted in burnt or caramelised glucose and no Hyg^R putative transformants grew on the selective medium. The recipe was changed and glucose was instead added after autoclaving (Gardiner & Wilson, revised by Elliot, 2005). All experiments that produced Hyg^R putative transformants were co-cultivated on such medium.

2.3.3. *A. tumefaciens* Strains and Binary Vectors

In order to choose the *A. tumefaciens* strain and binary vector that produced the highest rate of transformation efficiency, AGL1 and LBA4404 were transformed with the binary vectors, pBHt2, pPK2, or pCAMgfp (AGL1 only) (Table 2.9) Regardless of the plasmid, both strains generated a higher number of Hyg^R colonies after 3 days co-cultivation than after 2 days as seen in Table 2.7. AGL1 [pBHt2] produced a higher number Hyg^R colonies than LBA4404 [pBHt2], while LBA4404 [pPK2] produced a higher number of Hyg^R colonies than AGL1 [pPK2]. However, both strains generated a higher number of Hyg^R colonies with pBHt2 than with pPK2. AGL1 [pCAMgfp] produced the highest transformation efficiency.

Table 2.9. Effect of *A. tumefaciens* Strains and Binary Vectors on Transformation Efficiency.

***A. tumefaciens* Strains/Plasmids**	**2 days**	**3 days**
AGL1		
pBHt2	130	160
pPK2	0	10
pCAMgfp	170	230
LBA4404		
pBHt2	70	90
pPK2	50	60

AGL1 and LBA4404 were transformed with each of the three binary vectors, pBHt2, pPK2, or PCAMgfp. Co-cultivation of each strain [plasmid] combination with *T.basicola* endoconidia was carried out at 25^oC for 2 or 3 days. The three binary vectors contain a modified Hyg B resistant *hph* gene within the left and right border of the T-DNA. In pBHt2 and pCAMgfp the *hph* gene is under the control of the *Aspergillus nidulans* trpC promoter and in pPK2 this gene is under the control of the *A. nidulans* gpd promoter. Values represent the number Hyg^R colonies produced/ 10^6 endoconidia and are the means of 6 replicates, with the exception of AGL1 [pCAMgfp] values, which are the means from 3 replicates.

2.3.4. The Effect of *T. basicola* Cell Status on Transformation Efficiency

When testing spore age, Hyg^R putative transformants were obtained only when 4 day old endoconidia were transformed, but not 7 or 14 day old endoconidia. Transforming mycelia instead of endoconidia did not produce Hyg^R putative transformants. Table 2.10 and 2.7 indicate that AGL1 [pBHt2] generated a higher transformation efficiency when transforming germinating endoconidia than non-germinating endoconidia while LBA4404 [pBHt2] showed the reverse. As with transformation of non-germinating endoconidia (Tables 2.6, 2.7, and 2.8), AGL1 [pBHT2] had a higher transformation efficiency of germinating endoconidia than LBA4404 [pBHt2]. In addition, when transforming germinating

endocondia, AGL1 [pBHt2] generated a higher rate of transformation efficiency after 2 days co-cultivation rather than 3 days as was observed when this strain was transforming non-germinating conidia (Table 2.7).

Table 2.10. Effect of Germinating *T. basicola* Endoconidia on Transformation Efficiency.

***A. tumefaciens* Strain**	**2 days**	**3 days**
AGL1[pBHt2]	250	140
LBA4404[pBHt2]	10	80

Endoconidia were induced to germinate by growing them in liquid culture for 3hrs prior to co-cultivation with AGL1[pBHt2] or LBA4404 [pBHt2]. Co-cultivation medium were incubated at 25°C for 2 or 3 days. Values represent the number of Hyg^R colonies 10^6 endoconidia and are the means of 4 replicates.

2.3.5 Selection of HygB Resistant *T. basicola* Colonies

2.3.5.1. Transformation Efficiency using the Filter Transfer Method

A comparison was made of *T. basicola* growth on non-selective M-100 (1.5% agar) (a minimal medium) and ½ PDA (2.2% agar) (a rich medium). On this minimal medium, *T. basicola* had a ~50% reduction in growth by comparison to the growth observed on rich medium (Figure 2.7a). A comparison of colony morphology (Figure 2.7 b and c) indicated that *T. basicola* was greatly hindered when grown on M-100; vegetative growth was barely seen unless held up to the light, upon which, clear/white wispy mycelia could be seen dispersing from the centre stab and no chlamydospores were observed.

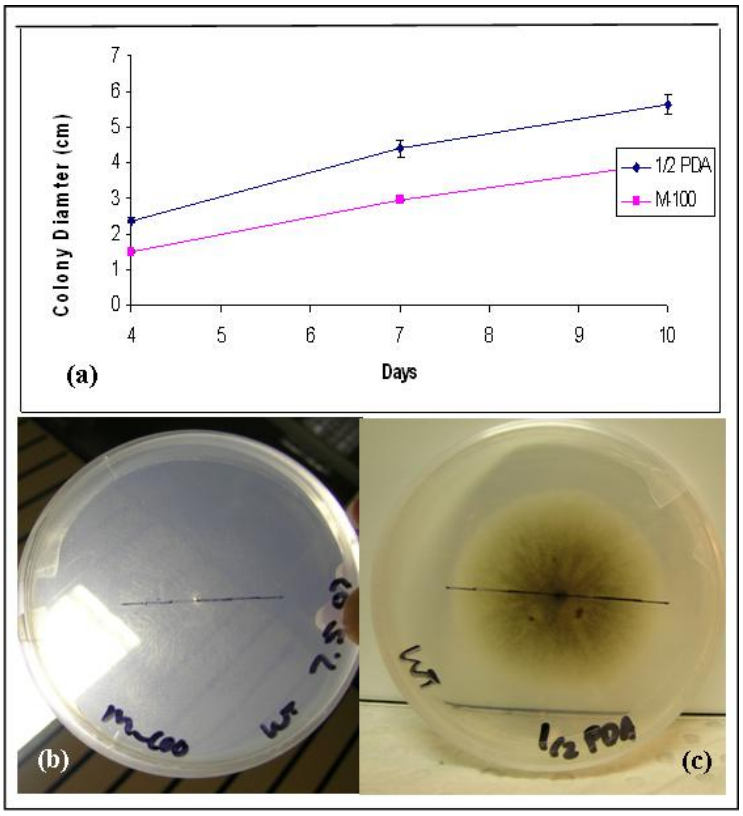

Figure 2.7. Vegetative Growth of WT *T. basicola* on M-100 (1.5% agar) vs ½ PDA (2.2% agar). Fungal cultures were stabbed into ½ PDA (2.2% agar) or M-100 (1.5% agar) and grown at 25°C for a total of 10 days. (a) Colony diameter was measured at 4, 7, and 10 days growth. (b-c) Comparison of WT *T. basicola* growth on (b) M-100 and (c) ½ PDA. Values represent the means of 3 replicates. Error bars represent standard errors.

When selecting Hyg^R putative transformants by filter transfer no colonies were ever observed when the filters (black or white) were transferred to M-100 (1.5% agar) selective medium. Some distinctive

white/light green colonies were observed when black filters were transferred onto selective ½ PDA (2.2% agar) medium but not when using white filters. After transferring Hyg^R putative transformants from these black filters onto fresh selective ½ PDA (1.2% agar) medium with 10µg/ml HygB no colonies ever grew (Figure 2.8).

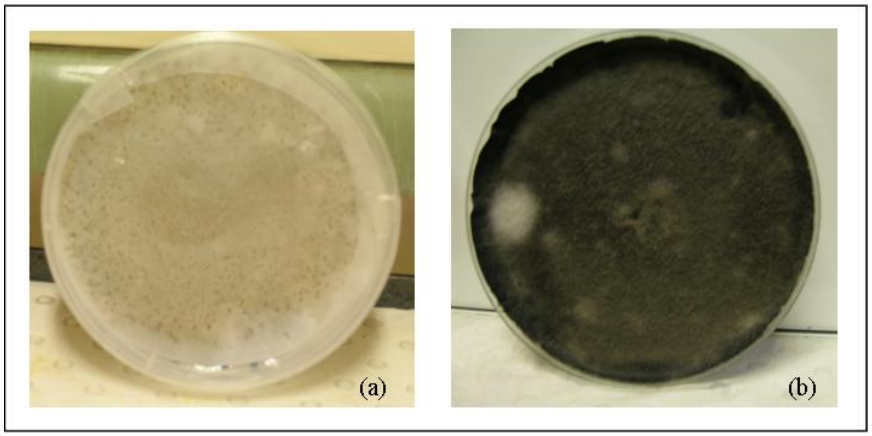

Figure 2.8. Selection by Filter Transfer. Comparison of fungal growth using (a) white and (b) black filters transferred onto ½ PDA (2.2% agar) amended with 10µg HygB/300µg Mef and incubated at 25°C for 5-10 days.

2.3.5.2 Selective Top Agar Method

To test the Hygromcin B minimal inhibitory concentration (i.e. the minimal antibiotic concentration above which WT *T. basicola* cannot grow; MIC) that would be required for selection, Hyg^R putative transformants using selective top agar, *T. basicola* was grown under conditions mimicking ATMT selection with top agar. Thick growth was seen through the top agar in the non-selective medium, but no growth was observed in any of the Hyg B selective medium having concentrations ≥ 5µg/ml.

For top agar selection, 20ml ½ PDA (2.2% agar) was used instead of 25ml since the latter was too great a volume for the size of the petri dishes and would often result in the top agar sticking to the lids of the plates. Both 10µg/ml and 25µg/ml Hyg B in the top agar, (taking into account the diffusion of the antibiotics into the bottom agar) produced Hyg^R colonies. However, since it took a shorter amount of time for $HygB^R$ colonies to appear in the top agar using 10µg/ml (e.g. 10 days before the first appearance of $HygB^R$ colonies when using 10µg/ml compared to ~21 days when using 25µg/ml), this concentration was found to be better suited. The concentration of Mef added to the top agar (300µg/ml) was not altered as this concentration sufficiently inhibited the growth of *A. tumefaciens;* no growth was ever observed on the selective top agar of either co-cultivation medium plates or negative controls (i.e. plates with *A. tumefaciens* only). Top agar selection plates were always incubated at 25°C for up to 24 days before subsequent isolation. *T. basicola*

Hyg^R putative fungal transformants show specific growth patterns when selected using top agar (Figure 2.9). In most cases, no colonies were observed until 10 days after incubation, at which time the Hyg^R

colonies began to "appear" in the top agar. Majority of the colonies were found within the agar overlay at varying distances from the surface. On first recordings, these colonies looked pale white, regularly shaped (i.e. circular), and small ~0.5-1 cm in diameter. By 14-21 days, chlamydospores appeared and the colonies became brown/green in colour. The colonies generally maintained their regular shape and increased in size as they continued to grow in the antibiotic amended medium; reaching diameters of ~1-3cm before transfer. The Hyg^R putative transformants migrated within the agar, progressing towards the surface of the overlay with time. By 21-24 days, majority of the colonies were either just below the surface of the top agar or had already broken through the overlay and began extending mycelial growth over the top agar surface.

In some instances, some colonies instead grew close to the agar interface i.e. the boundary that separates the bottom agar (IMAS co-cultivation agar) and the top agar overlay. These colonies were characteristically bright white, irregularly shaped, and very small in size (~0.1-0.2cm diameter). Regardless of incubation time, ~ 80% of such colonies never continued to grow. A few colonies appeared immediately on the surface of the top agar. These colonies were brown/green in colour and had typical "fluffy" mycelial growth and regular shape. At first recordings they were ~1-3 cm in diameter and by 21 days had grown to ~ 4-5cm. Several of these "surface" colonies actually displayed aerial hyphae growth, which in some cases grew high enough to touch the lid of the petri dish.

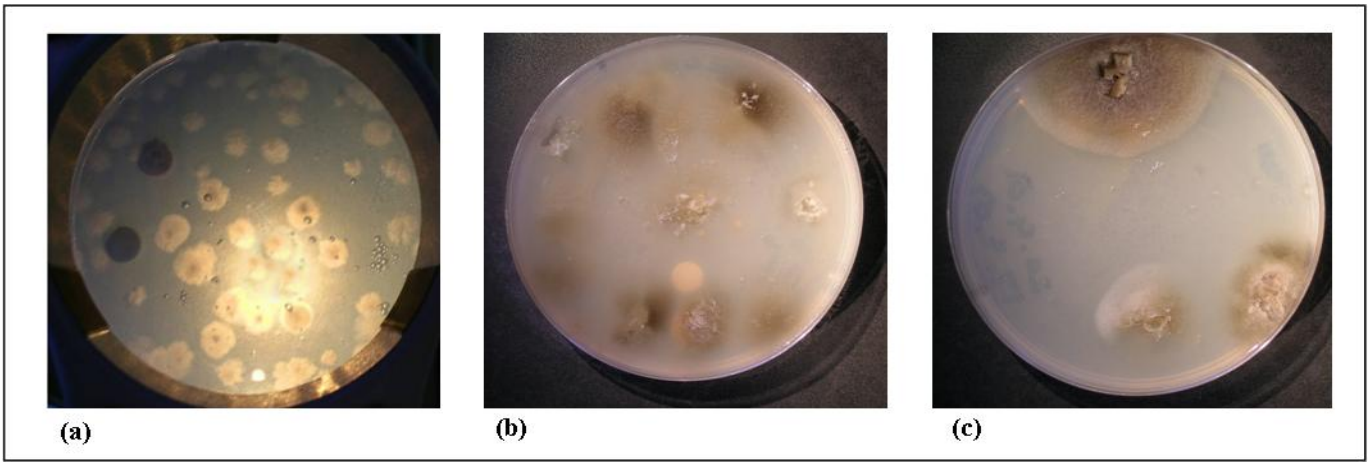

Figure 2.9. Selection of T. basicola Hyg^R Putative Transformants by Top Agar. $HygB^R$ colonies growing (a) in (b) just below surface/beginning to break through, and (c) on top agar. Twenty ml of amended top agar (10µg HygB/300µg Mef final concentration) were added to 2-3 day old co-cultivation medium plates, which were incubated at 25°C for 10-24 days.

Further selection on antibiotic amended medium was performed to confirm HygB resistance of the Hyg^R putative transformants and to isolate individual colonies for further manipulation and storage. It was not essential to wait until colonies grew through the surface of the top agar before transfer. After initial selection in top agar, Hyg^R putative transformants were excised and transferred onto ½ PDA (1.2% agar) with 0µg or 10µg/ml HygB (Figure 2.10). All colonies on non-selective medium showed thick mycelial growth and chlamydospores production within 2 days, while those on selective medium grew similarly, but within 2 weeks. The WT never grew on selective medium. Generally, the closer the fungal colony was to the surface of the top agar, the greater the number of spores produced and the faster the initial

growth after gel excision and transfer onto fresh Hyg B amended medium. No differences were observed in the viability or the level of antibiotic resistance between colonies transferred from within or on top of the selective overlay.

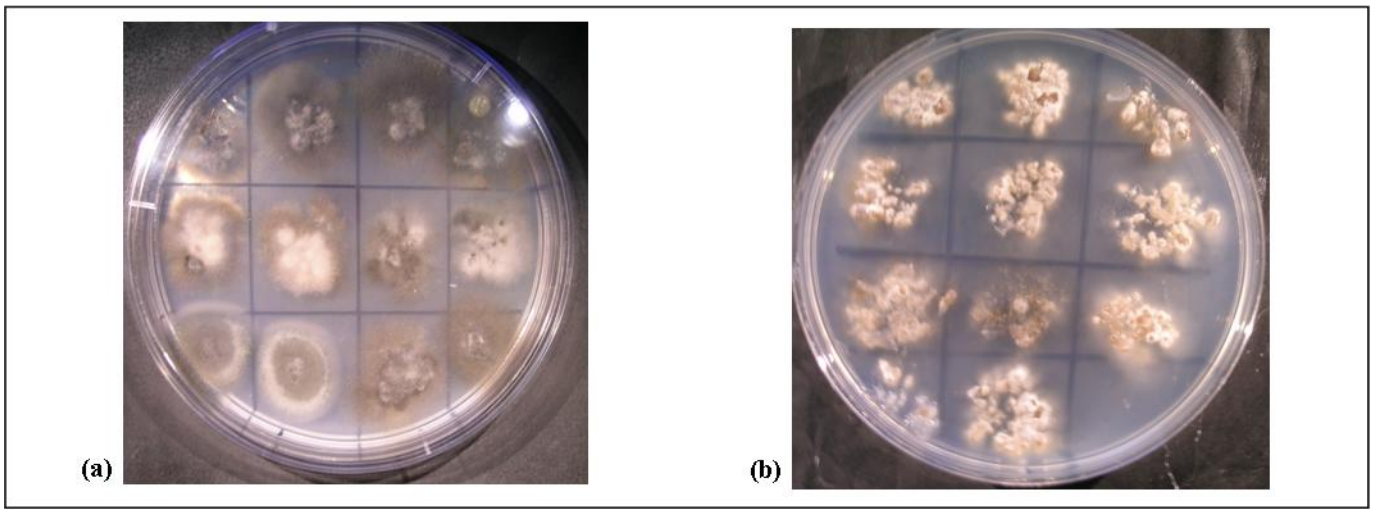

Figure 2.10. Gel Excision. Hyg^R putative transformants were transferred by gel excision from initial top agar selective medium onto ½ PDA (1.2%). (a) unamended (b)10 µg/ml HygB. Plates were incubated at 25°C for 2 days or 2 weeks respectively. WT control is in the bottom right hand corner.

To ensure antibiotic resistance at higher concentrations of HygB, all Hyg^R putative transformants were transferred to 25µg/ml HygB. Hyg^R putative transformants generally grew within 4 weeks. The WT never grew on this selective medium. For further analysis as well as storage, it is essential to use only pure fungal cultures. After growth of Hyg^R putative transformants on selective medium, a single colony was isolated (Figure 2.11 a) and then grown on ½ PDA (1.2% agar) medium amended with 10µg/ml Hyg B (Figure 2.11 b).

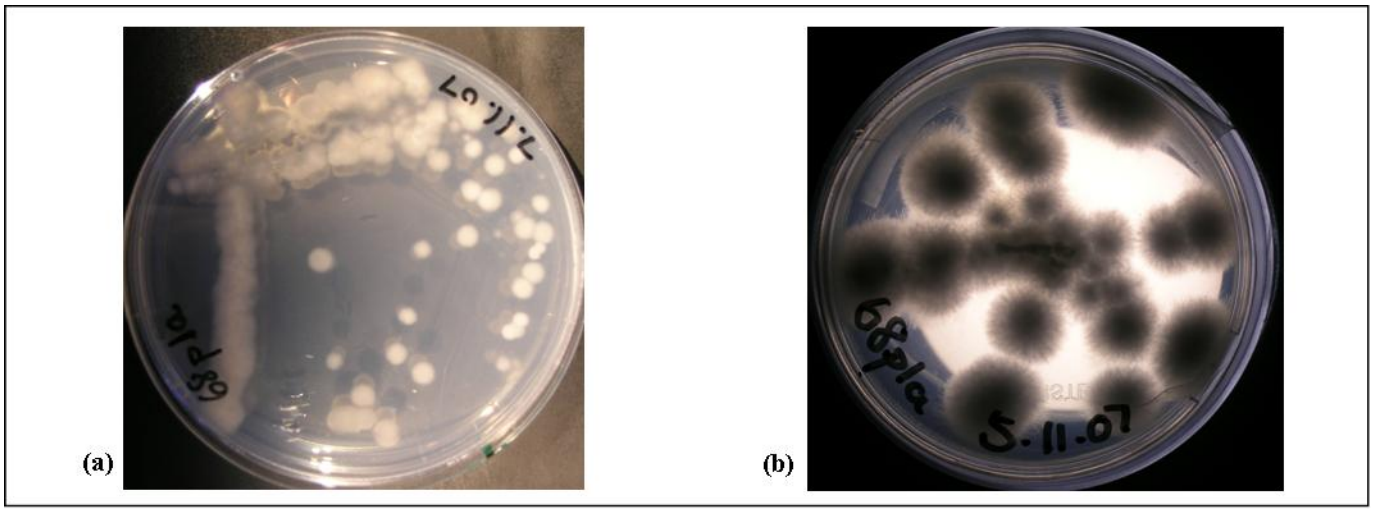

Figure 2.11. Isolated Streak Plate and Streak Plate. (a) To isolate a single colony, fungal transformants were streak isolated onto a ½ PDA (2.2% agar) medium and grown at 25°C for 2-4 days. (b)A single well isolated colony was always chosen for transfer (by criss-cross streaking) to ½ PDA (1.2%agar) supplemented with 10µg/ml Hyg B and incubated at 25°C for 2 weeks. Transformants could then be stored or used for further manipulation.

2.3.6. Confirmation of T-DNA Integration

2.3.6.1. Southern Blot Analysis of T-DNA Integration

PCR amplification of a region within the T-DNA of pBHt2 yielded the expected 1.8kb fragment to be used as the template DNA for the DIG-labelled hph probe (Figure 2.12).

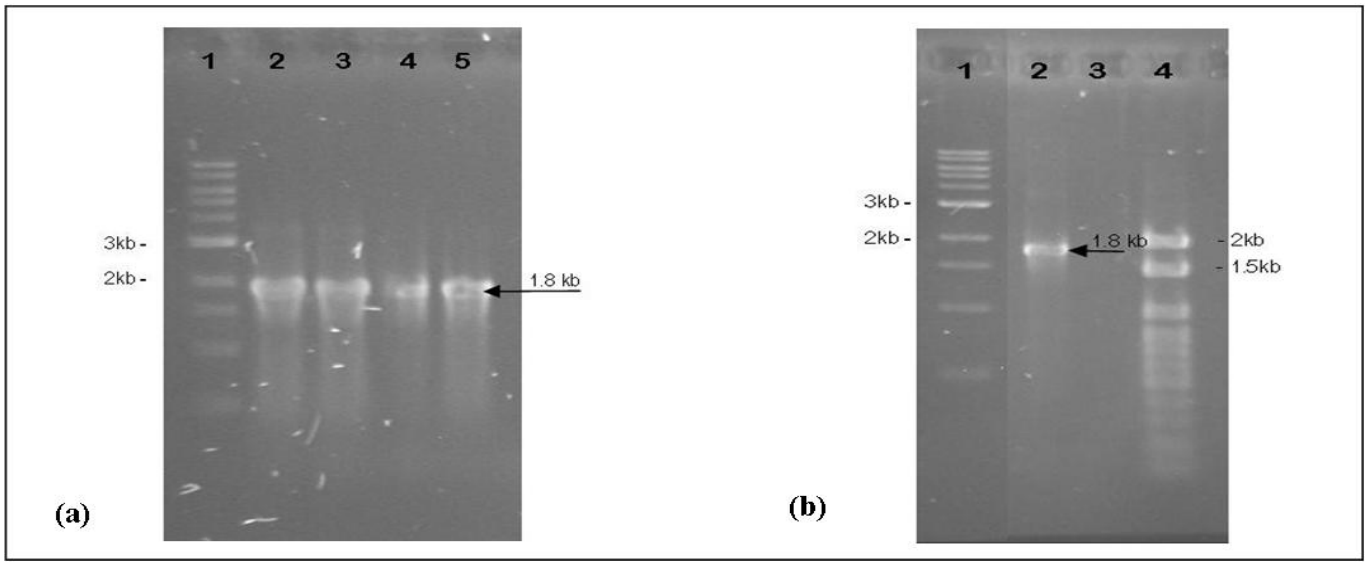

Figure 2.12. Gel Analysis of the DIG-Labelled hph Probe Template DNA. The hph probe template DNA prepared by PCR amplification of pBHt2 using the primers HYKasF1 (Forward) and LBKasR2 (Reverse).(a) lane 1: kb ladder, lanes 2-5 four amplicons of the 1.8kb hph probe template (b) lane 1: kb ladder, lane 6: combined and cleaned hph PCR amplicons

Double digestion of pBHt2 with *Nhe*I and *Sac*II, produced the expected a 5.5kb and 2.9kb fragments (Figure 2.13a). The 2.9kb fragment was used for preparation of the DIG-labelled Km probe (Figure 2.13 d).

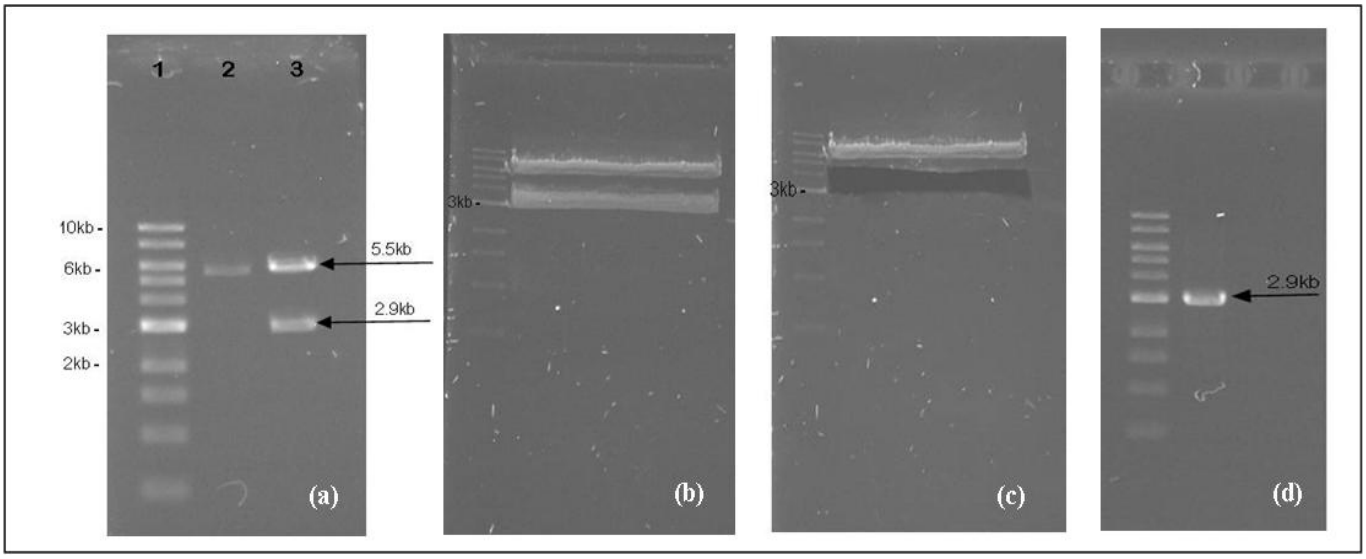

Figure 2.13. Gel Analysis and Preparation of the DIG-labelled Km Probe Template DNA. Template DNA was prepared by double digestion of pBHt2 with *Sac*II and *Nhe*I (a) Analytical gel of double digest. Lane 1: 1kb ladder, Lane2: uncut pBHt2 (supercoil) control, Lane 3: pBHt2 + *Nhe*I and *Sac*II digested 5.5kb and 2.9kb fragments. (b-c) preparative gel of pBht2 + *Nhe*I and *Sac*II digest (b) before gel excision (run in combined gel wells) and (c) after excision of 2.9kb fragment, (d) analytical gel of the 2.9kb fragment after gel clean and ethanol concentration.

Colour was detected for each of the two DIG-labelled probes (Figure 2.14) indicating that both probes had been successfully labelled. Based upon the results, the concentration of the two probes was estimated to be ~20ng/µl.

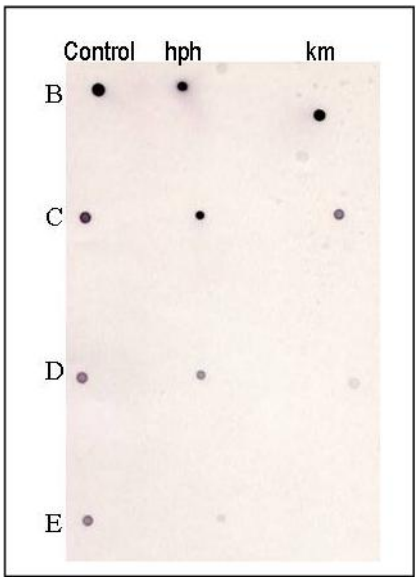

Figure 2.14. DIG-labelled Probe Check. Confirmation and concentration estimations achieved by serial dilution of the DIG labelled hph and Km probes and NBT/BCIP colour change detection. Lane 1: control DNA (Roche) dilutions, Lane 2: DIG-labelled hph probe

Fungal genomic DNA extracted from 20 Hyg^R putative transformants (11 showed in Figure 2.15) could be seen by the presence of single intense band of high molecular weight (~23kb) in each lane with the exception of 64p1a, and 58p1j in lanes 11 and 12 of gel (b), which lacked sufficient genomic DNA for further use in Southern blot analysis. Digested RNA was seen as a bright smear in between the two gels (and faintly at the bottom of gel b).

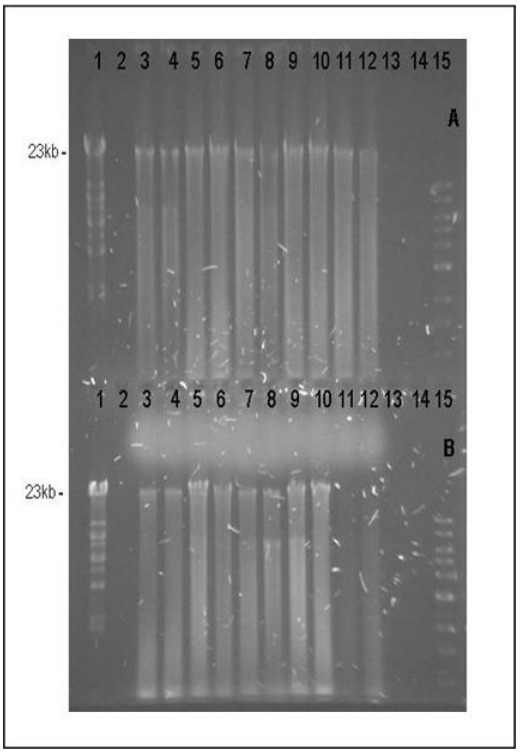

Figure 2.15. Gel Analysis of Extracted Fungal Genomic DNA digested with RNAse. Genomic DNA was extracted from 11 randomly chosen putative transformants and digested with RNAse 1hr prior to running the analytical gel (a) Lane 1: λHind III Ladder, Lanes 3-4: 43p3a, Lanes 5-6:72p1c, Lanes 7-8:66p4d, Lanes 9-10:35p1a, Lanes11-12:46p1c, Lane 15: 1kb ladder (b) Lane 1: λHind III Ladder, Lanes 3-4:33p3a, Lanes 5-6:42p3f, Lanes7-8 26p1b, *Lanes 9-10: 92p2c, Lane 11:64p1a, lane 12:58p1j, Lane 15: 1kb ladder. All transformants, were transformed with pBHt2, except for 9292c* which was transformed with pPK2.

Complete digestion of each sample of fungal genomic DNA digested with *Nhe*I was confirmed by the absence of the high molecular weight DNA bands. The two plasmid controls pBht2 and pPK2 can be faintly seen in Lanes 14 and 15 respectively (Figure 2.16).

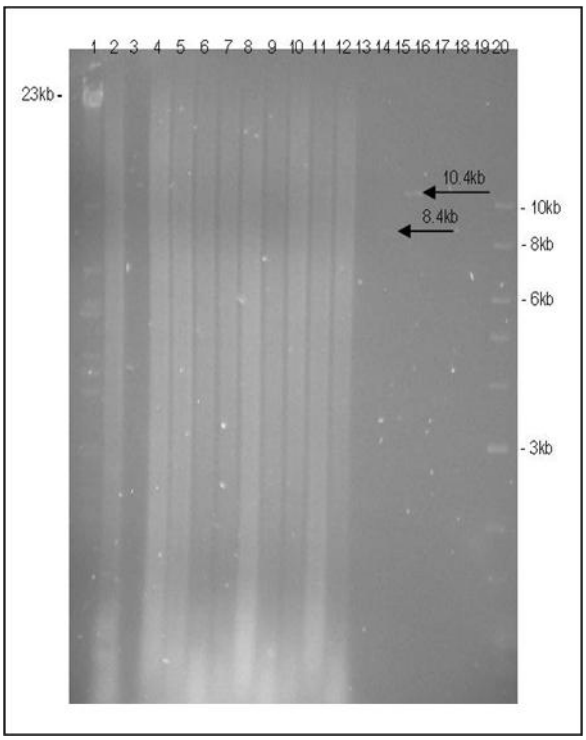

Figure 2.16. Gel Analysis of Digested Fungal Genomic DNA Prior to Southern Blot. Genomic DNA (1-1.5µg) from 9 ATMT mutants (plus WT) was digested O/N with *Nhe*I.RNase was added 1hr prior to running the samples for 16hrs on a preparative maxi gel for Southern blot analysis. Lane 1: λ *Hind* III ladder, Lane 2: WT, Lane 4: 43p3a, Lane 5: 66p4d, Lane 6: 71p1c, Lane 7 35p1c, Lane 8 46pc1, Lane 9: 33p3a, Lane 10: 42p3f, Lane 11: 26p1b, Lane 12: 92p2a, Lane 14: pBHt2, Lane 15: pPK2, Lane 16: hph probe template DNA, Lane 20: 1kb ladder.

Southern blot analysis of the 9 Hyg^R putative transformants, plus WT was performed using the DIG labelled hph probe under high stringency conditions. Two exposures of the film were taken. After 30min exposure (Figure 2.17 a), 3 bands were present (Lanes 14-16) at 8.4kb, 10.4kb, and 1.8kb corresponding to the expected sizes of the controls pBHt2, pPK2, and hph template. No bands were visible for any genomic DNA samples. After 2 days exposure (Figure 2.17 b), an ~8kb band was clearly seen in Lanes 5-12 and faintly in Lane 4 (hard to analyse due to the darkness of the film at this position). A second ~6kb band could be seen in Lanes 4, 5, 6, and 9. These 9 lanes corresponded to genomic DNA of the 9 Hyg^R putative transformants. It was unclear whether a similar band was present in the WT. The same control bands (corresponding to the digested plasmids and probe template DNA) were seen as for 30min exposure, except darker and more diffuse both within and across the lanes.

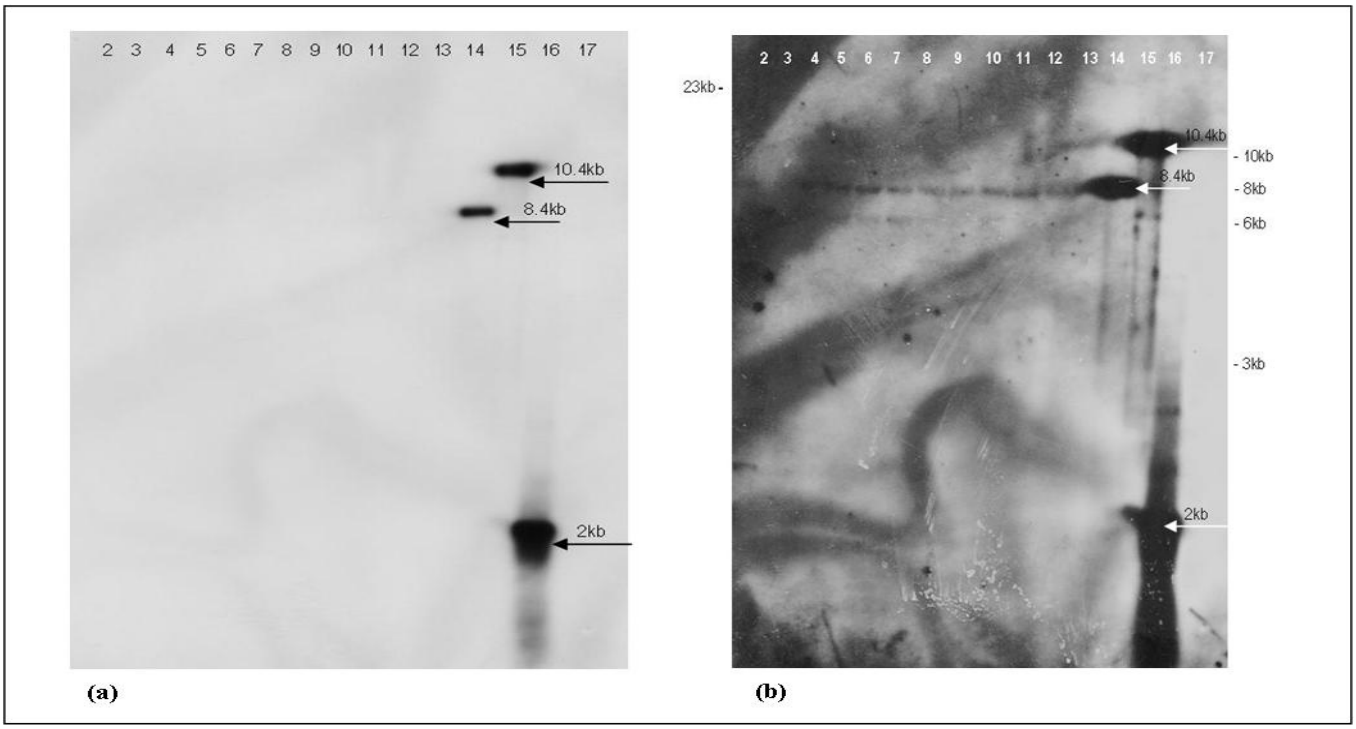

Figure 2.17 Southern Blot Analyses of Nine Putative ATMT Transformants Hybridised with the DIG-Labelled hph probe. Genomic DNA from the transformants was hybridised with the DIG-labelled hph probe under high stringency conditions. Films were exposed for (a) 30 min and (b) 2 days. Lane 2: WT, Lane 4: 43p3a, Lane 5: 66p4d, Lane 6: 71p1c, Lane 7 35p1c, Lane 8 46pc1, Lane 9: 33p3a, Lane 10: 42p3f, Lane 11: 26p1b, Lane 12: 92p2a, Lane 14: pBHt2, Lane 15: pPK2, Lane 16: hph probe template DNA.

2.3.6.2. PCR Analysis of T-DNA Integration

Genomic DNA of the same 9 Hyg^R putative transformants (plus WT) used in the Southern blot, as well as DNA from the two that did not produce high enough DNA concentrations, (Figure 2.15) were used in an attempt to amplify a region of the T-DNA constituting the LB and *hph* gene of pBHt2. The binary vector, pHBHt2 and the DIG-labelled hph probe template DNA were included as controls and can be seen in Lanes 13-15 of gel b (Figure 2.18). No bands are present in any of the transformants or WT.

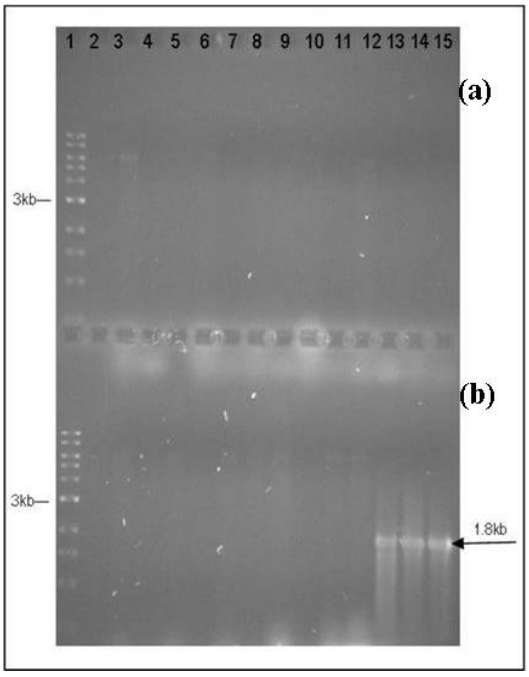

Figure 2.18. PCR Amplification of Fungal Genomic DNA of 11 ATMT Transformants. PCR amplification of fungal genomic LBKasR2, and reaction conditions as used to generate the DIG-labelled hph probe template DNA. Dilutions (1:10 and 1:100) were prepared for all DNA samples, with the exception of 64p1a and 58p1. (a) Lane 1: 1kb ladder, Lanes 3-4 WT, Lanes 5-6: 43p3a, Lanes 7-8: 66p4d, Lanes 9-10: 71p1c, Lanes 11-12: 35p1c, Lanes 13-14: 46p1c. (b) Lane 1: 1kb ladder, Lanes 3-4: 33p3a, Lanes 5-6:42p3f, lanes 7-8: 26p1b, Lanes Lanes 9-10: 92p2a, Lane 11: 64p1a, Lane 12: 58p1j, Lanes 13: hph probe template DNA, Lanes 14-15pBHt

2.3.7. Mitotic Stability

After 5 generations on non-selective ½ PDA (1.2% agar) medium, all 10 of the Hyg^R putative transformants tested for mitotic stability continued to grow on selective ½ PDA (1.2% agar) medium (10 and 25µg/ml HygB), whereas the WT did not (Figure 2.19).

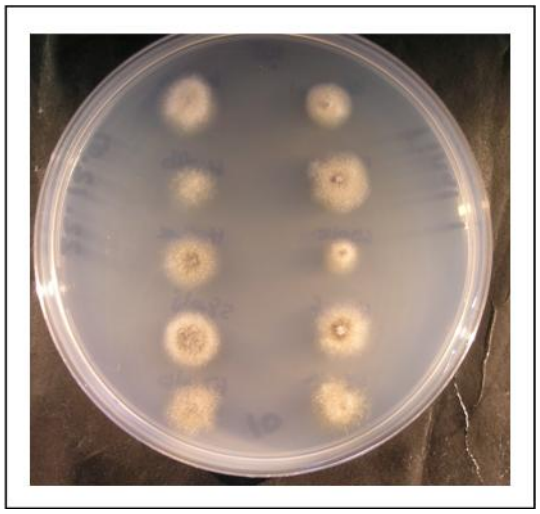

Figure 2.19. Mitotic Stability Test for 10 ATMT Hyg^R Putative Transformants . Transformants (plus wt) were grown for 5 generations on non-amended ½ PDA and then transferred to final selective medium amended with 10µg/ml and 25µg/ml Hyg B. Cultures were incubated at 25°C for 3 days (unamended) or 2-4 weeks (amended medium). Ten different mutants were inoculated per plate with five in each of two columns, (plus the wt at the top centre of the plate). From top to bottom: (a) Column 1: 64p1a-2, 66p4b, 66p4d, 58p1j, 67p1b. Column 2: 64p3a-1, 67p2c, 65p1e, 68p2f, 68p1a. (b) Column 1: 72p1c, 43p3a, 35p1a, 33p3a, 95p2a. Column 2: 42p3f-,46p1c-, 26p1b-92p2a, 95p3c-

2.3.8. Pathogenicity Screening

All seedlings infected with WT *T. basicola* or the 10 Hyg^R putative transformants showed fungal hyphae growing extensively on the epidermal root surface with chlamydospores nested in the mycelial mat and in some cases were seen protruding out of the root. Negative controls (seedlings dipped in water) showed no signs of fungal growth (Figure 2.20).

The positive control (i.e. WT *T. basicola*) had a high rate of pathogenicity with ~84% disease severity (Figure 2.20 a, b, Figure 2.21 and Table 2.11). The affected roots appeared medium to dark brown and displayed lesions on both root area and upper stalks. The roots appeared shrivelled, especially towards the tips. Root growth was stunted in comparison to negative control (i.e. uninfected roots) and showed growth of only the main tap root. The negative controls had 0% disease symptoms and roots appeared white/light yellow in colour. In addition to the main tap root, seedlings had numerous surface roots extending out all along the length of the tap root. The stems were long and green and leaves were large and fully open (Figure 2.20b, Figure 2.21).

Hyg^R putative transformants, 67p2c, 67p1b, 68p2f, 58p1j, 66p4b, 68p1a, and 64p1a-2 did not differ significantly ($P<0.5$) from the WT (Figure 2.21). These Hyg^R putative transformants produced symptoms of black root rot slightly greater than or comparable to those exhibited by the WT (Figure 2.20 c) and were classified as high to intermediate in pathogenicity (Table 2.11.). Three Hyg^R putative transformants, 64p3a-1, 65p1e, and 66p4d, showed a significant ($P<0.5$) reduced virulence in comparison to the WT (Figure 2.21). Both 64p3a-1 and 65p1e were weakly virulent displaying only ~25% disease severity

(Table 2.11.) The infected roots were yellow to light brown in colour with minimal root damage. Majority of the seedlings had surface roots in addition to their central tap root. Foliage appeared much healthier overall (Figure 2.20. d). Hyg^R putative transformant 66p4d was classified as non-pathogenic, showing ~15% root lesions (Figure 2.21 and Table 2.11.). Roots looked very similar to the negative control (Figure 2.20 e). Microscopy of the infected roots from these Hyg^R putative transformants with a reduction in pathogenicity did not reveal any apparent abnormality in the appearance of the chlamydospores.

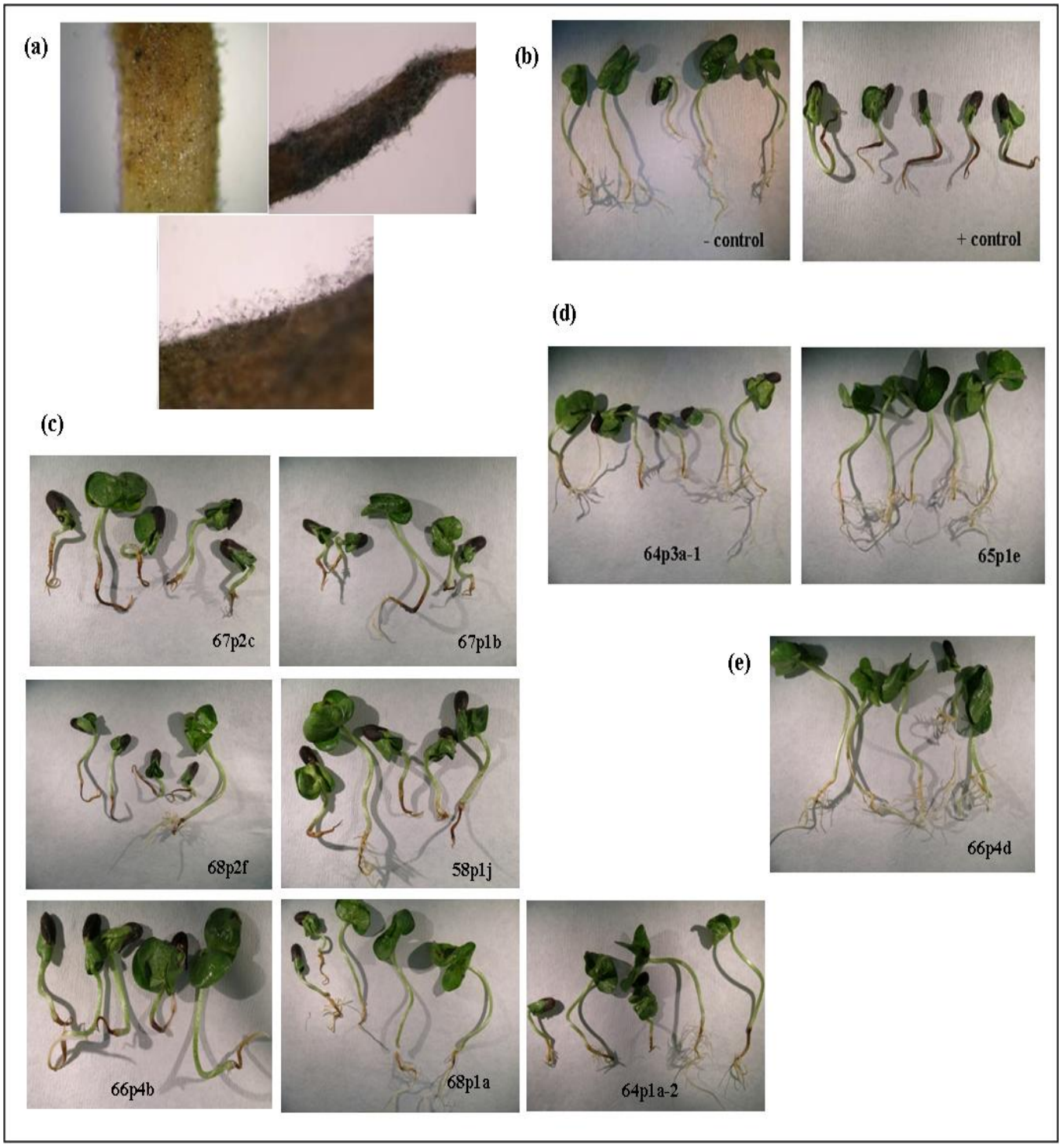

Figure 2.20. Disease Symptoms of Cotton Seedlings Infected with 10 ATMT Hyg^R Putative Transformants

(a) Microscopy of roots infected with WT (b) Controls: Positive (WT *T. basicola*) and Negative (water) (c) highly pathogenic transformants (d) weak/intermediate pathogenic transformants (e) non pathogenic transformant

Table. 2.11 *T. basicola* Pathogenicity Rating.

% Root Lesion	Pathogenicity Rating
0-20	Non-pathogenic
21-40	Weakly virulent
41-60	Intermediate in virulence
61-100	Highly Pathogenic

The extent of pathogenicity is rated according to the % root lesion exhibited. Table adopted from Al-Jaaidi (2007).

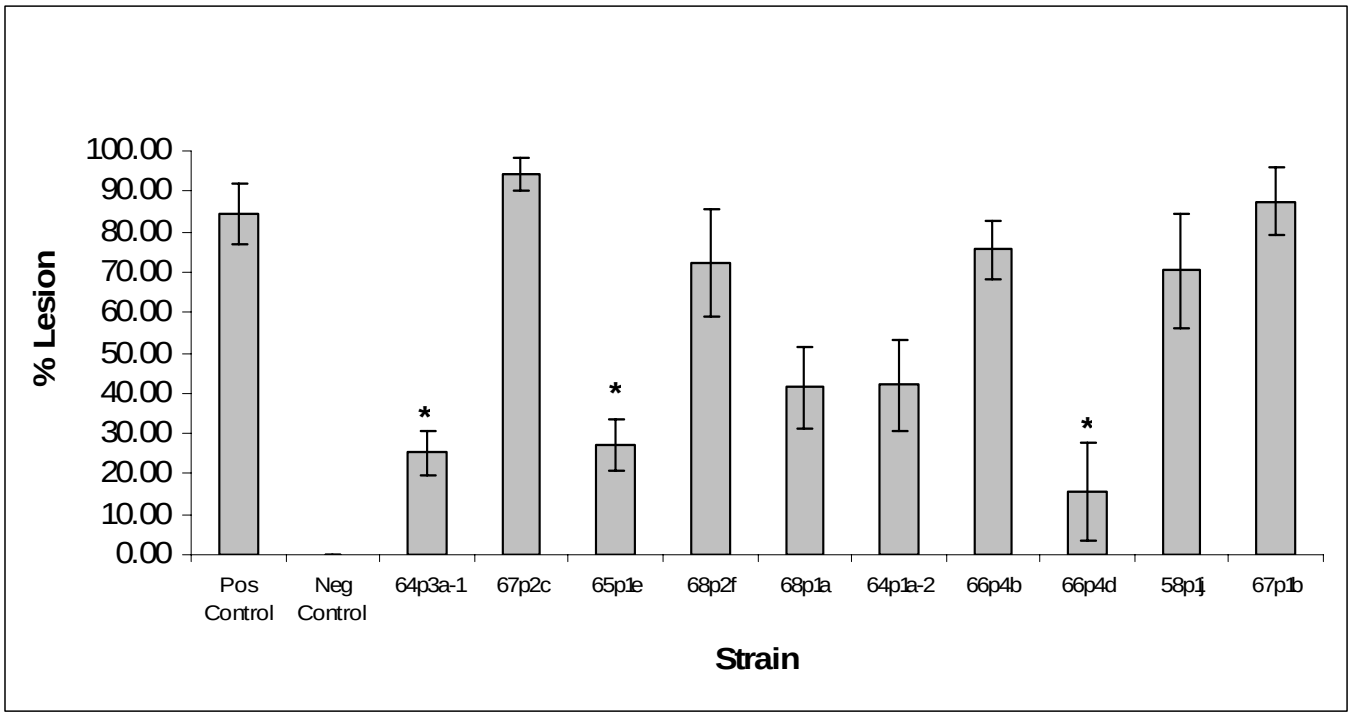

Figure 2.21. Pathogenicity Test of 10 Hyg^R Putative Transformants. Two-day old cotton seedlings were dipped in fungal suspensions and grown at 25^{o}C for 7 days. Total root length and length of root lesions was recorded (cm) and percent root lesion (lesion/total) calculated. Values represent the means of 5 replicates. To equalise variances arising from % values, numbers were first transformed to the Arcsin value prior to statistical analysis. Significance was calculated using Tukey's Post Hoc Test; those with asterisk (*) differ significantly ($P< 0.05$) from the WT. Error bars represent standard errors.

2.3.9. General Characteristics of Hyg^R Putative Transformants

2.3.9.1. Vegetative Growth

Four Hyg^R putative transformants, 64p3a-1, 67p2c, 68p1a, and 67p1b showed similar growth rate to the WT, while the other 7 tested, 65p1e, 68p2f, 64pa-1, 66p4b, 66p4d, and 58p1j showed a slight but significant reduction in growth. Variation in growth also existed between the Hyg^R putative transformants with the lowest growth by 65p1e and the highest growth by 67p2c (Figure 2.22).

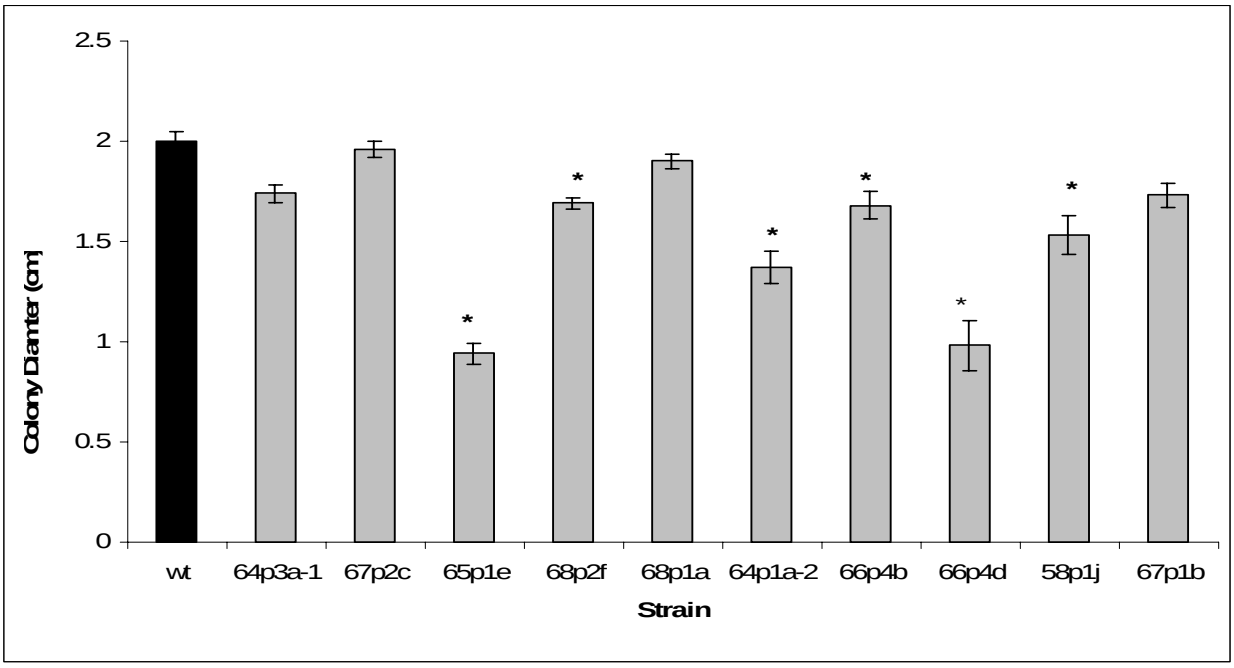

Figure 2. 22. Vegetative Growth of 10 ATMT Hyg^R putative transformants. Colony diameters measured after three day growth at 25^{o}C. Values represent means of 2 replicates from 5 experiments. Significance was calculated using Tukey's Post Hoc Test. Values marked with an asterisk (*) show a significant (<0.05) difference in growth compared to the WT. Error bars represent standard errors.

2.3.9.2. Colony Morphology

Differences in the colour, texture, and growth patterns in comparison to the WT (as well as to each other), were seen for some of the Hyg^R putative transformants (Figure 2.23) Both 64p1a-2 and 42p3f were very white and only a few chlamydospores were observed under the microscope. For 65p1e, though always showing reduced growth, numerous chlamydospores were produced. One Hyg^R putative transformant, 67p2c, had a velvety texture by comparison to the typical 'fluffy' appearance of WT *T. basicola* colonies. Hyg^R putative transformants, 68p1a, 46p1c and 65p1e, had a defined central ring that was always light brown/white and surrounded by a dark brown/green mycelial periphery. Both 95p2a and 95p3c- had light brown colouring and quickly lost the typical fluffy mycelia texture when handled, becoming flattened and moist in appearance.

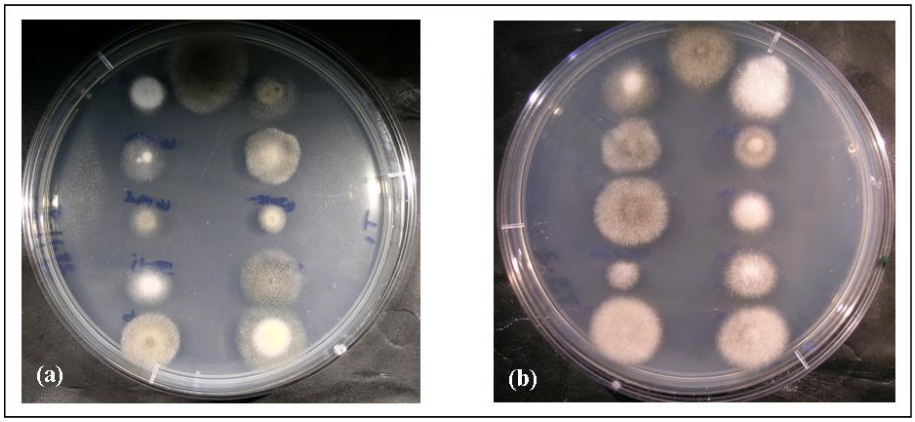

Figure 2.23. Colony Morphology of 10 ATMT Hyg^R Putative Transformants. Ten mutants were inoculated per plate with five in each of two columns, (plus the wt at the top centre of the plate). Cultures were grown at 25^{o}C for 3-4 days. From top to bottom: (a) Column 1: 64p1a-2, 66p4b, 66p4d, 58p1j, 67p1b. Column 2: 64p3a-1, 67p2c, 65p1e, 68p2f, 68p1a. (b) Column 1: 72p1c, 43p3a, 35p1a, 33p3a, 95p2a. Column 2: 42p3f-, 46p1c-, 26p1b-92p2a, 95p3c-

2.3.10. Hyg B Resistance of Putative Transformants

In all ATMT experiments performed, negative and positive control plates were prepared with WT *T. basicola* (and *A.tumefaciens*). On positive control plates, *T. basicola* rapidly grew through the non-amended top agar, whilst on negative control plates, *T. basicola* could not form a colony in the Hyg B amended top agar (10-25µg/ml). For all final selection plates (gel excision or otherwise), positive and negative *T. basicola* controls were also prepared. Again, on non-amended medium the WT strain (and Hyg^R putative transformants) easily grew, whilst when amended with Hyg B (10-25µg/ml), no growth of the WT was ever observed though Hyg^R putative transformants always grew. To ensure this, 10 randomly selected Hyg^R putative transformants, which had already shown resistance at concentrations of 10 and 25µg/ml HygB during both initial top agar selection and subsequent selection, were subjected to a Hyg^R growth test. The Hyg^R putative transformants were grown on ½ PDA (1.2% agar) medium amended with Hyg B (10µg/ml). This test was repeated five times (with two replicates per experiment) and in each experiment, the selective medium plates were freshly prepared no more than three to four days prior to use. Figure 2.24 shows that all 10 Hyg^R putative transformants grew on the selective medium, whilst the WT never grew.

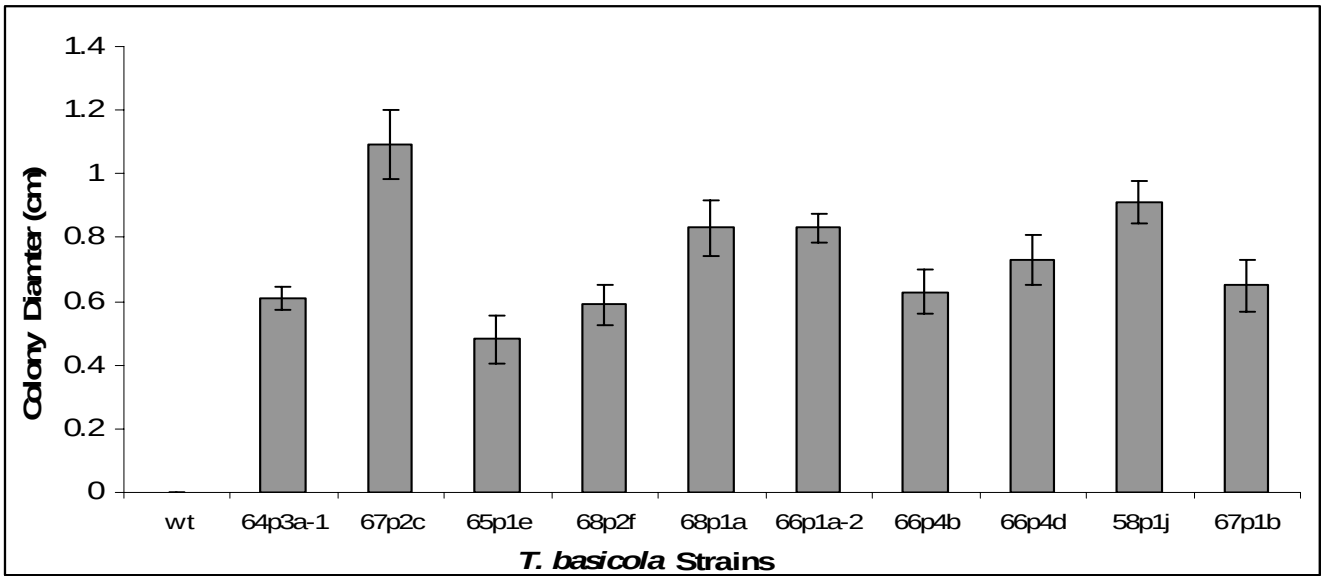

Figure 2.24.Hyg B Resistant Growth of 10 ATMT Hyg^R Putative Transformants. Hyg^R putative transformants were grown on ½ PDA (1.2% agar) medium supplemented with 10µg/ml and incubated at 25°C. Colony diameter was measured after 2 weeks growth. Values represent the means of 2 replicates from 5 experiments. Error bars indicate standard errors

2.3.11. Fluorescence Emission by Hyg^R Putative Tansformants

In the 10 Hyg^R putative transformants, (containing the binary vector pCAMgfp), tested for green fluorescence, a yellow/green autofluorescence was observed in some of the hyphae and endoconidia (Figure Figure 2.25 a and b, respectively) but never in the chlamydospores. Similar autofluorescence was seen in the WT but more yellow (Figure not shown). There was a high amount of autofluorescence seen surrounding the mycelia, which appeared as yellow, light green and red pieces of differing sizes. This was observed in low quantities and in only some of the Hyg^R putative transformants, but consistently and in higher quantities in the WT (Figure Figure 2.26 a and b respectively).

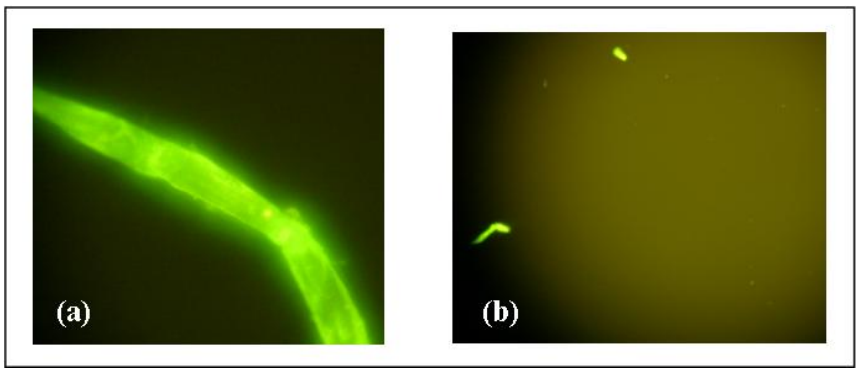

Figure 2.25. Fluorescence in *T. basicola* Transformed with pCAMgfp. Hyg^R putative transformants were first grown on ½ PDA (2.2% agar) medium at 25°C for 3-4 days and then in PDB liquid for ~5hrs prior to fluorescent microscopy. (a) Part of a hypha at x 40 objective. (b) endoconidia at x10 objective. Both figures presented are viewed under blue light.

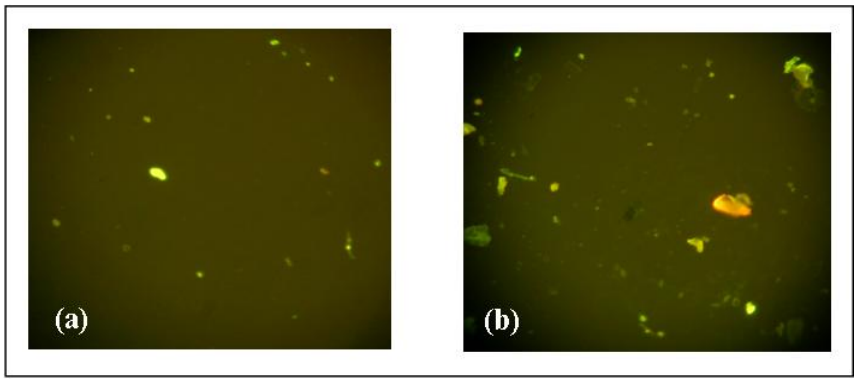

Figure 2.26. Fluorescence Surrounding *T. basicola* Transformed with pCAMgfp. Hyg^R putative transformants were first grown on ½ PDA (2.2% agar) medium at 25°C for 3-4 days and then in PDB liquid for ~5hrs prior to fluorescent microscopy. The surrounding medium for (a) one of the ten Hyg^R putative transformants and (b) WT. Both figures presented are viewed under blue light.

2.3.12. Plasmid Construction for Plasmid Rescue

Figure 2.27 (a and b) shows the expected 5kb and 3.4kb fragment produced from double digestion of pBHt2 with (1) *Eco*RI + *Nhe*I or (2) *Sma*I + *Nhe*I. The 5kb fragment from the *Eco*RI digest and the 3.4kb fragment from the *Sma*I digest were excised (Figure 2.27c) and used in the construction of pMAX.

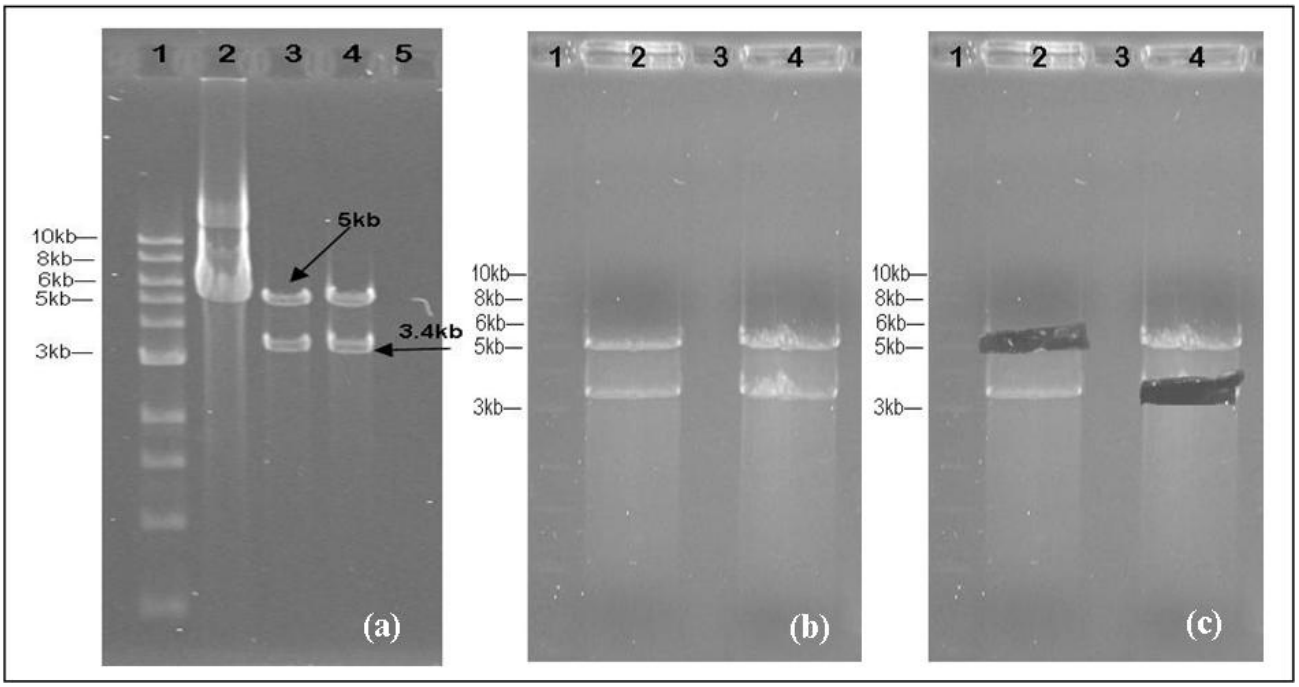

Figure 2.27. Gel Analysis and Preparation of pBHt2 Double Digestion Fragments. pBHt2 was double digested with two sets of restriction enzymes; *Eco*RI + *Nhe*I and *Sma*I + *Nhe*I. After gel analysis, the digests were run on a preparative gel and the 5kb *Eco*RI/*Nhe*I digest and the 3.4kb *Sma*I/ *Nhe*I digest were excised and cleaned. (a) Double Digestion of pBHt2. Lane 1: 1kb ladder, Lane 2: uncut pBHt2 control, Lane 3: pBHt2 double digested with *Eco*RI + *Nhe*I, Lane 4: pBHt2 double digested with *Sma*I + *Nhe*I, (b-c) pBHT2 digests before (b) and after (c) gel excision. Lane 1: 1kb ladder, Lane 2: pBHt2 double digested with *Eco*RI + *Nhe*I 5kb fragment excised, Lane 4: pBHt2 double digested with *Sma*I + *Nhe*I 3.4kb fragment excised.

Figure 2.28 shows the expected 2.2kb and 2kb fragments generated from double digestion of pBR322 with *Eco*RI and *BSa*AI. Due to the close proximity of the two bands, the 2kb fragment of interest, which contained the pBR322 Amp^R gene and ori, was not excised directly from the gel and the double digest solution was cleaned and used in the tri-ligation.

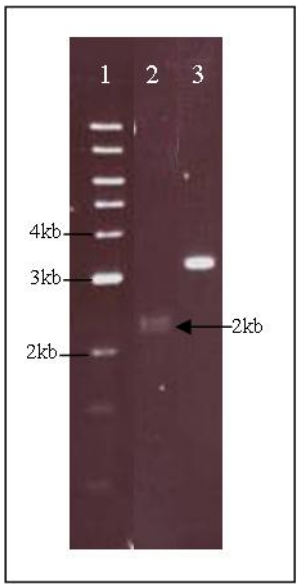

Figure 2.28. Gel Analysis of pBR322 Double Digestion. pBR322 was double digested with *Eco*RI and *Bsa*AI. After gel analysis, the digest was cleaned. Lane 1: 1kb ladder, Lane 2: pBR322 double digest, Lane 3: uncut pBR322 control.

For both *E. coli* strains, DH5α and JM109, transformation with the tri-ligation solution produced transformants on LB (1.6% agar) supplemented with Amp but not on Km or Amp+Km amended media (Table 2.12.).

Table 2.12. Antibiotic Resistant Colonies

Antibiotics in LB(1.6% agar)	**Resistant Colonies**	
	DH5α	***JM109***
Km	no	no
Amp	yes	yes
Km + Amp	no	no

E.coli DH5α and JM109 competent cells were transformed by heat shock with the tri-ligation solution. Colonies were plated on LB(1.6% agar) amended with (1) Km, (2) Amp, or (3) Km+Amp. Cultures were incubated at 37^{o}C O/N.

Gel analysis of the tri-ligation solution (Figure 2.29 Lane 2), showed 6 bands; a bright band at 3.4kb, a medium band 5kb, three faint bands at 7kb, 8.4kb, and 10.4kb, and a very faint band at 4.3kb. Controls were seen for uncut pBHt2 with the relaxed form at ~8.4kb (Lane 4) and the 5kb and 3.4kb fragments (Lanes 5-6 respectively). The 2kb fragment (Lane 7) is faintly visible. The gel broke down the lane which contained uncut pBR322 control.

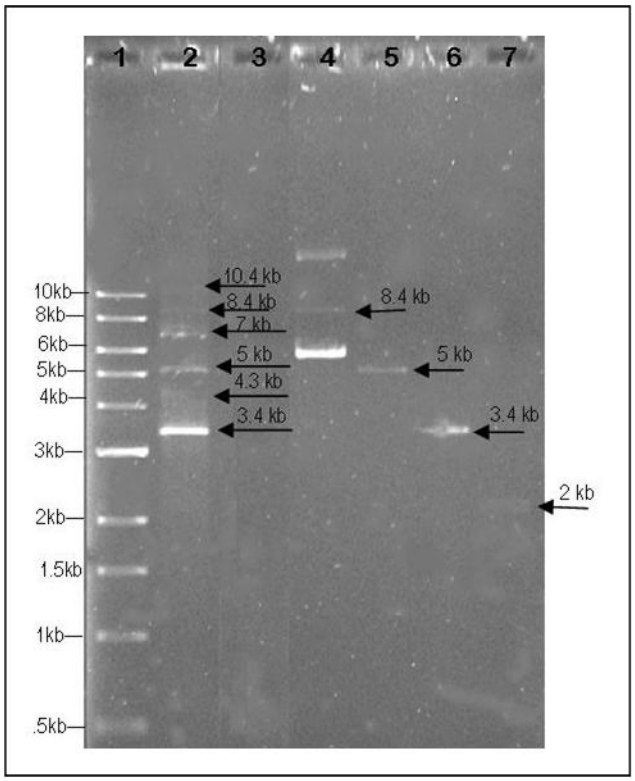

Figure 2.29 Gel Analysis of pMAX Tri-Ligation Solution. The 5kb, 3.4kb, and 2kb fragments were tri-ligated and run on an analytical gel along with uncut pBHt2 and the 3 ligation fragments as controls. Lane 1: 1kb ladder, Lane 2: Tri-ligation solution, Lane3: uncut pBHt2, Lane 4: 5kb fragment, Lane 5: 3.4kb fragment, Lane 6: 2kb

PCR amplification of a region within the binary vector pBHt2, yielded the expected 250bp linker to be used in construction of the binary vector pAIM3 for plasmid rescue (Figure 2.30 Lanes 2,4,5). Lane 6, which contained the negative control (i.e.miliQ) showed that no non-specific product was produced. Lane 7 showed an uncut control of pBHt2.

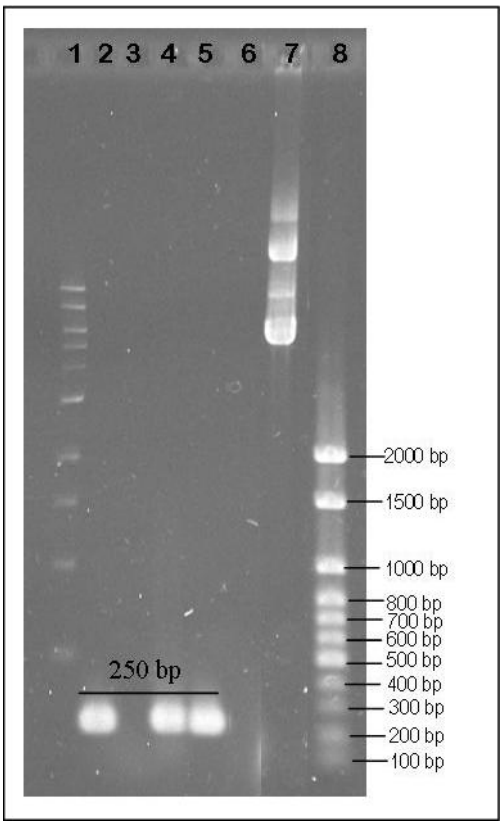

Figure 2.30 PCR amplification of the 250bp oligo for pAIM3 Construction.18.9.07. The 250bp oligo used in the construction of the plasmid pAIM3 was prepared by PCR amplification of a region within the plasmid pBHt2 using the forward primer LBEcoF1 and reverse primer LBKasR1. L1: 1kb ladder, L2, 4, and 5: 250 bp oligo amplicons, L6: PCR negative control, L7: uncut pBHt2 control, L8: 100bp ladder

2.4. Discussion

A total of 99 different ATMT experiments were conducted in this project (Appendix 2). These experiments were designed to test some of the most important variables found to play a role in successful transformation of filamentous fungi by ATMT. Appendix 3 shows the experimental conditions used by researchers in transforming other filamentous fungi using ATMT.

2.4.1. Optimal *A. tumefaciens* Pre-Cultivation Growth and Induction Conditions

For successful transformation, optimal A*grobacterium* growth conditions and induction prior to co-cultivation with *T. basicola* must be established. The *vir* genes are best expressed at 28^{o}C (Wei, et al., 2000) and growing *Agrobacterium* at higher temperatures may lead to loss of the binary vector (Elliot, personal communication). This is the optimal temperature found in numerous ATMT experiments using AGL1 (Mullins, et al., 2001; Leclerque, et al., 2003; Dobinson, et al., 2004) and LBA4404 (Gardiner & Howlett, 2004; Gardiner & Wilson, re-revised by Elliot 2006). As such, this temperature seemed best suited for both pre-cultivation and induction and was used for both strains (AGL1 and LBA4404) throughout this project.

The plant phenol acetosyringone (AS) induces *vir* gene expression, which is required for T-DNA transfer. As it is not produced by fungi, it must be added to the medium. For successful transformation, AS must be added to the co-cultivation plates (de Groot, et al., 1998; Combier, et al., 2003; Leclerque, et al, 2004; Betts, et al., 2007; Bundock, et al., 1995), however, its necessity in the induction medium (IM) is dependent upon the fungus being transformed. In *Hebeloma cylindrosporum,* and *Magnaporthe orzyae,* addition of AS to the pre-induction medium slightly lowered tansformation efficiency (Combier et al., 2003; Betts et al., 2007), while for *Beauveria bassiana* and *Fusarium oxysporum* it increased the efficiency of transformation at least 10 fold (Leclerque, et al., 2004; Mullins, et al., 2001). Pre-induction in the presence of AS increased the number of single T-DNA inserts in *H. cylindrosporum* and *B. bassiana* ATMT transformants (Leclerque, et al., 2004; Combier, et al., 2003). The number of transformants is found to be highly dependent upon the concentration of AS, with optimal results observed using 200µM-500µM (Leclerque, et al., 2004); this is the concentration of AS used in this study.

When determining optimal time and OD for induction, 6-8 hours growth after AS addition was the most important condition to ensure complete expression of the *vir* genes (Elliot, personal communication). This induction period, as well as the OD_{660nm} achieved (i.e. 0.6-0.8), compared to other researcher's experiments (Mullins, et al., 2001; Amey, et al., 2000; Dobinson, et al., 2004; Combier, et al., 2003; Meyer, et al., 2003; Betts, et al., 2007; Gardiner & Howlett, 2004; Gardiner & Wilson, re-revised by Elliot 2006)

2.4.2. Establishment of Optimal Co-Cultivation Conditions

A. tumefaciens and *T. basicola* are co-cultivated together on induction medium; (IMAS 1.5% agar). It is during this time that the T-DNA will be transferred into the fungal host's genome. During co-cultivation there are many variables that can effect the efficiency of transformation, including temperature, duration, cell ratios and media.

Co-cultivation temperature is a very important factor for efficient transformation. At 25^{o}C the number of Hyg^{R} *T. basicola* putative transformants generated was approximately doubled by comparison to the number generated at 28^{o}C. Since there was always a higher ratio of *Agrobacterium* to endoconidia cells used and *Agrobacteria* was found to naturally grow faster than *T. basicola* at both temperatures, it follows that the optimum co-cultivation temperature will be that which is best suited for enhancing *T. basicola* growth. These results are in agreement with those obtained by others when transforming *Leptosphaeria. maculans* and *Hebeloma cylindrosporum* (Gardiner & Howlett, 2004; Combier, et al., 2003).

Results from other groups showed that colonies of transformed cells are often first observed by 48hrs co-cultivation (with very few ever appearing before this time), with transformation efficiency increasing between 2-4 days. Beyond this period, there is either no significant increase in the number of transformants or a decline may occur (Combier, et al., 2003; Betts, et al., 2007; Leclerque, et al., 2004; Meyer, et al., 2003). This pattern compares well to the results obtained in this study in which Hyg^{R} putative transformants generated by AGL1 and LBA4404 containing the plasmid pBHt2 were observed at two days and increased to an optimum by three days. After seven days, the number of Hyg^{R} putative transformants generated by AGL1 [pBHT2] declined to almost 30% of the three-day optimal transformation efficiency.

The ratio of *A. tumefaciens* cells to fungal endoconidia has been found to have significant bearing on the efficiency of transformation (Combier, et al., 2003; Betts, et al., 2007; Meyer, et al., 2003). For co-cultivation, *Agrobacteria* cells and fungal conidia are spread on solid IMAS. Since it is necessary for at least one bacterial cell to be in close enough proximity with a spore to integrate the foreign T-DNA into the fungal host's genome, it is reasonable to conclude that increasing the number of bacterial cells per conidium would increase the chance of contact and thus transformation. When the ratio of bacterial to fungal cells becomes too high, this can inhibit successful transformation due to competition for space and nutrients. This is evidenced in this work by the higher number of Hyg^{R} *T. basicola* putative transformants obtained at lower ratios compared to higher ratios. These effects of *A. tumefaciens* cell concentration on transformation efficiency is in agreement with those obtained for transformation of other fungi (Combier, et al., 2003; Betts, et al., 2007; Meyer, et al., 2003). The optimal number of transformed colonies, which resulted from a 1:250 ratio of *T. basicola* endoconidium to AGL1 [pBHt2], corresponds very well to the

results obtained by Betts et al., (2007) when using the same strain and plasmid to transfor the filamentous fungus *B. bassiana.*

IMAS (1.5% agar) medium is used for co-cultivation. An acidic pH is required for expression of the *vir* genes (Wei, et al., 2000), which is why IMAS medium normally has pH 6 (this work). Certain fungi were successfully transformed after suppressing *Agrobacterium* growth by lowering the pH by 1-2 degrees (Kang, personal communication). Such an acidic growth environment is not favourable for *T. basicola*, as evidenced by the diminished mycelia and rapid dense accumulation of chlamydospores generated for long-term survival when conditions are harsh.

The increase in transformation efficiency that resulted when glucose was added to the IM after autoclaving is reasonable since sugars are needed for successful expression of the *vir* genes (Wei, et al., 2000, Elliot, personal communication). In a *virA* defective strain, it was found that the addition of glucose to the medium produced a 1,500 fold increase in the level of *virB* expression. Also, the addition of sugars to the solid IMAS was found to greatly reduce the background effect (i.e. false positives) during selection of *Magnaporthe grisea* transformants (Betts, et al., 2007).

Plating endoconidia on agarose slows germination, causing the cells to swell without releasing a germ tube; for *Leptosphaeria maculans* this effect on endoconidia produced some of the highest numbers of transformants during ATMT (Elliot, personal communication). However, this is in contrast to the results in this work where no Hyg^R *T. basicola* putative transformants were generated when using agarose in the media. In fact the opposite was actually observed, in which germinated endoconidia grown on IMAS medium with agar generated the highest number of Hyg^R putative transformants.

2.4.3. Optimal Combination of *A. tumefaciens* Strains and Binary Vectors

A. tumefaciens strains show varying levels of virulence towards their hosts, which in turn varies the efficiency of T-DNA integration. Virulence is determined by the Ti plasmid. The supervirulent strain A281 harbours the Ti plasmid pTiBo542, which confers high levels of virulence by the *virG* locus, inducing increased *vir* gene expression (Wei, et al., 2000, Covert, et al., 2001). AGL1 is a commonly used hypervirulent strain, containing the helper plasmid pTiBo542ΔT, which is a disarmed derivative of pTiBo542, lacking one of the border sequences, but maintaining the *vir* genes (Wei, et al., 2000, Covert, et al., 2001; Combier, et al., 2003; Betts, et al., 2007; Mullins, et al., 2001). LBA4404 (and other strains of the LBA series) is also used in many transformations of filamentous fungi (Hoekema, et al., 1983; Bundock, et al., 1995; Wei et al., 2000; Gardiner & Howlett, 2004; Gardiner & Wilson, re-revised by Elliot, 2006). However, LBA4404 is not a hypervirulent strain and contains a Ti helper plasmid lacking the entire T-DNA region. These differences between the two strains are seen in the high *T. basicola* transformation efficiency that resulted for AGL1 [pBHt2] compared with LBA4404 [pBHt2].

Interestingly, LBA4404 [pPK2] generated a higher number of Hyg^R *T. basicola* putative transformant than did AGL1 [pPK2], and AGL1 [pCAMgfp] generated the highest number of Hyg^R *T. basicola* putative transformants. This indicates that not only the strain, but also the combination of strain with plasmid effects transformation efficiency. This has also been observed by others using multiple *A. tumefaciens* strains and plasmid combinations (Wei, et al., 2000, Covert, et al., 2001; Betts, et al., 2007).

For *T. basicola,* as well as many other filamentous fungi, the *hygromycin phosphotransferase* (*hph*) gene is used as the antibiotic selectable marker on the binary vector (Bailey, et al., 2002; Dobinson, et al., 2004; Covert, et al., 2001; Leclerque, et al., 2004; Sexton & Howlett, 2001; Amey, et al., 2002). As such, the promoter used to express the *hph* gene plays an important role in overall success of ATMT. If the *hph* promoter is not recognized or does not allow sufficient expression, then even when T-DNA is successfully integrated into the fungal genome, transformants will not have resistance when selected with Hygromycin B. As such, fungal promoters with constitutive strong expression, often from *Aspergillus nidulans,* are used. Both pBHt2 and pCAMgfp use the *A. nidulans* trpC promoter for expression of the *hph* gene (Mullins, et al., 2001; Sesma, 2005), while pPK2 uses the *A. nidulans* gpd promoter (Covert, et al., 2000); both promoters are constitutively expressed (Appendix 3). For both AGL1 and LBA4404, using the binary vector pBHt2 results in higher number of Hyg^R *T. basicola* putative transformants than with pPK2 and the highest results overall, were obtained with AGL1 [pCAMgfp]. This may indicate that in *T. basicola* the trpC promoter drives stronger expression of the *hph* gene than does the gpd promoter. These results compare well to the differences in transformation efficiency of *F. oxysporum* obtained when using these two promoters (Mullins et al., 2001).

2.4.4. *T. basicola* Cell Status for Optimal Transformation

Though endoconidia are most commonly transformed, *Agrobacterium* has also proven efficient in transforming protoplasts, hypha and mycelia (Glick & Pasternak, 2003:514-520; Bundock, et al., 2002; de Groot, et al., 1998; Covert et al., 2000; Hoekema, et al., 1983; Leclerque, et al., 2003; Mullins, et al., 2001; Amey, et al., 2002). In this work, spores were usually harvested from 4 day old plates, as this produced the highest number of spores and best mycelial growth conditions for preparing spore suspensions. To see if any difference resulted in the efficiency of transformation when using older spores, fungal cultures were instead grown for 7 and 14 days prior to co-cultivation. However, no transformants were produced. It seems that as the spore age increases, the cells become more resistant to bacterial infection; possibly due to changes in spore structure, melanin content, and or metabolic processes.

Transformation efficiency of germinating *T. basicola* endoconidia using AGL1 [pBHt2] is higher than with non-germinating conidia. This corresponds to the results obtained when transforming *B. bassiana* (Leclerque, et al., 2004). Interestingly, co-cultivation for two days produces higher number of Hyg^R putative transformants than does three days. A hypothesis put forward by Leclerque et al., (2004) (but still

requiring further investigation) is that "conidiation is a prerequisite of transformation in that a change in cellular state preceding the formation of microconidia might increase fungal transformation competence." During germination of endoconidia, the germ tube ruptures through the spore wall, changing the status of the cell from a quiescent state to an actively expressing cell that begins to divide rapidly. Interestingly, *A. tumefaciens* preferentially integrates its T-DNA into transcription active genes and requires dividing host cells (Wei, et al., 2000). This effect was also observed with the pathogenic fungus *Coccidioides immitis*, in which germinating spores significantly increased transformation efficiency (Aboudeh, et al., 2000, referenced by Meyer, et al., 2003). For transformation of germinating endoconidia by LBA4404 [pBHt2], optimal transformation efficiency was still seen after three days co-cultivation with *T. basicola*. This may be since LBA4404 grows much slower than AGL1 (this work; Elliot, personal communication) and thus still requires this extra co-cultivation time. In addition, the number of Hyg^R *T. basicola* putative transformants generated by this strain is slightly lower for germinated than for non-germinated conidia.

2.4.5. Optimal Selection Procedure for *T. basicola* ATMT Transformants

Even after successful T-DNA integration, suitable selective pressure must be applied to isolate and select for *T. basicola* transformants. There are a number of selection variables, which play a role in the success of ATMT and include the method of selection, type of medium, antibiotic concentrations, temperature, and duration of selection.

Filter transfer is the most common method used for selection of transformants generated by ATMT (Mullins, et al., 2001; Covert, et al., 2000). The rationale for this method is the "ease" with which transformants can be transferred (Elliot, personal communication). This was not found to be an effective or suitable method for selecting *T. basicola* Hyg^R putative transformants even when a suitable selection medium was used (i.e. ½ PDA vs M-100). Black filters produced some distinct white colonies (which could not be observed on the white filters), but were always surrounded by a high amount of background growth. Determining which colonies were actually growing due to having antibiotic resistance from integration was very difficult and produced many false positives. While typical HygB concentrations used for selection are 75µg/ml-300µg/ml (Mullins, et al., 2001; Combier, et al, 2003; Gardiner & Howlett, 2004; Betts, et al., 2007) in the case of the *B. bassiana*, a concentration as high as 1mg/ml Hyg B was still insufficient to inhibit fungal growth on filters (Leclerque, et al., 2004). The filters seem to convey a protective effect for *T. basicola* from the hygromycin B in the selective plates, which has also been observed in *B. bassiana, Aspergillus niger*, and *Colletotrichum gloeosporiodes* (Leclerque, et al., 2004; deGroot, et al, 1998).

Top agar selection proved to be more reliable and a better alternative to filter transfer. All *T. basicola* Hyg^R putative transformants in this work were produced using this selection method. In earlier work, when stable mutants were generated by PEG transformation, 25 µg/ml Hygromycin B was used for

selection (Al-Jaaidi, 2007). ATMT Hyg^R putative transformants did grow in 25µg/ml selective top agar, but the rate of growth was very slow. Using a concentration of 10µg/ml Hygromycin B for the selective top agar proved most effective. This concentration allows the Hyg^R putative transformants to grow at a reasonable rate before transfer, yet is still well above the MIC (i.e. ≤ 5µg/ml) thereby effectively maintaining selective pressure (wild type controls did not grow under these conditions).

The temperature for selection was not manipulated since it is reasonable to conclude that optimal growth of Hyg^R putative transformants should be at the most favourable temperature for *T. basicola* growth i.e. 25^oC (Al-Jaaidi, 2007). In many ATMT experiments by other researchers, the time allowed for selection was 5-7 days (Appendix 3), however colonies of *T. basicola* began to appear in the top agar after 10 days. Selection was often continued for up to 24 days before colony transfer; by this time, colonies had increased in size, mycelial growth, and migration close to (or on top of) the surface of the top agar, making it easier to excise the colonies from the gel.

Further selection of *T. basicola* Hyg^R putative transformants on HygB amended medium performed in this work ensured that (1) all Hyg^R putative transformants were viable and (2) that selective pressures were maintained (i.e. growth at 10-25µg/ml HygB) throughout the isolation of the colonies. This is important in establishing a transformation protocol, especially before T-DNA integration and mitotic stability are confirmed. After the HygB resistance and mitotic stability of putative transformants is well established, the transformants can simply be excised from the gel and grown on non-selective medium to obtain an isolated colony used for subsequent storage, analyses (e.g. Southern blot, pathogenicity screening, phenotypic tests), and manipulations (e.g. recovery of tagged sequences by TAIL-PCR) (this work; Elliot, Personal communications).

If the colonies produced on selective top agar were from the spores of the same *T. basicola* colony, e.g. due to top agar spread, then they would have the T-DNA integrated at the same location and thus the same tagged sequence. This would not be beneficial since the aim is to tag as many different *T. basicola* genes as possible in order to identify those conferring pathogenicity. However, the Hyg^R putative transformants 66p4b and 66p4d, taken from the same plate and in very close proximity showed marked differences in vegetative growth, morphology, and pathogenicity tests (see Figures 2.13, 14, 16, and 17), indicating that such colonies are not likely to arise from the same spore.

2.4.6. Transformation Efficiency of ATMT in Filamentous Fungi

The variability observed in the number of *T. basicola* Hyg^R putative transformants is commonly occurrence observed in the transformation of many filamentous fungi using ATMT (Mullins, et al., 2001; de Groot et al., 1998; Covert, et al., 2000; Combier, et al., 2003; Leclerque, et al. 2003) and may simply be due to uneven spreading of the cells during co-cultivation plating. ATMT has been found to produce a high rate of transformation efficiency in a number of filamentous fungi (Table 2.3). When using the optimal conditions established, the efficiency of ATMT in *T. basicola* (i.e. 300-770 Hyg^R putative transformants / 10^6 endocondia) is within the range of efficiency found for *Fusarium circinatum, Verticillium dahliae, Neurospora crassa,* and *Magnaporthe orzyae*, and above that found in *Aspergillus niger, Colleotrichum gloeosporioides, Fusarium venatum, Fusarium oxysporum, Maganporthe grisea, Hebeloma, cylindrosporum, Beauveria bassiana, Tirchoderma reesie,* and *Agaricus bisporus* (Table 2.3).

Table 2.3. Transformation Efficiency of ATMT in Filamentous Fungi

Species	**No. of Hyg^R colonies/10^6 endoconidia**	**Reference**
Aspergillus niger	0.5	de Groot, et al., 1998
Colleotrichum gloeosporioides	90	de Groot, et al., 1998
Fusarium venatum	2.5-10	de Groot, et al., 1998; Mullins, et al., 2001
Fusarium oxysporum	40-200	Mullins, et al., 2001
Fusarium circinatum	20-1,500	Covert, et al., 2000
Maganporthe grisea	200	Mullins, et al., 2001
Verticillium dahliae	50-500	Mullins, et al., 2001
Hebeloma cylindrosporum	60-80	Combier, et al., 2003
Beauveria bassiana	100-200	Leclerque, et al., 2003
Tirchoderma reesie	70	de Groot, et al., 1998
Neurospora crassa	500	de Groot, et al., 1998
Agaricus bisporus	0.5	de Groot, et al., 1998
Magnaporthe orzyae	500	Betts, et al., 2007

As this method of transformation is simple to perform and not very time consuming, the number of transformants generated can be further scaled up by simply increasing the number of co-cultivation plates. In the PEG mediated transformation system, used to generate *T. basicola* transformants thus far, an average 2.5 transformants/10^6 endoconidia were produced (Al-Jaaidi, 2007). Thus ATMT can be viewed as an excellent method of random mutagenesis for the generation of *T.basicola* mutants.

2.4.7. Confirmation of T-DNA Integration

2.4.7.1. Southern Blot Analysis of Hyg^R Putative Transformants

The DIG-labelled hph probe constitutes the Hygromycin B resistance *hph* gene cassette and left border of the T-DNA from plasmid pBHt2. The probe is thus designed to confirm successful insertion of the T-DNA into the genome of Hyg^R *T. basicola* putative transformants. It is expected that a band would be present in the genomic DNA of transformants, whilst being absent from the wild type, which lacks the *hph* gene. Hybridization with this probe will allow further conclusions to be drawn as to the nature of the T-DNA insertions. When digesting the genomic DNA with specific restriction enzymes, the number of bands present reveals how many insertion events have occurred in the genomic DNA of each transformant. It is most favourable that a transformation system produce single insertion events at a single locus, as this will minimise any difficulties with later recovery of the tagged sequences. The band sizes indicate randomness of integration and may also give some idea as to whether tandem repeats have occurred (depending upon which restriction enzymes are used). It is important that a successful transformation system result in random integration in the host genome to increase the chances of tagging *T. basicola* pathogenicity genes. Unless homologous or highly repetitive sequences are present in the host genome, T-DNA integration will generally produce non-tandem, single or low copy number inserts at random locations within the genome. (Covert, et al., 2000; de Groot, et al., 1998; Dobinson, et al., 2004; Bundock, et al., 1999 and 2002; Shi, et al., 1994; Kahmann & Basse, 1999; Meyer et al., 2000).

Only the T-DNA should be integrated into the fungal genome during ATMT. However, on several occasions, sequences of the Ti plasmid located outside the T-DNA are inserted (Covert et al., 2000; Combier, et al., 2003). Therefore, in this work, the Km probe was constructed, constituting a fragment of the plasmid pBHt2 that includes the Km^R gene, which is located outside of the T-DNA. Since genomic DNA was digested with the restriction enzyme *Nhe*I, hybridisation with the hph probe should result in a single band if the T-DNA is inserted once as expected.

The three bands corresponding to the controls pBHt2, pPK2, and the hph template DNA, which are visible after 30min exposure, indicate that successful hybridisation of the DIG-labelled hph probe has taken place with complementary sequences having high homology (i.e. pBHt2, the hph template DNA, and pPK2) The absence of a band for the fourth control (i.e. the Km probe template DNA) is expected since the hph probe has no complementary sequences with this DNA. This shows that the high stringency conditions used for the blot prevent non-specific binding of the probe. No band in the WT genomic DNA is expected, but the absence of bands for the 9 Hyg^R *T. basicola* putative transformants was not expected.

After two days exposure, some interesting results were observed. In this work it was found that *T. basicola* contains a high concentration of RNA, characteristically appearing ~8kb and ~6kb, as well as some very low molecular weight bands ~100-300bp. The high molecular weight RNA was always present in extracted genomic DNA and often still faintly observed even after RNAse digestion for 1hr prior to gel analysis. Thus, binding of the hph probe to these RNA sequences would produce uniform bands at ~8kb and ~ 6kb, which is exactly what was observed in the blot. Since the integrated T-DNA contains the *hph* gene, which must be expressed in order to convey the HygromycinB^R observed in the transformants, then hph mRNA would also be present in the fungal cells. Thus, if no band is present in the WT genomic DNA, then a plausible theory is that these bands are the result of the probe binding to mRNA, rather than integrated DNA. However, it is unclear (due to the background on the blot) if there is a band in the WT; if there was then it is still likely to be the result of probe binding to RNA but the reason for this binding is uncertain.

It is possible that the concentration of DNA used in the blot was too low for detection of T-DNA inserts, though high enough to detect RNA. In several experiments attempted to extract suitable concentrations of fungal genomic DNA, no high molecular weight DNA bands were observed, though the typical 8kb and 6kb RNA bands were still clearly visible (figure not shown). In this work, 1-1.5µg of fungal genomic DNA was used, as this concentration was sufficient for Southern analysis of the *T. basicola* PEG transformants (this work chapter 3; Al-Jaaidi, 2007). However, Southern hybridisations with ATMT mutants can require genomic DNA concentration as high as 5-10µg (Amey, et al., 2002; Bundock, et al., 1995). Genomic DNA is present, as evidenced by gel analysis prior to the blot, but perhaps insufficient transfer to the membrane occurred, (although sufficient for the controls).

One other possibility is the probe used for detection. For the 5 PEG transformants a DIG-labelled probe was used for hybridising, which consisted of the entire 6.9kb plasmid (this work; Al-Jaaidi, 2007), compared with a 1.8kb fragment used here. There would likely be more DIG molecules incorporated into this larger probe, thus not requiring as a high concentration for detection. It may be that a more suitable probe labelling method is required; radioactive labelled probes (e.g. ^{32}P- dNTPs) are commonly used for detecting T-DNA inserts in the genomic DNA of ATMT mutants (Amey, et al., 2002, Betts, et al., 2007; Meyer, et al., 2003; Combier, et al., 2003; Mullins, et al., 2001).

Low DNA and/or probe concentrations would be especially relevant if single and non-tandem inserts had occurred as expected. However, due to limited time, higher concentrations of pure DNA and radioactive probe labelling were not able to be tested.

2.4.7.2. Polymerase Chain Reaction

PCR is often used for confirmation of DNA insertions, however this technique does not allow for the same extent of information about the nature of the insert like the Southern blot. Primers are designed to detect sequences within the T-DNA, in complement to the *hph* gene. Verification is thus achieved by amplification products the same size as *hph* gene (Dobinson, et al., 2004; Combier, et al., 2003; Leclerque, et al., 2004). The most likely reason for the lack of amplification product in this work is due the primers used. In this project, the primers for amplification had complimentary sequences to the *trpC* promoter of the *hph* gene and the left border of the T-DNA. Though the forward primer was well within the right border of the T-DNA, the reverse primer matched the sequence of the left border. It is common that part or even all of the left and/or right border sequences can be deleted during integration of the T-DNA (Leclerque, et al., 2004; Bundock, et al., 1995; Combier, et al., 2003). As a result, the reverse primer probably had no or insufficient complementing sequence with which to bind and thus the inability for amplification of the fragment. These primers were used since they were the only available primers supplied at the time of this experiment. In addition, the genomic DNA used in this work was not pure. Optimal conditions for extracting high concentrations of clean genomic DNA from *T. basicola* have not yet been established. The DNA contains high concentrations of cellular contaminants like carbohydrates and proteins. The cellular contaminants may have inhibited the action of Taq polymerase. Due to limited time the use of better suited primers and optimal DNA purification conditions were not able to be tested.

Despite the inconclusive evidence exhibited by genetic analyses of the Hyg^R putative transformants, a number of phenoyptic properties, which included, mitotic stability, level of pathogenicity, general characterisation (e.g. vegetative growth and colony morphology) and HygB resistant growth suggest that they are indeed genetically modified.

2.4.8. Mitotic Stability of *T. basicola* Putative Transformants

A high rate of abortive transformants, due to loss of the T-DNA insert, would prove difficult in the subsequent manipulations of transformants required to elucidate tagged pathogenic genes. As such, for a transformation system to be of any use, consistent generation of mitotically stable transformants is of great importance. Since the integrated DNA contains a selectable marker, after loss of the insert, mitotically unstable mutants will be unable to maintain growth on selective medium. To establish mitotic stability the fungal transformants are grown for several generations on unamended plates lacking the selective pressure and then transferred back to selective medium. Those transformants possessing stable integration will retain their ability to grow under selective conditions whilst those with unstable insertions will have lost the insert and fail to grow. The frequency with which mitotic instability occurs depends largely upon the method of transformation and the particular fungal host. Loss of the T-DNA insert is not a common occurrence in ATMT and mitotic stability of the Hyg^R putative transformants in this work is in accordance with the findings by other researchers for other filamentous fungi transformed by ATMT (Mullins, et al., 2001; de Groot, et al., 1998; Meyer, et al., 2003).

2.4.9. Analysis of Reduced Virulence in the *T. basicola* ATMT Putative Transformants

Though numerous *T. basicola* transformants could be produced by ATMT, those exhibiting reduction in pathogenicity are of most value in elucidating the molecular events that govern pathogenesis of black root rot in cotton. Since all transformants need to be screened for reduced virulence, the test needs to be rapid, yet sensitive, and easy to reproduce. As such, the root dip assay lends itself as a convenient pathogenicity test. The idea is to directly expose cotton seedling roots to a fungal suspension and then examine the extent of root damage and general seedling growth after a given time. The browning and percent lesion of the roots (length of root lesion/total root length) infected by *T. basicola* transformants are compared with positive (WT *T. basicola*) and negative (water) controls. Seedlings showing a reduction in disease symptoms are indicative of *T. basicola* transformants having reduced pathogenicity, possibly due to disruption of a key pathogenic gene. A rating of pathogenicity can be assigned to each transformant which is in direct correlation to the percent of root lesion exhibited.

According to the pathogenicity rating, two of the 10 Hyg^R putative transformants tested, 68p1a and 64p1a-2, are classified as intermediate in virulence. However, these Hyg^R putative transformants showed variability in the disease symptoms exhibited between each root and as a result were not classed as differing significantly from the WT. This is likely because this was a preliminary pathogenicity test and further confirmation would be needed. Secondary tests should be performed using a specific concentration of endoconidia ($3.5x10^4$ has been shown to give good results) and growing the seedlings both on media plates as well as in soil (Al-Jaaidi, 2007; Bailey, et al., 2002; Dobinson, et al., 2004; Sweigard, et al., 1998). The three Hyg^R putative transformants, 64p3a-1, 65p1e, and 66p4d did show a significant reduction in pathogenicity making them good candidates for the recovery of tagged pathogenicity genes; in particular, 64p3a-1 as it has similar growth rate to that of the WT on rich medium (thus the possible mutation is probably not due to mutation of a general "housekeeping" gene).

2.4.10. Vegetative Growth and Colony Morphology Analyses of Hyg^R Putative Transformants

Standard vegetative growth is the typical growth exhibited by a fungi under standard conditions i.e. for *T. basicola* ½ PDA (1.2% agar) rich medium incubated at 25°C. The reduced growth rate seen for six of the Hyg^R putative transformants in comparison to WT *T. basicola* may be due to the metabolic burden placed on the Hyg^R putative transformants from the constitutive expression of the *hph* gene under the trpC or gpd promoter. This was also observed for the five *T. basicola* reduced pathogenicity PEG mutants, which have constitutive expression of the *hph* gene under the trpC promoter. Interestingly, 64p3a-1, which showed significantly reduced pathogenicity, displays vegetative growth that does not differ significantly to WT *T. basicola*. This indicates that, the reduction in disease severity does not necessarily correlate to a reduction in growth rate, which further validates that reduced pathogenicity exhibited by these Hyg^R putative transformants is attributed to a real reduction in virulence due to a mutated pathogenic gene.

The white colouring observed for both 64p1a-2 and 42p3f indicates a reduction in melanin content, this is likely because both of the Hyg^R putative transformants produced fewer chlamydospores by comparison to the WT. It is possible that the disrupted gene may be involved in the regulation of chlamydospore production or development. Interestingly, 64p1a-2 was one of the Hyg^R putative transformants that showed reduced disease severity. For 67p2c, the velvety texture seems to indicate an increase in hyphal growth, which correlates well with the high vegetative growth seen in this Hyg^R putative transformant (compared to the other 9 Hyg^R putative transformant tested, as well as the WT). Interestingly, disruption of *CPMK1*, a mitogen activated protein kinase (MAPK) encoding gene in *Clavicep purpurea* resulted in a similar increased vegetative growth. Such genes have also been implicated in pathogenicity for many filamentous fungi, (Tudzynski & Sharon, 2003), though this is not likely the gene disruption for 67p2c, since this Hyg^R putative transformant did not show any reduction in pathogenicity. Two of the three Hyg^R putative transformants that had a defined central ring, (68p1a and 65p1e) also showed reduced disease severity (46p1c was not tested for reduced virulence) and though 65p1e showed decreased vegetative growth, 68p1a grew at a comparable rate to the WT. The texture of both 95p2a and 95p3c-, has been described as an "easily wettable mycelia" and may be due to a reduction in the hydrophobicity of the fungal surface. In *Schizophyllum commune* disruption to the gene *MPG1*, which encodes for a hydrophobin like protein, similarly resulted in this easily wettable appearance and a reduction in pathogenicity for this fungus. These two Hyg^R putative transformants have not yet been tested for reduced pathogenicity; interestingly however, hyrophobins are differentially expressed during fungal pathogenesis and have been implicated to play a role in initial host contact and appressorium formation (Howard & Valent, 1996).

2.4.11. Fluorescence of pCAMgfp Transformed *T. basicola*

The *gfp* gene originates from the jellyfish *Aequorea victoria* and encodes for GFP, a 238-amino acid green fluorescent protein. GFP and its derivatives have been successfully expressed in a wide array of organisms including filamentous fungi (Amey, et al., 2002; Liu, et al., 2002; Sweigard & Ebbole, et al., 2001; Sexton & Howlett, 2001). Expression of *gfp* can be observed in individual cells, cell clusters or populations and whole organisms. To observe fluorescence, it is necessary that the gene can be expressed and functional and in filamentous fungi this requires a suitable fungal promoter and *gfp* derivative with optimized codon usage for fungi. The Ti plasmid pCAMgfp used in this work (Appendix 3), expresses the SGFP protein, most often used for transformation of filamentous fungi. This *gfp* derivative contains a serine-to-threonine substitution at amino acid position 65 (S65T), which increases the fluorescence and solubility of the protein as well as causing a red shift in the excitation maxima. In addition, *SGFP* has plant optimized codon usage, which increases translation efficiency in fungi (Lorang et al., 2001). In pCAMgfp, this gene is under the control of the *Pyrenaphora tritici-repentis ToxA* promoter, which shows a high level of constitutive expression in filamentous fungi (Manning & Ciufetti, et al., 2005; Lorang, et al., 2005).

The ability to successfully express the green fluorescent protein in *T. basicola* could greatly aid in studying the function and time of expression for isolated putative pathoegenic genes and their roles in host fungus interactions. In *Magnaporthe grisea*, fusion of *EGP1* to *CAMmg* (a calmodulin gene required for appressorium development) showed that expression of this gene requires conidial attachment to the host surface and can be induced by adding plant waxes (Liu & Kolattukudy, 1999). In *Colletotrichum lindemuthianum* fusion of SGFP to *clpg2* (encoding an endopolygalactourunase gene) was expressed during early germination of endoconidia and the formation of appressoria (Dumas, 1999).

When using the plasmid pCT74, which also carries the *SGFP* under the control of the *ToxA* promoter, high levels of green fluorescent protein expression was observed in 8 filamentous fungi, all members of the Ascomycota (Lorang, et al., 2001). These results are promising and since some endoconidia and hyphae of Hyg^R *T. basicola* putative transformants tested in this work did show a light green colouring, further attempts to obtain green fluorescence with *T. basicola* transformed with pCAMgfp may prove rewarding. Since there is a two hour lag time for auto-activation of the green fluorescent protein's chromophore (Lorang et al., 2001), the Hyg^R putative transformants tested were grown in liquid culture for 5 hours prior to fluorescent analysis. However, these conditions may simply not have been optimal for expression of the green fluorescent protein in *T. basicola.* In addition, though integration of the *GFP* gene may be successful, it seems not all transformants will necessarily express the protein or levels strong enough to be visualised and thus a large number of transformants may need to be screened. In *Verticillium fungicola*, only three of the 12 putative GFP transformants expressed the green fluorescent protein and only 1 showed sufficient fluorescence for subsequent analysis of the fungus in host tissue during infection (Amey, et al., 2002).

However, even if no green fluorescence is observed in *T. basicola*, the autofluorescence exhibited may prove even more beneficial in tracking the disease progress in cotton, since no special staining or green fluorescent tagging would be required; provided that cotton does not omit the same level or colour of autofluorescence. The high amount of autofluorescence seen in the surrounding medium, is likely the result of some excreted fungal metabolites (Wu & Warren, 1984). It may be interesting to see if the amount/brightness of this autofluorescence in WT *T. basicola* increases during infection of cotton compared to normal growth conditions. This may give some indication as to whether *T. basicola* secretes products used during pathogenesis, such as fungal toxins, hydrophobins, or secondary metabolites involved in host recognition (Sexton & Howlett, 2000). During pathogenesis by *Brassica-Leptosphaeria maculans,* yellow autofluorescence was observed ahead of the hyphal advance, which likely resulted from the enzymatic degradation of the host cell walls (Sexton & Howlett, 2000).

2.4.12. Recovery of T-DNA Tagged Pathogenicity Genes

Since the sequence of the T-DNA is known, integration into genomic DNA will result in *T. basicola* transformants with "tagged" gene/s. Recovery of tagged genes from reduced pathogenicity mutants is essential for subsequent genetic identification and analysis of these putative pathogenicity genes. If time permitted, recovery of tagged genes would have been attempted by TAIL-PCR and plasmid rescue (see Chapter 4 for details).

Since the three Ti plasmids used in this work (pBHt2, pPK2, and pCAMgfp) contain the bacterial selectable marker and ori outside of the T-DNA (Appendix 3), for analysis of tagged genes using the technique of plasmid rescue, a suitable plasmid first had to be constructed. To confirm successful construction and functionality of pMAX, a binary vector built from the backbone of pBHt2, selection was made on Amp+ Km amended media since the 2kb insert contains an Amp^R gene and pBHt2 already has a Km^R gene.

The bands observed at 3.4kb and 5kb correspond to the two pBHt2 ligation fragments, while the remaining 4 fragments correspond to various ligation products. The 4.4kb band corresponds to the expected size of pBR322 and indicates that ligation of the two pBR322 fragments (i.e. 2.2kb and 2kb) has occurred to regenerate this plasmid. This is confirmed by growth of Amp resistant *E.coli* transformants. Another band is visible at ~7kb, which is likely due to ligation of the sticky *Eco*RI termini from the *Eco*RI+*Nhe*I 5kb fragment with the *Eco*RI+*Bsa*AI 2kb fragment. The 8.4kb band is due to re-generation of pBHt2 from ligation of the sticky *Nhe*I termini from the *Eco*RI+*Nhe*I 5kb fragment with the 3.4kb *Sma*I+ *Nhe*I fragment. A correspondence of this band with the relaxed form of the pBHt2 plasmid control and an absence of Km resistant transformants indicates that this is the linear form of the plasmid. This confirms successful digestion of the two closely related RE sites, *Eco*RI and *Sma*I in pBHT2 MCS has occurred.

The final band at ~10.4kb corresponds with the expected size of pMAX and indicates that the 3 fragments have ligated. However, no Km +Amp resistant colonies were observed. This may be due to the bacterial cells having been transformed with an insufficient quantity of pMAX, however, since only a single functional plasmid is required to enter the cells, this is not likely to be the case. Another possibility is that generation of pMAX results in a vector that has two ori of replication, both derived from pBR322, (Mullins, et al. 2001; New England BioLabs Catalogue 2002-2003). It may due to incompatibility as the presence of two ori's on a single plasmid may hinder its successful replication. The most plausible explanation however is that this is the linear form of the plasmid, resulting from ligation between the sticky *Nhe*I and *Eco*RI termini, but not the blunt ended *Bsa*AI and *Sma*I termini. This is further supported by the absence of a ~5.4kb fragment in the ligation mix, which would result from the joining of the 3.4kb

and 2kb fragments via these two termini. Further attempts to successfully construct pMAX were not performed due to limited time.

As an alternative to pMAX, construction of a second vector, pAIM3, also based on the backbone of pBHt2, has started. Amplification of the 250bp linker and 2kb hph and LB insert from pBHt2 was successfully achieved. However, due to limited time, construction of this vector was not able to be completed.

2.4.13. Conclusions

A number of transformation systems are available for filamentous fungi however, not all will be suitable for the fungus of interest and choosing the right system will prove critical if efficient transformation is to be achieved. Although a PEG-mediated transformation system has been established for *T. basicola*, producing some pathogenicity Hyg^R putative transformants, the protocol has proven unreliable, giving inconsistent results regarding protoplast production (often depending on hydrolytic enzyme batch etc.) and resulting in multiple, often tandem insertions, preventing efficient genetic analysis. If a large number of *T. basicola* reduced pathogenicity mutants are to be obtained, a high rate of transformation efficiency is required. Furthermore, for the recovery and analysis of those tagged sequences, stable, random, and single insertions are most favourable. For a number of filamentous fungi, including *T. basicola,* ATMT has proven an efficient, more reliable, and less labour intensive method of transformation than PEG/$CaCl_2$. However, this is the first report for the development of a successful ATMT transformation system for *T. basicola*.

The success of ATMT in filamentous fungi is dependent upon the effective development and optimisation of the technique for the specific fungus being transformed. In this part of the project, an attempt to establish a successful and efficient ATMT transformation system for *T. basicola* was made. The efficiency of transformation was found to be strongly influenced by the experimental conditions prior to, during, and after transformation. Successful transformation of *T. basicola* required optimising (1) growth and induction conditions of *A. tumefaciens,* including the temperature, duration of growth and addition of AS, (2) co-cultivation conditions including temperature, duration of co-cultivation, fungus:bacteria cell ratios, and media, (3) selection of the *A. tumefaciens* strain, AGL1 or LBA4404 in combination with the binary vector pBHt2, pPK2, or pCAMgfp, (4) *T. basicola* cell status, including germinating or non-germinating endoconidia, different aged spores, and mycelia, (5) selection conditions, including the method of selection (filter transfer vs top agar), HygB concentration, media, temperature, and duration.

On the basis of these findings, an optimised ATMT protocol for *T. basicola* was established, a summary of which is presented below: pre-cultivating *A.tumafaciens* AGL1 [pBHt2] at OD_{660nm} 0.15 in liquid IM with 200µM AS at 28^{o}C for 7hrs to reach a final OD_{660nm} of 0.7. Then, co-cultivating 100µl of the

induced bacteria with an equal volume of 1.0 x 10^6 germinating endoconidia/ml (~1 conidium: 250 bacteria) on IMAS (1.5%agar) pH 6 (Appendix 1) at 25^{o}C for 2 days. Putative Hyg^R putative transformants are then selected with 20ml ½ PDA (1.2% agar) top agar, supplemented with 10µg/ml HygB and 300µg/ml Mefoxin and incubated at 25^{o}C for up to 24 days. Individual colonies are transferred by gel excision from the top agar onto ½ PDA (1.2% agar) supplemented with 10µg/ml HygB, and incubated at 25^{o}C for 2 weeks. Isolated colonies are grown on ½ PDA (1.2% agar), supplemented with 10µg/ml HygB for subsequent long term storage and further analysis.

Though in early stages, the number of *T. basicola* putative Hyg^R putative transformants generated using these optimal conditions is already within the range found for other filamentous fungi and well above the efficiency found using the PEG/$CaCl_2$ method.

Confirmation of successful T-DNA insertion (as well as the nature of the insertion), using Southern blot analysis and PCR, was not completed due to limited time. Genetic analysis will be required to add *T. basicola* to the growing list of filamentous fungi successfully transformed by ATMT and to ensure that this transformation system indeed generates single and random insertions of only the T-DNA region in *T. basicola*. Southern blot analysis with the DIG- labelled hph and Km probes developed in this project, will be need to be repeated, but using higher concentrations of purer DNA. In addition, PCR can be used to confirm the success of integration, though better suited primers will be required which complement sequences located further within the T-DNA left and right borders; preferably flanking an internal sequence of the *hph* gene.

Other phenotypic tests performed on a subset of putative transformants, including mitotic stability, primary pathogenicity screening, vegetative growth, colony morphology, and HygB resistant growth indicated that these Hyg^R putative transformants are most likely real transformants as they showed different phenotype to the WT. In addition, valuable information about the efficacy of ATMT for use in transforming *T. basicola* was provided by the confirmation that all 10 Hyg^R putative transformants tested, showed mitotic stability (i.e. 100% stability). It is promising, that three of the ten Hyg^R putative transformants that underwent primary pathogenicity screening, (64p3a-1, 66p4d, and 65p1e) showed a significant reduction in virulence, in particular where a reduction in pathogenicity did not correlate to reduced growth (i.e. 64p3a-1).

These findings further validate the benefit that will result from using the ATMT transformation system of random mutagenesis to elucidate key pathogenic genes responsible for the molecular interactions that exist between *T. basicola* and cotton.

Chapter 3: Analyses of Five *T. basicola* Reduced Pathogenicity Mutants Generated by Polyethylene Glycol/$CaCl_2$ Mediated Transformation

3.1. Introduction

After a fungal pathogen has been successfully transformed by random mutagenesis and confirmation has been made that the mutants have both stable inserts and reduced virulence, retrieval of information about the tagged gene is desired. Various molecular techniques have been developed in order to reveal the nature of the disrupted pathogenicity gene and involve the direct recovery of the tagged gene using methods like plasmid rescue and TAIL-PCR (Amey, et al., 2002; Al-Jaaidi, 2007; Ruiz-Diez, 2002; Shi, et al., 1995; Meyer et al, 2003; Liu, et al., 1995; Liu, et al., 1993; Liu & Huang).

In earlier research, (Al-Jaaidi, 2007), five *T. basicola* reduced pathogenicity mutants p16, p737, p849, p888, and p954 were generated by transformation of *T. basicola* protoplasts with the plasmid pGpdGFP using the method of polyethylene glycol (PEG)/$CaCl_2$ mediated transformation (see section 2.1. for details of the PEG/$CaCl_2$ method and Figure 3.1 below).

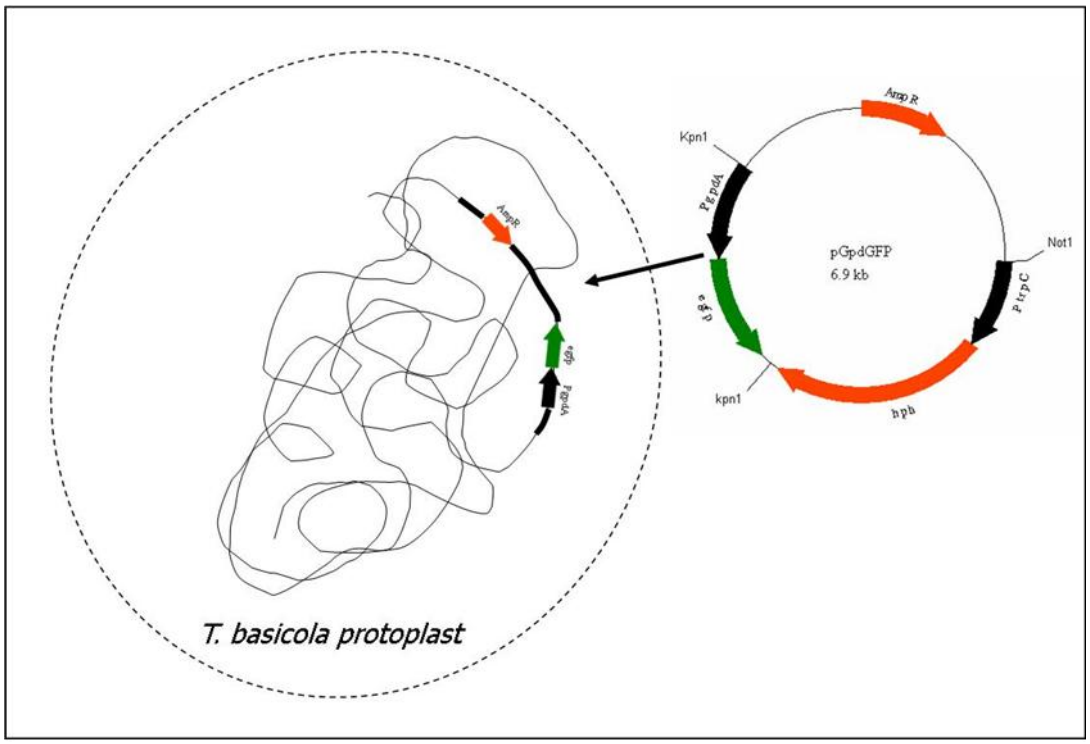

Figure 3.1. Polyethylene Glycol/ $CaCl_2$ - Mediated Transformation of *T. basicola* protoplast.

As discussed in section 2.1., recovering the disrupted pathogenicity genes from fungal transformants generated by PEG/$CaCl_2$ mediated transformation can be difficult due to the nature of plasmid integration when using this method, which often results in multiple insertions of the plasmid in more than one locus.

Southern blot analysis is used, not only to confirm successful transformation by integration of transgenic DNA, but also to provide insight into the nature of that integration; including the number of copies of the inserted DNA, whether insertion was random or specific, and if multiple inserts did occur, whether they were single or in tandem. From this information it can be determined whether it will be possible to recover the tagged sequences from the genome of the reduced pathogenicity mutants.

When direct genetic analyses cannot be performed or when further information about the identity of the pathogenicity gene and the phenotype it controls is required, phenotypic tests can be used. Phenotypic tests are designed to allow comparison of the wild type with mutants under specific growth conditions and thereby allow understanding the nature of the disrupted pathogenicity gene.

3.1.1. Objectives

The aim of the work presented in this chapter was to reveal genotypic and phenotypic properties of five *T. basicola* reduced pathogenicity mutants previously generated by PEG/$CaCl_2$ transformation, in particular, pathogenicity related phenotypes.

Southern blot analysis of these mutants using a novel strategy would provide new information as to the nature of plasmid integration. This will aid in determining how efficient PEG mediated transformation is and also provide some important information as to whether gene recovery can be performed, which will assist in pathogenicity gene identification. Phenotypic analyses would elucidate the nature of the disrupted pathogenic genes by investigating traits that relate to pathogenicity in other fungi and also to confirm mutant properties.

3.1.2. Research Strategy

To assess the nature of plasmid insertion by PEG mediated transformation, genotypic analysis of the mutants was performed by Southern blot of the genomic DNA using the plasmid pGpdGFP as the probe. In addition to digesting genomic DNA with *Nhe*I, which does not cut within the plasmid and *Xba*I, which cuts once within the plasmid, another enzyme, *Kpn*I, which cuts twice within the plasmid was used for the first time. Southern blot on *Kpn*I digested genomic DNA, will allow further insight into the integration pattern of the plasmid pGpdGFP into each *T. basicola* mutant. The plasmid pGpdGFP, digested with (1) *Kpn*I and (2) *Not* I, which cuts once within the plasmid, was used as control in the Southern blot analysis.

Phenotypic analyses were performed on five pathogenicity mutants (and wild type) to test their growth rate on rich medium with no amendment, osmotic tolerance on different salt concentrations, pH tolerance on different pH concentrations, and growth at a range of temperatures. Other mutant fungal phenotypes noted were colony colour and morphology, number of endoconidia, endocondidia and chlamydospore morphology, and melanin content.

3.2. Methods

3.2.1. Southern Blot Analysis

3.2.1.1. Preparation of the pGpdGFP DIG-labelled Probe

The pGpdGFP DIG labelled probe consisted of the entire 6.9kb plasmid, pGpdGFP. Midi preparation of pGpdGFP was performed as described in section 2.2.8.2. Five µg of plasmid DNA was linearised by digestion with *Not*I in an 80µl total reaction and incubated O/N at 37°C. After gel analysis to confirm complete digestion, the reaction was terminated by heat inactivation at 65°C for 20min. The linearised plasmid DNA was purified using the QIAquick PCR Purification Kit for microcentrifuge (according to manufacturer's instructions) and eluted in 50µl of 10mM Tris-HCl (pH8.5).

Labelling of the pGpdGFP template DNA was performed as described in section 2.2.10.1, except that a small scale labelling reaction of 20µl total volume was instead used with reagents added in the following order:

10x Hexanucleotide mix (Roche)
10x DIG DNA Labelling Mix (Roche)
bring to19µl with sterile miliQ water
1µl Klenow enzyme 1U/µl (Pharmacia)

Confirmation of successful DIG labelling and estimation of DIG labelled probe concentration was performed as described in section 2.2.10.1.

3.2.1.2. Preparation of Genomic DNA from T. basicola PEG-Mediated Reduced Pathogenicity Mutants

Growth, harvesting, and freeze-drying of mycelia was performed as described in section 2.2.10.2.

The dried mycelia were ground to a fine powder, using a mortar and pestle, and transferred into a sterile 15ml disposable centrifuge tubes (no more than 3ml per tube). After adding 3ml of 50mM EDTA pH8/ 0.2% SDS and thoroughly mixing by vortexing to ensure that all mycelia was completely moistened, tubes were incubated in a water bath at 65°C for 15min.

Samples were spun in a swinging bucket rotor at 3,000rpm for 15 min at room temperature and the supernatant then transferred to a clean 15ml tube. 0.6ml of 5M KAc pH4.8 (Appendix 1) was then added to the supernatant, mixed by inversion, and incubated on ice for ~1 hr. Samples were then spun at 3,000rpm for 15 min at room temperature and the supernatant carefully transferred (leaving a small layer

of supernatant at the interface between the pellet and the upper supernatant) to a fresh 15ml centrifuge tube. An equal volume of isopropanol was added to the supernatant, mixed by inverting, and centrifuged at 3,000rpm for 10min at room temperature. The majority of the supernatant was discarded, leaving only enough to allow efficient uptake of the soft DNA pellet. The pellet was transferred using a 1ml pipette into a sterile 2ml microcentrifuge tube containing 1ml of chilled 70% ethanol and centrifuged at high speed for ~30sec. The ethanol was decanted from the tube and the 70% ethanol wash was repeated 2-3 times. DNA pellets were dried and dissolved in a final volume of 50-100µl 1xTE as described in section 2.2.10.2.

3.2.1.3. Digestion of Genomic and Plasmid DNA

One µg of fungal genomic DNA was digested with (1) *Nhe*I (10U/µl New England Biolabs), (2) *Xba*I (10U/µl New England Biolabs), and (3) *Kpn*I (10U/µl Pharmacia). Restriction digests were prepared (following manufacturer's guidelines) in 25µl total volumes and incubated O/N at 37^{o}C.

Restriction digestions of the plasmid pGpdGFP, to be used as positive controls in Southern blot analysis were performed using (1) *Not*I (10U/µl New England Biolabs) and (2) *Kpn*I (10U/µl Pharmacia). For digestion with *Kpn*I, 500ng pGpdGFP was prepared in 20µl total volume and incubated O/N at 37^{o}C. For digestion with *Not*I, the same stock of linearised pGpdGFP used for the DIG probe template (section 3.2.1.1) was used, but diluted 1:20.

Two µl aliquots of each genomic DNA digest were tested on a midi gel in order to confirm complete digestion. The remaining 23µl of each genomic DNA digest and 1-2µl of control plasmid digests (each constituting ~5ng of plasmid DNA) were run on a maxi gel at 38v for 16-18 hours.

The gel was stained in ethidium bromide and trimmed as described in section 2.2.8.5.

3.2.1.4. Southern Blot Procedure

The Southern blot procedure was performed as described in section 2.2.10.3.

3.2.2. Phenotypic Characterisation Tests of the Five *T. basicola* PEG Reduced Pathogenicity Mutants

3.2.2.1. Colony Growth and Morphology

Stab cultures were prepared for the five PEG mutants and WT *T. basicola* as described in section 2.2.4. Colony growth rate was determined by measuring colony diameter (using a ruler) at 4, 7, 10, and 14 days after inoculation. Colony morphology was assessed at 4, 7, 10, and 14 days, using both naked eye and stereomicroscope at 1.0-6.3 x magnification. Characteristics examined included colony colour, vegetative growth appearance/texture, colony growth patterns, chlamydospore production, and any other distinctive features were noted.

3.2.2.2. Endoconidia Concentrations

To determine endoconidium concentration (endoconidia/ml), a suspension was prepared by scraping 1/3 (from the centre stab to the periphery) of a 14 day old stab culture (section 2.2.7) into 3ml sterile dH_2O in a McCartney bottle, and vortexing for 1min. Endoconidia were separated from mycelia and chlamydospores by filtering the suspension through a layer of sterile mira cloth. The number of endoconidia was estimated by placing 15µl of endoconidial suspension onto a hameocytochamber counter viewed under the compound microscope at 40x objective.

3.2.2.3. Spore Morphology

To observe endoconidum morphologies, 1-2 drops of spore suspension (from the same suspension prepared for estimating the endoconidia concentrations) was placed onto a microscope slide and viewed under the compound microscope at 20x and 40x objective. Shape, size, and melanin content were noted.

To examine chlamydospore morphology, an inoculation loop full of a 14 day old stab culture (section 2.2.7.) was placed on a microscope slide with 1-2 drops of sterile dH_2O and viewed under the compound microscope at 20x and 40x objective. Individual chlamydospore shape and size, chain length and shape, number of basal and apical cells and melanin content were noted.

3.2.3. Tolerance Tests

The effect of NaCl concentration, pH and temperature ranges on the vegetative growth of the five *T. basicola* PEG mutants and WT on amended media, was tested using stab cultures described in section 2.2.7. For osmotic tolerance tests, ½ PDA (2.2% agar) medium was amended with 0%, 0.5%, 1.0%, 1.5%, or 2.0% NaCl (Appendix 1) and for pH tolerance tests, media plates had a pH 5, 7, 8, or 10 (Appendix 1). For temperature tolerance tests, media plates were unamended but incubated at 10, 15, 20, 28, 30, and 37^{o}C. In all tests, colony growth rate and morphology were assessed as in section 3.2.2.

3. 3. Results

3.3.1 Southern Blot Analysis

The entire 6.9kb plasmid, pGpdGFP, which was used to transform *T. basicola* was used as a probe for Southern blot in the genetic analysis of the five PEG mutants, p16, p737, p888, p849, and p954. Figure 3.2. shows the expected 6.9kb band that resulted from linearising pGpdGFP using *Not*I.

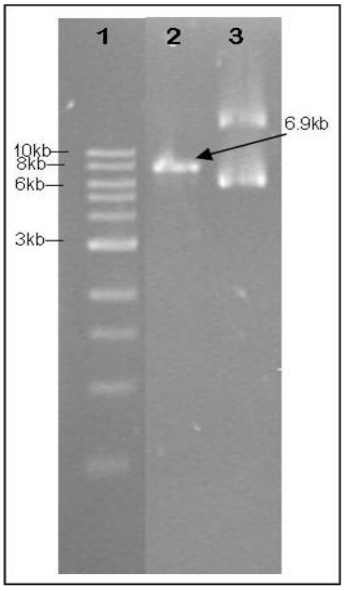

Figure 3.2. Gel Analysis of digested pGpdGFP Template DNA used for Preparation of the DIG-Labelled Probe To prepare the pGpdGFP probe template DNA, pGpdGFp as first linearised by digestion with the restriction enzyme *Not*I. After gel analysis, the linearised plasmid was cleaned. Lane 1: 1kb ladder, Lane 2: pGpdGFP + *Not*I, Lane 3: uncut pGpdGFP control.

Colour was detected for the probe check (Figure 3.3.) indicating that the probe had been successfully labelled with DIG. Based on the results the concentration of DIG-labelled probe was estimated to be ~10-14ng/µl.

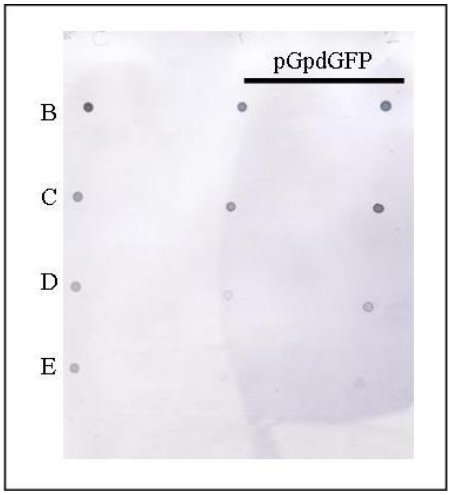

Figure 3.3. DIG-labelled Probe Check. Confirmation of successful DIG labelling and estimation of DIG-labelled probe concentrations was achieved by serial dilution of the DIG labelled probe and NBT/BCIP colour change detection. Lane 1: control DNA (Roche) dilutions, Lane 2 and 3: DIG-labelled pGpdGFP probe dilutions.

Fungal genomic DNA extracted from the WT, p16, p737, and p888 was seen as a single intense band of high molecular weight (~23kb). For both p849 and p954 there was insufficient genomic DNA for further use in Southern blot analysis (Figure 3.4).

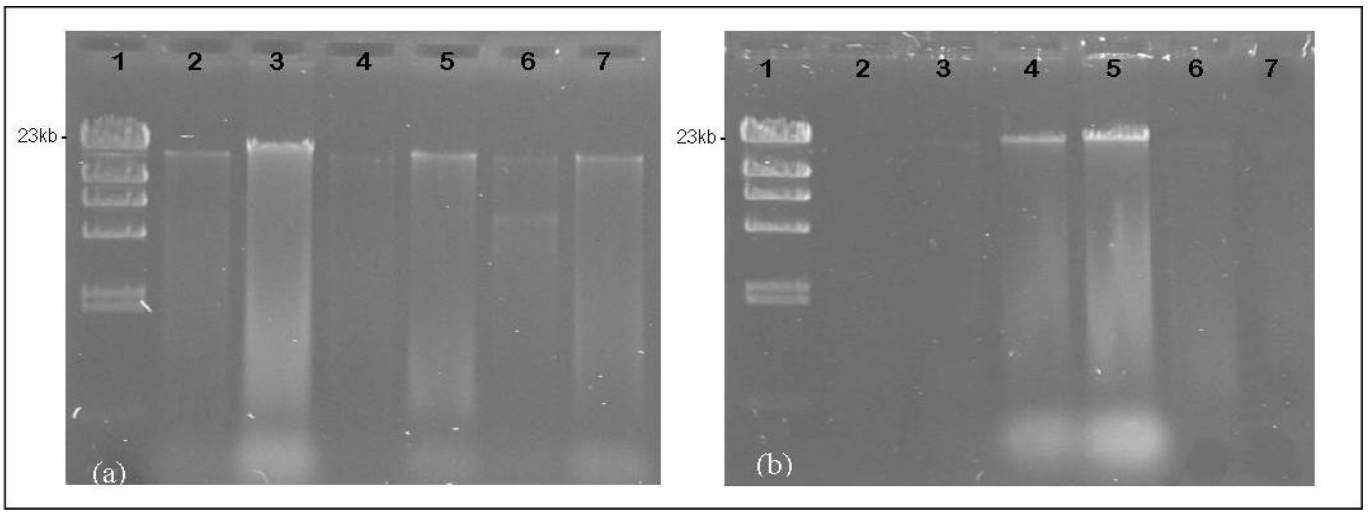

Figure 3.4. Gel Analysis of Extracted Fungal Genomic DNA Digested with RNAseA. Genomic DNA was extracted from five PEG mutants and concentrated by ethanol precipitation. Samples were digested with RNAaseA prior to gel analysis and two different volumes (0.5µl and 1µl) were run for each sample except p954 (1µl only). (a) Lane 1: λ *Hind*III Ladder, Lanes 2-3: WT, Lanes 4-5: p16, Lanes 6-7: p737. (b) Lane 1: λ *Hind*III Ladder, Lanes 2-3: p849, Lanes 4-5: p888, Lanes 6: p954.

Complete digestion of the fungal genomic DNA prepared for Southern blot analysis using the restriction enzymes, *Not*I, *Xba*I, or *Kpn*I was confirmed by the absence of the high molecular weight DNA bands (Figure 3.5). The plasmid pGpdGFP, digested with *Not*I or *Kpn*I, and run on the gel as control, was not seen since only 5ng was used.

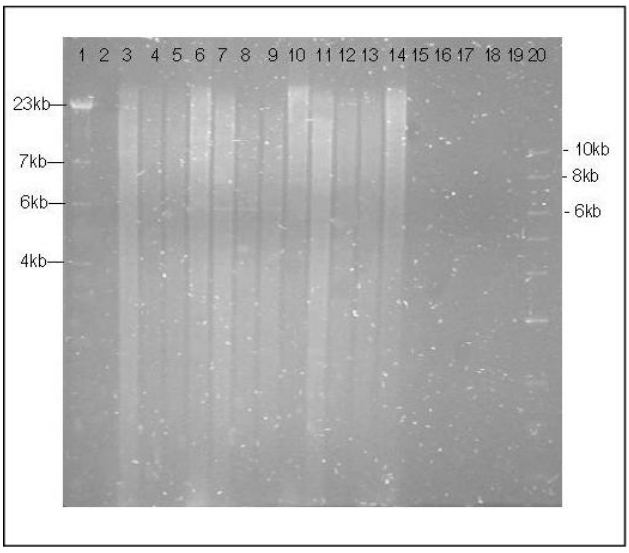

Figure 3.5. Gel Analysis of Digested Fungal Genomic DNA Prior to Southern Blot Analysis. Genomic DNA from the five PEG mutants (plus wt) was digested O/N with *Nhe*I, *Xba*I, or *Kpn*I. RNaseA was added 1hr prior to running the samples for 18hrs on a preparative maxi gel for Southern blot analysis. Lane 1: λ HindIII Ladder, Lanes 3-6: WT, p16, p737,p888 digested with *Nhe*I, Lanes 7-10: WT, p16, p737, p888 digested with *Xba*I, Lanes 11-14: WT, p16, p737, p888 digested with *Kpn*I, Lane 17:pGpdGFP digested with *Kpn*I, Lanes 18-19: pGpdGFP digested with *Not*I, Lane 20: 1kb ladder.

Southern blot analysis of three p16, p737, p888 and WT *T. basicola*, was performed using the DIG labelled pGpdGFP probe under high stringency conditions. With the exception of p16 and p737 DNA digested with *Nhe*I, multiple hybridisation bands of varying intensity could be seen for the genomic DNA of all three mutants digested with each restriction enzyme (Figure 3.6 and Table 3.1). No bands were seen in any of the WT digests. Control bands of pGpdGFP digested with *Not*I or *Kpn*I were seen at the expected single band of 6.9kb and double bands of 5kb and 1.9kb, respectively (Figure 3.6.).

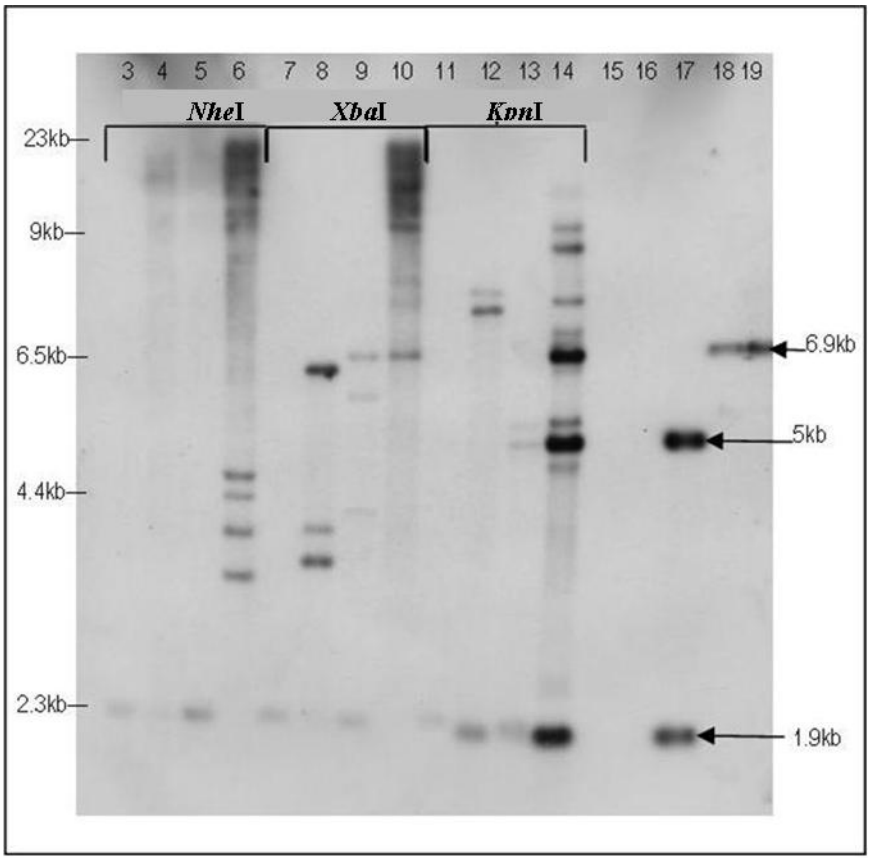

Figure 3.6. Southern Blot Analysis of 3 PEG Mutants Hybridised with the DIG-Labelled pGpdGF. Digested Genomic DNA from the transformants was hybridised with the DIG-labelled hph probe under high stringency conditions. The film was exposed to the membrane for 2hrs. Lanes 3-6:WT, p16, p737, p888 digested with *Nhe*I, Lanes 7-10: WT, p16, p737, p888 digested with *Xba*I, Lanes 11-14: WT, p16, p737, p888 digested with KpnI, Lane 17: pGpdGFP digested with *Kpn*I, Lanes 18-19: pGpdGFP digested with *Not*I.

Table 3.1. Fragment Sizes of Digested Genomic DNA from p16, p737, and p888 Hybridised by the DIG-Labelled pGpdGFP Probe.

Mutant	*Nhe*I	*Xba*I	*Kpn*I
p16	Not conclusive	6.6, 4, 3.4	11, 9.6, 1.9
p737	Not conclusive	6.9, 6.2	5.4, 5, 1.9
p888	21.4, 4.6, 4.4, 3.8, 3.2	17.4, 12, 9.6, 6.9	22.4, 18.6, 16, 10, 8, 7, 5.4, 5, 4.8, 1.9

Genomic DNA from p16, p737, and p888 was digested with N*he*I, *Xba*I, or *Kpn*I. Values indicate approximate band sizes (kb) of genomic DNA fragments hybridised with the DIG-labelled pGpdGFP probe.

3.3.2. Characteristic Phenotype Analyses of the Five PEG Reduced Pathogenicity Mutants

3.3.2.1. Spore Concentration, Morphology, and Melanin Content

Estimating endocondial number in 10 day old cultures revealed that for the mutant p849, there was a significant increase in endoconidial concentration in comparison to the WT (Figure 3.7).

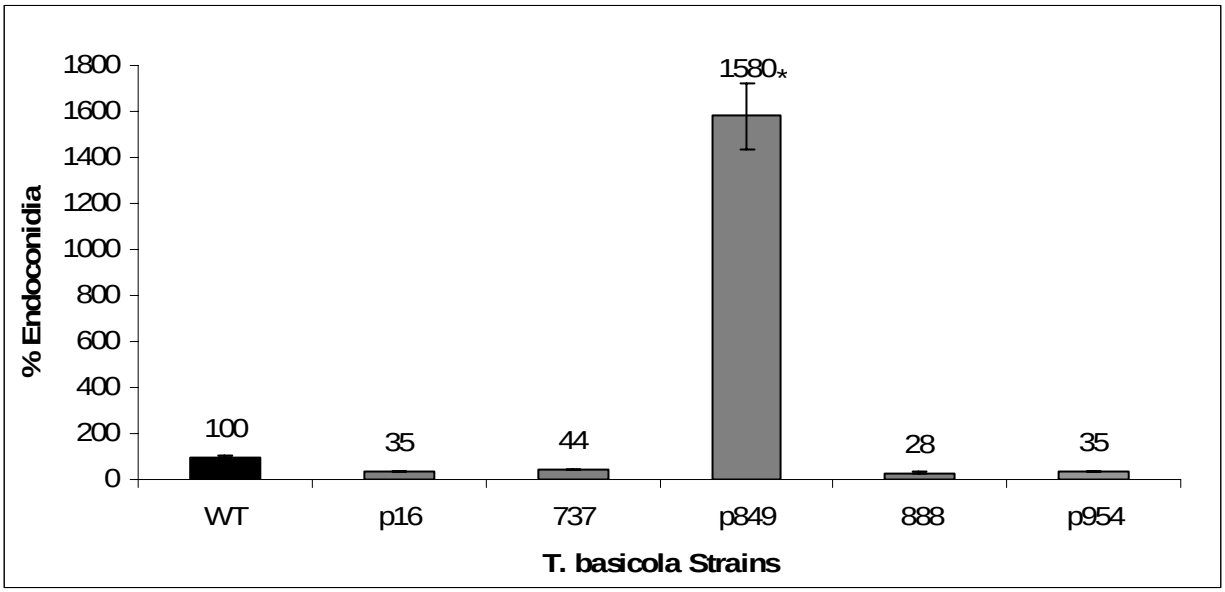

Figure 3.7. Endoconidia Concentration of the 5 PEG Mutants. A haemocytochamber counter was used to estimate the concentration of endoconidia (conidia/ml) from endocondial suspensions prepared for the five PEG mutants, plus the wt. Endocondial concentrations are expressed as percentages with the WT being 100%. Due to some counts having zero values all values were first transformed to log+1 to allow subsequently statistical analysis Levene's homogenity test showed no significance for experimental or interaction effects, allowing the 2 experiments to be combined. Significance was calculated Tukeys Post Hoc Test. The (*) indicates those values that differ significantly from the WT. Values represent the means of 6 replicates from two experiments. Error bars represent standard errors.

Observation of endoconidia and chlamydospores from the five PEG mutants viewed under the compound microscope (20-40x objectives) revealed that mutants p737, p849, and p888 produced endoconidia that appeared very similar to the WT; they were cylindrical with flat ends and had a thick dark periphery that surrounded a clear centre. Both p16 and p954, though producing typical spores, also synthesised a large number of abnormal endoconidia that were ~ ½ the size of normal endoconidia, appear swollen, and seemed to have a reduction in the thickness of the outer spore wall (Figure 3.8)

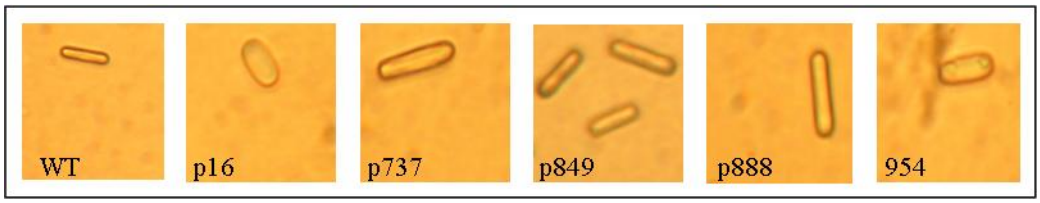

Figure 3.8. Endoconidia Morphology of the Five PEG Mutants. Endoconidia of the 5 PEG mutants and WT were viewed under the compound microscope at x40 objective. The abnormal spores for p16 and 954 are shown here.

WT *T. basicola* chlamydospores, when in a chain, had a typical circular but compressed shape and thick dark walls could be seen surrounding each spore of the chain (usually 2-4 spores/chain). In some cases, an overall envelope could be seen surrounding the entire chain. The most striking difference in chlamydospore morphology and melanin production was seen by p16, which typically produced ~5-15 spores per chain, each appearing smaller in diameter and more rounded than the WT spores, and lacking the normal compressed appearance. These spores didn't appear to be melanised. For p737, there was often only one chlamydospore per chain however, they appeared normal in morphology and still showed

comparable melanin content to the WT. For p849, a large number of chlamydospores were always seen tightly clustered together. Some of these spores appeared abnormal, having only one swollen spore per chain which showed reduced melanin content. The chlamydospores of p888, resembled the WT in morphology, but had higher melanin content. For p954, there were tyically1-2 spores per chain, which were much more dispersed. The overall melanin content of the spores also seemed reduced (Figure 3.9)

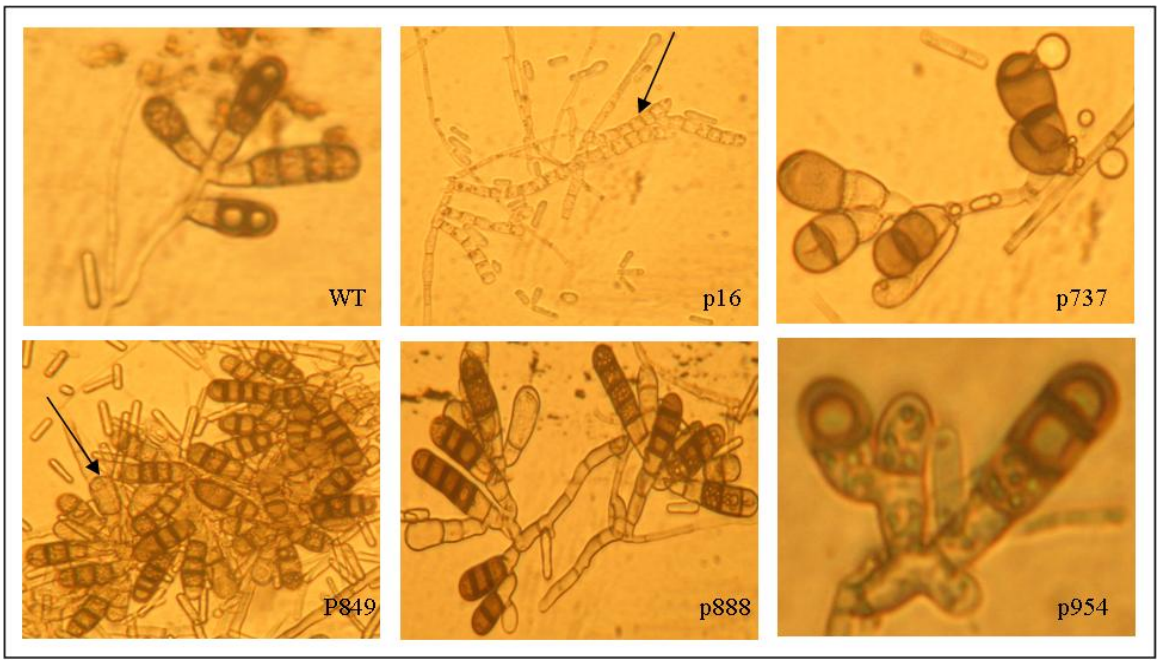

Figure 3.9. Chlamydospore Morphology and Melanin Content of the Five PEG Mutants.
Chlamydospores of the 5 PEG mutants and wt were viewed under the compound microscope at x40 objective. Arrows point at abnormal Chlamydospores.

3.3.2.2. Vegetative Growth and Colony Morphology

After 10 days, measurement of the colony diameter for all mutants indicated a slight but significant reduction in their vegetative growth compared to the WT. Growth of the mutants in comparison to each other consistently showed that p16 grew the slowest, p737 and p849 were intermediate and p888 grew the fastest. Mutant p954 exhibited varying levels of growth ranging from slow to medium (Figure 3.10).

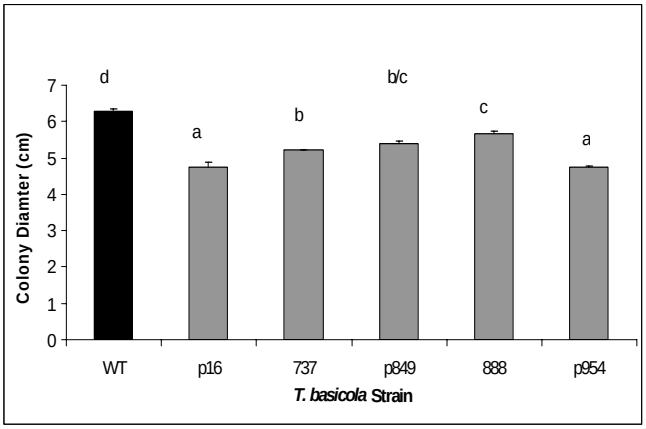

Figure 3.10. Vegetative Growth of the Five PEG Mutants.
The five mutants, plus WT, were stab inoculated onto ½ PDA and grown at 25°C for 14 days. Colony diameter was measured at 4, 7,10, and 14 days. Values represent the colony diameter at 10 days growth as this was found to produce the steadiest and most accurate growth period. The experiment was repeated twice. Due to experiment-isolate interaction for p954 (varied in growth from slow to medium), as determined by Levene's homogeneity test, the experiments were not combined. Significance was calculated using Tukey's Post Hoc Test and columns with the same labels do not differ significantly ($p<0.05$). Values represent the means from 6 combined replicates. Error bars represent standard error.

The colony of the WT was dark olive green to brown and the colony appeared "fluffy" in growth with a light white hyphal ring ~1/2 way from the centre. P16 had light fawn colouring in the centre of the colony, which became darker towards the colony periphery; fluffy white hyphae formed over most of the colony. Mutant p737 was similar in colouring and general appearance to the WT but had a slightly more "peppery appearance" due to the clustering of chlamydospores. Colonies of p849 were medium olive green with a brown/grey tinge. This mutant had a more pronounced fluffy appearance and whiter hyphal ring than the WT. Mutant p888 had very dark green/black colouring and an especially peppery appearance. p954 was fawn coloured but slightly darker than p16, especially in the centre (where chlamydospores were the thickest). The growth pattern for WT and p16 was often an evenly distributed circle extending from the centre plate. For p737, p849, and p888 this pattern was intermediate, with a circular appearance present in most cases, but less pronounced. By comparison, p954 often lacked the typical circular pattern growing with a more uneven appearance, especially at the peripheries (Figure 3.11).

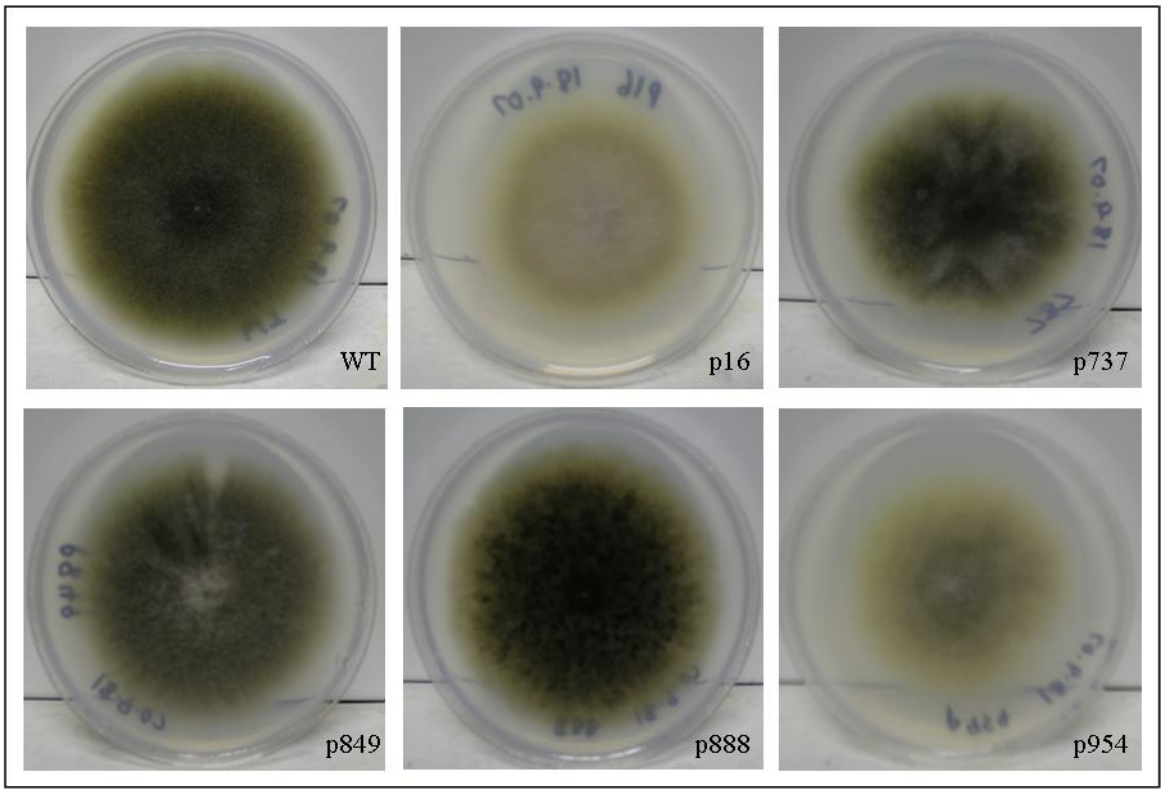

Figure 3.11. Colony Morphology of the Five PEG Mutants. The five mutants as well as the wt were stabbed into ½ PDA (2.2% agar) and grown at 25°C for 14 days. Colony morphology was observed at 4, 7,10, and 14 days. Plates shown here are from 10 days growth.

3.3.2.3. Osmotic Tolerance

When tested for osmotic tolerance on ½ PDA medium supplemented with 0%, 0.5%, 1%, 1.5%, or 2% NaCl, both the WT and five mutants showed a decline in growth as NaCl concentration increased (Figure 3.12). Mutants p737, p849, and p888 consistently grew slower than the WT at all salt concentrations. However, p16 and p954 did not follow this same pattern; as the concentration of NaCl increased beyond 1%, these two mutants actually grew faster than WT. For both p16 and p954, colonies on the 1.5% medium showed a marked increase in fluffy, thick, white hyphal growth (Figure 3.12 and Figure 3.13. a

and b). Mutant p888, showed a very similar growth pattern to the WT at all concentrations, but grew the slowest of all mutants, particularly at 1% NaCl. At 1% NaCl, p888 showed a dark brown/grey colouring and the typical peppery appearance was not so pronounced (Figure 3.12 and Figure 3.13 c).

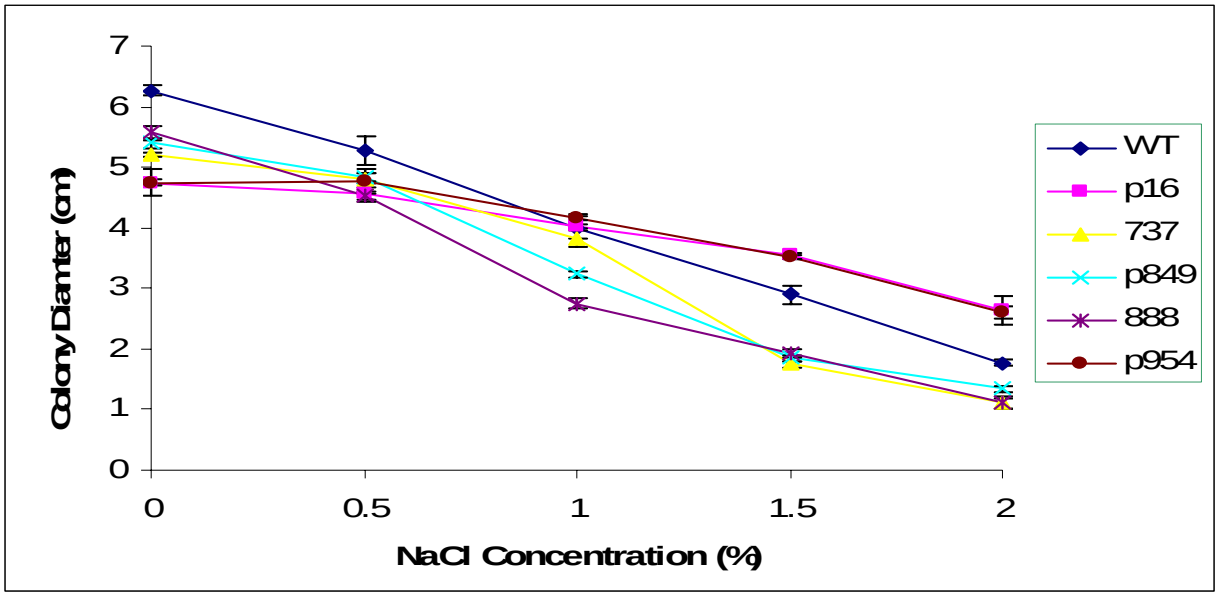

Figure 3.12. Osmotic Tolerance Test of the 5 PEG Mutants. The five mutants as well as the WT were stabbed into ½ PDA (2.2% agar) supplemented with NaCl (0%-2%) and grown at 25°C for 14 days. Colony diameter (cm) was measured at 4,7,10, and 14 days. Graph represents 10 days growth. Values represent means from two experiments with three replicates in each experiment. Error bars represent standard error.

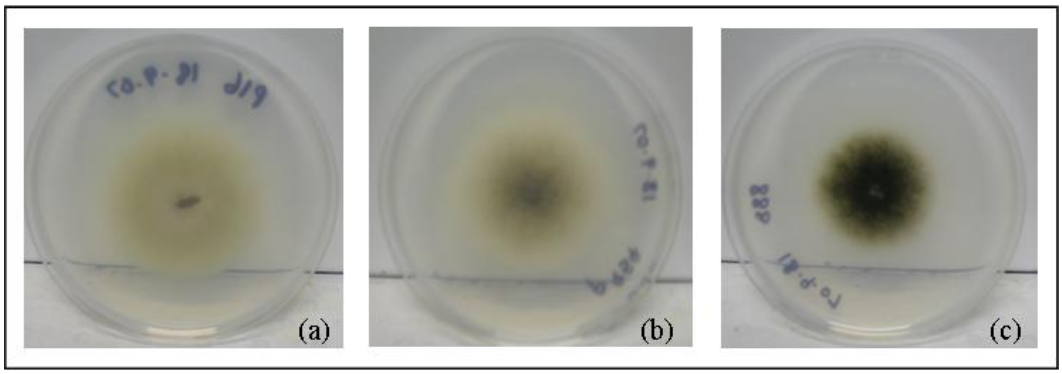

Figure 3.13. Differing Colony Morphology for p16, p888, and p954 on Selected Osmotic Medium (a) p16 on ½ PDA (2.2% agar) amended with 1.5% NaCl. (b) p954 on ½ PDA (2.2% agar) amended with 1.5% NaCl. (c) p888 on ½ PDA (2.2% agar) amended with 1.0% NaCl.

3.3.2.4. pH Tolerance

When tested for pH tolerance on ½ PDA solid medium at pH of 5, 7, 8, or 10, the WT showed a higher rate of growth than any of the mutants (Figure 3.14). A pH of 5 seemed similarly suppressive for all mutants. As the pH increased, the mutants generally followed the same trends seen for standard vegetative growth, i.e. p16 showed slowest growth, p888 the highest, and p737, p849, and p954 were intermediate. For all strains, the slightly alkaline pH 8 exhibited optimum growth. Colony morphology for each mutant and WT was similar to that observed in standard vegetative growth, with the exception of p737 at pH 10. At the extreme alkaline pH this mutant appeared to have inhibited filamentous growth and light brown colouring (Figure 3.15).

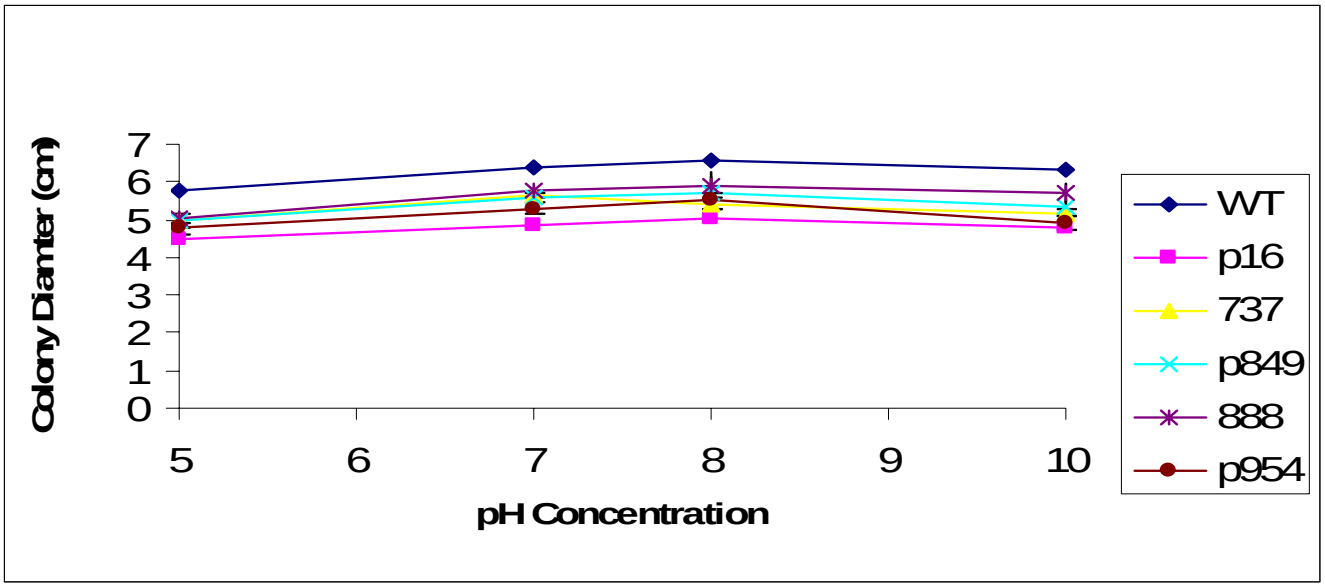

Figure 3.14. pH Tolerance Test for the Five PEG Mutants. The 5 mutants as well as the wt were stabbed into ½ PDA (2.2% agar) with varying pH (5-10) and grown at 25°C for 14 days. Colony diameter (cm) was measured at 4,7,10, and 14 days. Graph represents 10 days growth Values represent means from two experiments with three replicates in each experiment. Error bars represent standard error.

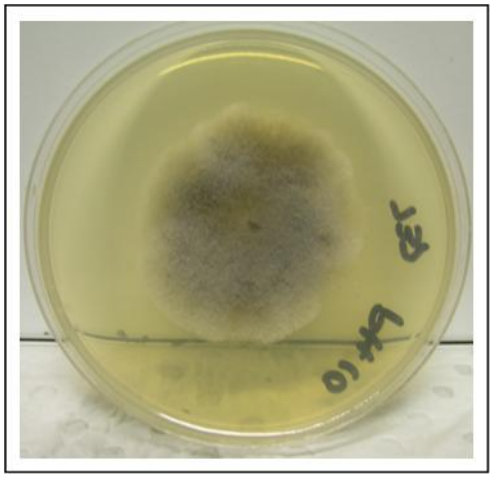

Figure 3.15. Differing Colony Morphology of p737 on Selected pH Medium. p737 on ½ PDA (2.2% agar) at pH 10.

3.3.2.5. Temperature Tolerance

When tested for temperature tolerance on ½ PDA medium incubated at 10, 15, 20, 28, 30, or 37°C, *T. basicola* strains showed minimal growth at 10°C, which increased to a maximum at 28 °C, and had declined by 30 °C with no growth seen at 37 °C (Figure 3.16). Overall, p737, p888 and 954 followed a similar trend and growth rate as the WT at all temperatures tested. The exceptions were p737 and p16 which did not show the marked increase in colony diameter from 20°C to 28 °C. Mutant p849 exhibited slightly slower growth except at optimal temperature (28°C). At 10 °C, p16 grew slightly faster than all strains, including the WT; at this temperature, the other 4 mutants had barely grown at all. By 15 °C, this mutant was similar in growth compared to the other mutants (and WT) and at the optimal temperature grew the slowest. However, as the temperature became unfavourable, p16 appeared to increase in growth; showing a larger diameter than p737 and p849. The colony morphology of p16 varied at different temperatures. At 10 °C, the colony was a light olive green in the centre and microscopy showed the presence many scattered chlamydospores not normally observed under normal vegetative growth (not

shown). At 15 °C the colony had a dark brown centre, extending to a fluffy white periphery. Chlamydospores were present but much fewer and more scattered. The colony morphology then appeared normal until 30 °C is reached, where alternating brown and white colouring patches of chlamydospores could be seen under the stereomicroscope (Figure 3.17).

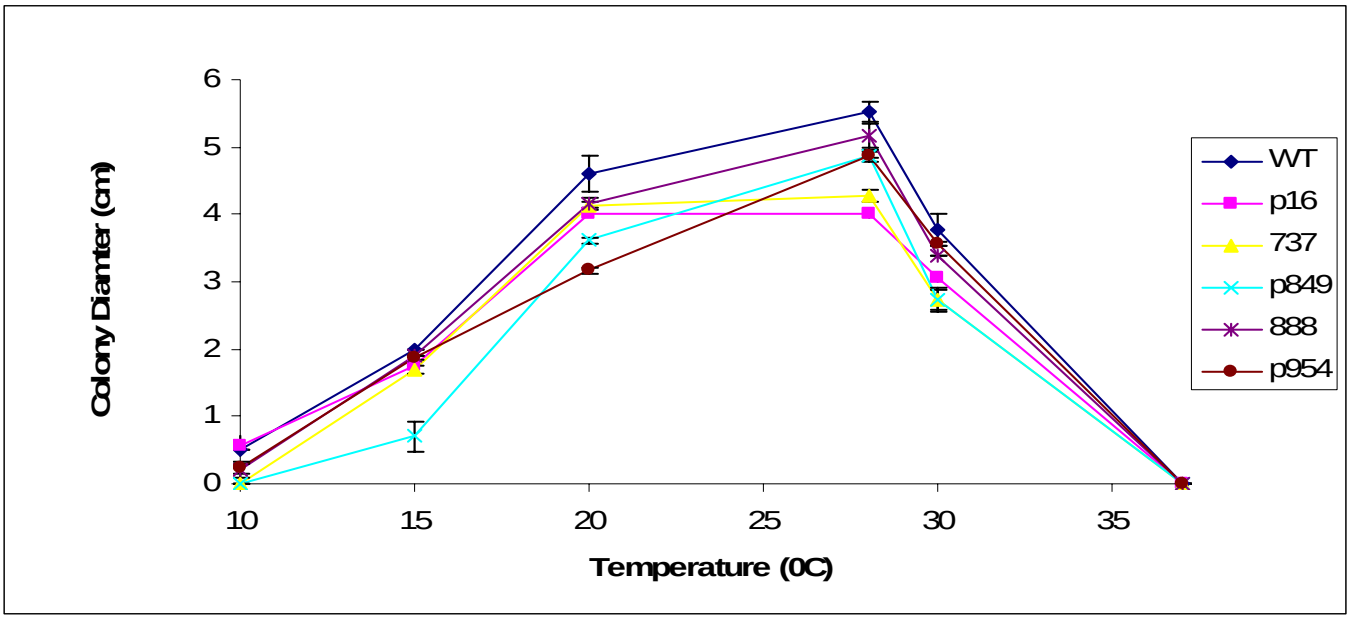

Figure 3.16. Temperature Tolerance Test of the Five PEG Mutants. The five mutants as well as the WT were stabbed into ½ PDA (2.2% agar) and incubated at temperatures ranging from 10-37°C for 14 days. Colony diameter (cm) was measured at 4,7,10, and 14 days. Graph represents 10 days growth Values represent means from 3 replicates. Experiment was performed twice. Error bars represent standard error.

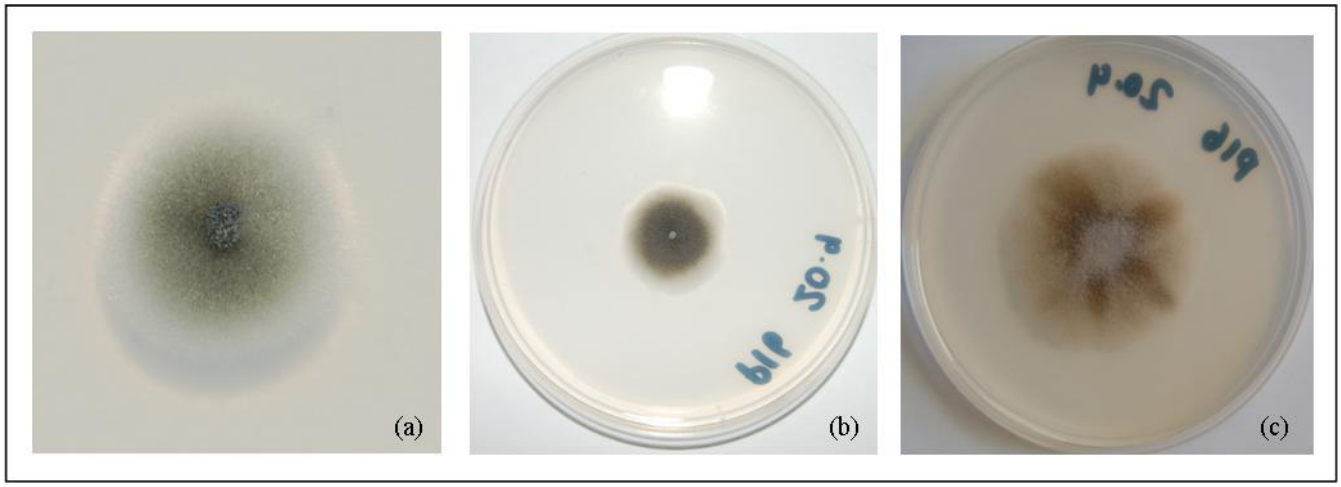

Figure 3.17. Differing Colony Morphology of p16 at Selected Temperatures. p16 on ½ PDA (2.2% agar) medium incubated at (a) 10°C, (b) 15°C, and (c) 30°C

3.4. Discussion

3.4.1. Plasmid Integration Pattern in *T. basicola* PEG Mutants

In PEG mediated transformation, the plasmid pGpdGFP was integrated into the genomic DNA of the five *T. basicola* PEG mutants. By digesting the genomic DNA with the three restriction enzymes, *NheI*, *XbaI*, or *KpnI*, and using the entire plasmid as the probe, one can conclude (1) whether the plasmid has integrated into the genomic DNA of the mutants, (2) how many insertions have taken place at different genomic loci, and (3) whether these insertions have occurred in tandem.

Since *NheI* does not cut within pGpdGFP, if a single copy integrated into the fungal genome, one band would be expected. If random integration has occurred in the different mutants, the bands should be seen at different positions for the five mutants. The dark smears at the top of all three *Nhe*I digests, is probably due to probe binding to undigested genomic DNA. This indicates that the plasmid has integrated into the chromosomal DNA of the 3 mutants and Hygromycin resistance is not due to autonomous replication of the pGpdGFP. *Nhe*I digestion for p16 and p737 prove inconclusive as to the nature of plasmid integration. For p888, 4 distinct bands are present between 4.6kb- 3.2kb and a fifth band appears faintly at 21kb. The exact nature of these smaller bands is unclear and maybe due to overdigestion of the DNA. However, it seems that integration at more than one location has occurred. These results compare well to those found by Al-Jaaidi (2007).

Since *Xba*I cuts once within pGpdGFP, if a single integration occurred, two bands would be expected. The sizes of the bands would depend upon their location of integration within the genome. A band the same size as the plasmid (i.e. 6.9kb) would indicate that at least two copies of the plasmid have been inserted in tandem. For p16, two intense bands are seen at 6.6kb and 3.4kb and a third medium intense band at 4kb.The presence and relative intensities of these three bands compares to past results (Al-Jaaidi, 2007). Since there are three bands and the sum of any two of the three is greater than that of the plasmid, then the plasmid may have integrated at more than one locus. However, it is more likely that it has integrated once and the extra band is due to star activity of *Xba*I; also seen in past results (Al-Jaaidi, 2007). Since none of these bands are the size of the plasmid, it indicates that insertion is not tandem. This is incongruous with the past results for p16, which indicated that the integration was in tandem due to the presence of a weak hybridisation band at 6.9kb (Al-Jaaidi, 2007). For p737, there is a band the same size as the plasmid, plus 2 smaller bands. This indicates that this mutant has multiple inserts of the plasmid at a single locus, with at least one insert occurring in tandem. For p888, a band also corresponds to the size of pGpdGFP, plus 3 additional bands, all larger than the plasmid; two of these bands, 12kb and 17kb were also observed in past analysis (Al-Jaaidi, 2007). This indicates that the plasmid has integrated at multiple loci within the genome, and insertion in at least one locus, has occurred in tandem.

*Kpn*I cuts twice within pGpdGFP to produce two bands with sizes of 5kb and 1.9kb. Digestion of genomic DNA with this enzyme would result in three bands if a single insertion has occurred. Sizes of two of the bands will vary depending upon the location of the insertion within the genome and how the plasmid integrated. Mutant p16 shows the 1.9kb band but not the 5kb band, indicative that the plasmid has integrated at some point within the 5kb region. Two other bands are observed at 9.6kb and 11kb, which would be expected by flanking fungal sequences and which further indicate that a single non-tandem integration has occurred in this mutant. For p737 and p888 both the 1.8kb and 5.1kb bands are present (as well as fainter 5.4kb band). Thus, the point of plasmid integration cannot be determined for these two mutants. The sizes of these bands further indicate that tandem insertion has occurred. Again, mutant p888 shows numerous bands of medium intensity all greater in size than pGpdGFP; indicative of multiple insertions. Interestingly, in p888, very intense bands are also observed at the sizes of both pGpdGFP *Kpn*I fragments, possibly indicating a second site or more copies of tandem insertion.

The multiple insertions of pGpdGFP into the genomic DNA of p737 and p888 can be explained by the typical nature of plasmid integration often obtained when using the method of PEG mediated transformation (Amey, et al., 2001; Meyer et al, 2003; Ruiz-Diez, 2002; Shi, et al., 1994). As a result, further attempts to recover the tagged sequences from these mutants may prove difficult. On the other hand, since the results indicate that a single non-tandem insertion has occurred in p16, further attempts to recover the tagged fungal sequences from this mutant may prove successful. The Southern blot indicates that integration could not have taken place within the 1.9 kb *Kpn*I fragment and since p16 is Hygromycin resistant, integration could not have occurred within the 2.1kb *hph* gene cassette. This leaves the 2.9kb region of the plasmid that contains the Amp^R gene. If integration occurred within either of the sequences flanking the Amp^R gene and ori, then plasmid rescue would be possible. However, if integration did take place within the gene itself or ori, then plasmid rescue would not be possible, as there would be no way to select resistant bacterial colonies containing the recovered plasmid. In this case, TAIL-PCR could be tried (4.3.3.). Since integration would be at an unknown point with the Amp^R gene, long nested primers would need to be designed that complement sequences just outside of the disrupted gene.

3.4.2. Phenotypic Properties of the Five PEG Mutants Possibly Related to Pathogenicity

The altered phenotypes for each of the five PEG mutants may have resulted from either direct disruption of more than one gene or by disruption to a regulatory gene. Such genes, encoding for transcription factors, cAMP dependent protein kinases, MAPK cascade enzymes, and GTP-binding proteins, have all been found to play an important role in pathogenicity in various filamentous fungi like *Magnaporthe grisea*, *Ustilago maydis*, *Colletrotrichum lagenarium, Fusarium oxysporum,* and *Pyrenaphora teres* (Idnurm & Howlett, 2001; Kahmann & Basse, 2003; Tudzynski & Sharon, 2003; Li, et al., 2006).

3.4.2.1. Spore Formation and its Relation to Pathogenicity

Since sporulation is essential for successful *T. basicola* survival and pathogenesis, altered characteristics of the mutant's endoconidia and chlamydospores may provide useful insight into the nature of the disrupted pathogenic genes. The significant increase in the number of endoconidia observed in p849 was also seen in past experiments; and showed that this same mutant had a significant increase in chlamydospore production as well (Al-Jaaidi, 2007). It is possible that this mutant has a disruption in a gene involved in regulation of spore synthesis or development and thus appears less virulent (i.e. smaller lesions) on cotton roots.

Endoconidia of *T .basicola* can appear abnormal and swollen under starvation conditions, (Stover, 1950; Hammil, 1974; Cole, 1991; Rigs & Mimms 2000; Hood & Shew, 1997). Interestingly, p16 shows lowest growth in both vegetative growth tests, while p954 shows low to medium growth and both produced abnormal swollen spores. It is possible that these two mutants have a disruption in a gene encoding a hydrolytic enzyme such as a chitinase, cellulase, or pectinase, which play a role in carbohydrate degradation, and thus appear starved. Such enzymes can also play an important role in host penetration and necrotrophy. Hydrolytic enzymes are also required for successful spore germination, both under normal conditions and during pathogenesis, and reduction in germination would also lead to decreased vegetative growth (Idnurm & Howlett, 2001, Kahmann and Basse, 2001 and references therein). In addition, in *Magnaporthe grisea*, gene mutations which result in abnormal endoconidia morphology can also result in abnormal appressorium formation and thus reduced pathogenicity (Howard & Valent, 1996; Idnurm & Howlett, 2001).

The highly abnormal chlamydospore morphology for p16 was also observed in past experiments (Al-Jaaidi, 2007). These spores actually look similar to the basal cells typically observed in a chlamydospore chain, which characteristically lack melanin and provide the branch point from which multiple chains develop (Riggs & Mims 2000; Cole, 1991; Stover, 1950). It may be that this mutant has a gene disruption that prevents the chlamydospores from correctly differentiating into typical apical cells. Mutant p849 shows a reduced melanin content, especially in the chlamydospores that appear abnormal morphology and not fully developed. Since chlamydospores only become melanized after the chain is complete (Riggs & Mims 2000; Cole, 1991; Stover, 1950), this may indicate that p849 has a disrupted gene involved the development and or maturation of the chlamydospores.

3.4.2.2. Melanin as a Possible Factor in T. basicola Pathogenicity

Melanins are a ubiquitous class of complex polymers that are typically black or brown. Melanin is involved in numerous mechanisms, which link it as an important determinant in pathogenicity. Melanin depositions in the spore walls confer rigidity, which helps protect the fungus from physical and biological stresses and promotes survival. Melanin has profound effects on interactions between a pathogenic fungus and its host. During host invasion melanin can protect the fungus from host defence toxins and also plays a role in maintaining high turgour pressure of infection structures during host penetration (Butler, et al., 2001; Howard & Valent, 1996; Gomez & Nosanchuk, 2003). Disruption to melanin genes often results in a reduction or loss of pathogenicity (Idnurnm & Howlett, 2001; Gomez & Nosanchuk, 2003; Butler, et al., 2001). The melanin content for each of the five PEG mutants compares well to past results obtained both by microscopy and liquid culture analysis (Al-Jaaidi, 2007). Reduction in pathogenicity for mutants p16, p849, and p954, may be due to disruption of a key gene involved in regulation or biosynthesis of melanin production. The resulting reduction in melanin may be thus related to one of the pathogenic properties provided by melanin. However, the increase in melanin content for p888 may also be responsible, at least in part, for its reduced pathogenicity. Increased melanin decreases cell wall permeability, which may have a significant effect on the ability of this mutant to secrete cell wall degrading enzymes, signal molecules, toxins, and secondary metabolites needed for successful pathogenesis (Howard & Valent, 1996).

3.4.2.3. Possible Metabolic Burden on T. basicola Transformants from pGpdGFP Integration

The type of promoter used to express the *hph* gene may explain the slight but significant reduction in growth seen by all five mutants in comparison to the WT. Since the plasmid pGpdGFP uses the constitutively expressed *Aspergillus nidulans* trpC promoter, this may result in an increased metabolic burden on the mutants (Jones et al., 1999). The growth of the mutants in comparison to the WT as well to each other is fairly consistent with the data obtained in the past (Al-Jaaidi, 2007). Under standard conditions, p16 shows the slowest growth rate, yet this mutant had only one plasmid integration, whilst p888 showed the fastest growth but had multiple inserts at multiple loci. This indicates that though constitutive expression of the *hph* gene by trpC promoter slows overall growth rate, there does not seem to be a correlation between the increased number of trpC promoters and growth rate. Other factors also play a role in determining the growth of the mutants, e.g. in p16 and p954 the presence of swollen endocondia, may indicate conditions of starvation, which could account for reduced growth.

3.4.2.4. Possible Relation between Osmotic Tolerance and Pathogenicity in T. basicola

The overall decline in fungal growth as the NaCl concentration increases is expected, since the higher the salt concentration, the greater the osmotic stress on the cell membranes and increased chance of cell lysis (Howard & Valent, 1996). Interestingly, though p888 demonstrates slightly higher standard vegetative growth and higher melanin content than the other four PEG mutants, this mutant consistently grows the slowest in salt-amended media. On the other hand, p16 and p954 have reduced melanin and grow the slowest under standard conditions, yet appear to grow fastest at high salt concentrations. Increased melanin content can decrease the porosity of cell walls, which prevents exogenous solutes (such as NaCl) from permeating into the cell but still allows water to freely pass through (Howard & Valent, 1996; Silverthorn, 2004). Thus, hyperosmotic conditions, can cause such highly melanised cells to collapse (i.e. cytorrhysis) (Howard & Valent, 1996). This may explain the slow rate of growth that was exhibited by p888 during osmotic stress. For p16 and p954, the lowered melanin content, would increase cell porosity, thereby preventing cytorrhysis and explaining the relatively higher growth rate exhibited by these mutants under osmotic stress. This "osmo sensitivity" exhibited by p888 may also be accounted for by disruption to a mitogen-activated protein kinase (MAPK) gene or a gene with similar function. Disruption to such a gene in *Magnaporthe grisea* (the *MPS1* gene) results in sensitivity to osmotic stress, defective cell-wall synthesis, and reduced pathogenicity (Tudzynski & Sharon, 2003).

3.4.2.5. A Possible Connection between pH Sensitive Genes and Pathogenicity in T. basicola

Overall, altered pH does not seem to exhibit much effect on colony growth for the WT or any of the mutants; showing their resilience to pH changes and further indicating that any effects of pH on *T. basicola* growth are usually associated with other factors e.g. ammonia or aluminium levels (Meyer & Shew 1991; Meyer, et al., 1994; Harrison & Shew 1999). However, the inhibited filamentous growth indicated by the colony morphology exhibited by p737 at pH 10 may have some correlation to its disrupted pathogenicity gene. In *Aspergillus nidulans,* pH sensing genes can be divided into those encoding secretory enzymes, permeases, or enzymes that synthesise secondary metabolites. Mutation to one such gene, *pacC,* which is preferentially expressed at alkaline pH, resulted in poor filamentous growth at alkaline pH and reduced pathogenicity (Penalva & Herbert, 2002).

Secretory enzymes, permeases, or enzymes that synthesise secondary metabolites are commonly implicated in fungal pathogenicity (Idnurm & Howlett, 2001) and disruption to such a gene may result in the decreased pathogenicity exhibited by p737. An alternative is that the disrupted gene encodes an ambient pH sensing receptor or transcription factor. Disruption to this gene may prevent necessary cellular regulation at alkaline pH, resulting in altered growth morphology for this mutant. Regulation of gene expression by ambient pH receptors in filamentous fungi has also been implicated to play a key role in fungal pathogenicity; controlling the production of toxins and secreted enzymes necessary for successful pathogenesis (Penalva & Herbert, 2002).

3.4.2.6. Expression of Some Pathogenicity Related Genes may be Temperature Dependent

For p16, the production of numerous typical ("WT-like") chlamydospores at extreme temperatures (i.e. 10^{o}C and 30^{o}C) compared to the abnormal chlamydsopres usually seen under standard growth is very interesting. This indicates that the ability of the mutant to produce typical chlamydospores with sufficient melanin content is possibly temperature dependent. This mutant may have a disrupted gene that encodes for a temperature dependent receptor or enzyme involved in chlamydospore synthesis and or maturation.

3.4.3 Conclusions

Genetic and phenotypic analyses shed light on the nature of the mutation of each PEG reduced pathogenicity mutant and the relation of phenotypic properties to pathogenicity in *T. basicola.*

Mutant p16 had a single insert of the plasmid pGpdGFP at a single locus within the genome. Insertion has taken place outside the *hph* and *gfp* genes and occurs somewhere within the 2.9kb region containing the *Amp* gene and ori. This mutant grows slowest under standard conditions but fastest under osmotic stress and extreme temperatures. It produces endoconidia and chlamydospores with abnormal morphology and shows a low melanin content under standard growth conditions but normal melanised spores at extreme temperatures. This mutant may have disruption in a gene involved in (1) regulation or biosynthesis of melanin production, (2) chlamydospore development/maturation, (3) a hydrolytic enzyme, or (4) development of penetration hyphae. This may be a temperature dependent/sensing gene.

Mutant p737 has multiple inserts (in tandem) of pGpdGFP at a locus within its genome. With the exception of abnormal colony morphology when grown at pH 10, this mutant does not show many alterations from the WT. The disrupted gene in p737 may encode (1) a regulatory receptor or transcription factor or (2) an alkaline pH sensing gene encoding enzymes which may play a role in pathogenesis.

Mutant p849 has excessive endoconidia and chlamydospore production and many of these chlamydospores have an abnormal morphology and low melanin content. The growth of this mutant is particularly slow at all temperatures tested besides the optimum. For p849, a disruption may occur in a gene involved in (1) regulation of melanin production or (2) regulation of spore production. This may be a temperature sensing/dependent gene.

In mutant p888, tandem insertions of pGpdGFP had occurred at 1-2 loci and additional single inserts at multiple loci. This mutant has increased melanin content and grows relatively fast under standard conditions but slowest under osmotic stress. For p888, one of the disrupted genes may play a role in (1) the regulation or biosynthesis of melanin production or (2) encode a MAPK gene.

Mutant p954 has slow to medium growth under standard conditions, abnormal endoconidia morphology and low melanin content in the chlamydospores. It is possible that this mutant has a disrupted pathogenicity gene involved in (1) regulation or biosynthesis of melanin production, (2) a hydrolytic enzyme or (3) development of penetration hyphae.

For each mutant the disrupted gene may be directly involved in pathogenesis or may instead be a regulatory gene.

For the reduced pathogenicity *T. basicola* PEG mutants containing a single insert, attempts to recover the tagged sequences could be performed. Past attempts at plasmid rescue were inconclusive, due to time limitations, (Al-Jaaidi, 2007), however in light of the information obtained in this study, it may prove beneficial to try this method and or TAIL-PCR, especially for p16.

In addition to the osmotic, pH, and temperature tolerance tests used to analyse these PEG mutants other phenotypic tests such as carbon and nitrogen utilisation and protease activity may provide some more information into the nature of the disrupted pathogenicity genes (Pereg-Gerk, personal communication).

Chapter 4. General Discussion

The diseases caused by plant pathogenic filamentous fungi, such as vascular and leaf wilt by *Fusarium oxysporum*, blast diseases by *Magnaporthe oryzae*, root, leaf, and stem rot by *Rhizoctonia solani*, tan spot necrosis by *Pyrenaphora tritici-repentis*, and black root rot by *T. basicola* are a continuous problem to global crop productions including rice, wheat, tobacco, and cotton (Mullins et al., 2001; Healy, 1923:100-103; Wrather, et al., 2002). A vast amount of research, time, effort, and resources are invested into the development and application of effective chemical, biological, cultural, and genetic control measures against these fungi and the diseases they cause (Abawi & Widmer 2000; Candole & Rothrock, 1996; Rothrock et al 1994; Manning & Ciufetti, 2005; Betts, et al., 2007; Nehl, et al., 2000; Kristyanne, et al., 1997).

One of the most effective ways to develop new disease control strategies is to have a good understanding of the underlying mechanisms by which the disease progresses. This project is part of a multidisciplinary research programme which aims to identify factors involved in the pathogenesis by *T. basicola* towards cotton causing Black root rot. Armed with this information, improved crop protection can be achieved by developing more effective control measures against both the fungal pathogen (e.g. fungicides) as well as increasing the resistance of the cultivars against the disease (e.g. genetically modified cotton). The overall research ultimately aims to achieve an increase in Australian cotton crop yields and other plants affected by Black root rot.

Despite this, for many pathogenic filamentous fungi, including *T. basicola*, the knowledge on these mechanisms that result in such widespread diseases, is very limited. An effective way to study disease-causing mechanisms is by gene disruption and analysis of those mutants exhibiting reduced virulence. Transformation techniques, both targeted and random, are by far the most common employed for such analysis. Generation of mutants exhibiting reduced pathogenicity, performed by random insertional mutagenesis strategies have proven effective in identification of a large number of virulence-related genes in pathogenic filamentous fungi including *Magnaporthe grisea, Fusarium oxysporum, Leptosphaeria maculans, Colletotrichum lagenarium, Pyrenaphora tritici-repentis,* and *Verticillium dahliae* (Sweigard, et al., 1998; Dobinson, et al., 2003; Gardiner & Howlett, 2003; reviewed by Sweigard & Ebbole, 2001 and Idnurm & Howlett, 2001; Ciuffetti & Manning, 2005). The main aim of this project was to develop an efficient random transformation protocol for *T. basicola*, which could easily generate a large number of reduced pathogenicity mutants towards cotton, and to recover and identify the nature/function of these tagged pathogenic genes.

Ideally, identification of reduced pathogenicity genes requires that a sufficient number of fungal transformants are generated in order to increase the probability that every pathogenicity gene in the fungal genome will be targeted. As such, the transformation system used needs to be capable of generating a high frequency of transformed colonies with single, stable inserts at random locations within the genome. Evidence from this work as well as many others (Leclerque, et al., 2004; Covert, et al., 2001; de Groot, et al., 1998; Betts et al., 2007; Mullins, et al., 2001; Combier, et al., 2003) shows that the method of ATMT is capable of meeting such expectations and is superior to other methods of random insertional mutagenesis like PEG/$CaCl_2$ and REMI (this work; Meyer, et al., 2003; Amey, et al.).

In developing a protocol for transforming *T. basicola* using ATMT, it was found that like all transformation protocols, the transformation efficiency with ATMT is strongly influenced by the experimental conditions, which must be optimised for each filamentous fungus (Mullins & Kang, 2001). As evidenced in this work, and published protocols for other filamentous fungi (Appendix 3) conditions of pre-cultivation and induction, co-cultivation, and selection, as well as the *A. tumefaciens* strains and binary vectors and the status of the fungus, all play a role in producing the most optimal results of transformation.

After successful confirmation of T-DNA insertion into *T. basicola*, the optimal conditions established in this project can be used to generate a large number of mutants that can be screened for reduced pathogenicity. It may be possible to identify a larger number of reduced pathogenicity transformants. In combination with the root dipping technique, it is common in our laboratory to perform a directional growth test in which the spore suspension is instead placed close by the roots rather than directly on them. This allows for the detection of any transformants that have mutations in pathogenicity genes involved in fungal growth towards the root or root contact (Al-Jaaidi, 2007). One possible way to increase the actual number of reduced pathogenicity mutants produced by ATMT, is based upon previous findings that *Agrobacterium* preferentially inserts its T-DNA into actively expressed genes (Wei, et al., 2000). As pathogenicity genes will be actively expressed at the highest rate during infection of host plant, one possible way to greatly increase the number of tagged pathogenicity genes could be to perform ATMT either (1) directly during *T. basicola* pathogenesis of cotton or (2) in the presence of cotton exudates or extract.

For *T. basicola* ATMT reduced pathogenicity $HygB^R$ colonies, 64p3a-1, 65p1e, and 66p4d, as well as the many reduced pathogenicity transformants which will be generated in the future, and the five PEG mutants analysed in this study (Al-Jaaidi, 2007), recovery of the tagged pathogenic sequences will be of great priori if *T. basicola* pathogenicity genes are to be elucidated. Two methods that are commonly used for recovery of tagged sequences are Thermal Asymmetric Interalced - PCR (TAIL-PCR) and plasmid rescue.

Thermal Asymmetrically Interlaced – PCR

This powerful method of recovery has proven a technical advancement showing highly efficient, rapid, and specific recovery of tagged pathogenic sequences, particularly when adjacent to T-DNA inserts. The technique relies upon the use of three nested long specific primers complimentary to sequences located close to the left or right border sequence of the T-DNA and a short arbitrary degenerate (AD) primer consisting of a random DNA sequence. The nested primers are used in 3 successive reactions (primary, secondary, and tertiary) together with the AD. Each reaction constitutes high and low melting points to allow high stringency annealing conditions for the long specific primers and low stringency annealing conditions for the short arbitrary primer. This "thermal asymmetry" allows target sequences to be amplified preferentially over non-specific products. After tertiary PCR, a highly specific tertiary amplicons is produced, which can be clearly differentiated from the secondary amplicon due to an exact size reduction that results from the precise positioning of the second and third specific primers. These highly specific amplicons usually range in size from 300bp-2,000bp and provide excellent DNA templates that can be used directly to sequence the flanking pathogenicity gene (Liu, et al., 1995; Liu, et al., 1993; Liu & Huang).

Due to limited time, TAIL-PCR could not be attempted on *T. basicola* reduced pathogenicity mutants however this method is very successfully used for recovering tagged pathogenic sequences from ATMT transformants of other filamentous fungi (Mullins, et al., 2001; Betts, et al., 2007; Combier, et al., 2003; Hoekema, et al., 1983; Leclerque, et al., 2004).

Plasmid Rescue

Plasmid rescue is a second method of recovering tagged pathogenic sequences, which could be performed on ATMT and PEG *T. basicola* transformants. For recovery by this method, the binary vector used in ATMT must contain an *E. coli* ori and selectable marker sequence within the left and right border repeats of the T-DNA. In this approach, the fungal genomic DNA is digested with a RE that does not cut within the T-DNA (or if it does, then at a site that will not lose any sequences required for the rescue procedure, such as bacterial antibiotic resistance gene and ori). The resulting fragment, consisting of the DNA insert and flanking fungal genomic sequences, are ligated to produce a circular plasmid containing an *E. coli* selectable marker and ori, which is inserted into *E. coli* cells. The plasmid is retrieved from antibiotic resistant colonies and can then be used directly for sequencing (Kahmann & Basse, 1999; Sweigard, et al., 1998; de Groot, et al., 1998; Mullins, et al., 2000). The construction of two plasmids pMAX and pAIM3 (Leclerque, et al., 2004), for use in plasmid rescue has started in this project, however, due to time limitations, neither could be completed.

After sequencing the disrupted pathogenicity genes recovered from either technique, sequence analysis should be performed to identify the function of the gene product (Sweigard & Ebbole, 2001).

A great deal of progress has been made towards understanding these underlying mechanisms that govern fungal pathogenesis for a wide range of filamentous fungi like *Maganporthe grisea, Fusarium oxysporum, Leptosphaeria maculans*. According to Idnurm & Howlett, (2001), 79 fungal pathogenicity genes have already been identified, disruption to which have resulted in a mutant phenotypes with a reduction or complete loss to pathogenicity. Pathogenicity genes from phytopathogenic fungi have great diversity of function including those involved in (1) the formation of infection structures and hydrophobins, (2) spore development and germination (3) regulation and biosynthesis of melanin, (4) cuticle and cell wall degrading hydrolytic enzymes, and (4) regulatory proteins, including transcription factors, receptors, G proteins, and enzymes (Idnurm & Howlett, 2001; Mendgen, et al., 1996; Sweigard & Ebbole, 2001; Tudzynski & Sharon, 2003).

Identification of pathogenicity genes from *T. basicola* is still in early stages, with no genes having yet been found. However, based upon the types of pathogenicity genes identified in other pathogenic filamentous fungi thus far, the phenotypic tests performed in this study, on the subset of ATMT Hyg^R putative transformants and the five PEG mutants, gave some insight into the possible nature of these disrupted genes. This included *T. basicola* mutants with altered phenotypes that indicated possible disruption to genes involved in (1) synthesis of hydrophobins (e.g. 95p2a and 95p3c-), hydrolytic enzymes (e.g. p16, p954, and 737), melanin regulation and biosynthesis (e.g. p16, p954, p849, p888,), chlamydospore development and maturation (p16, p849, 64p1a-2 and 42p3f), development of infection hyphae (e.g. p16, p954, 95p2a and 95p3c-), and transcription factors, regulator receptors and enzymes, MAPKs, or G - proteins (e.g. 67p2c, p16, p737, p849, and p954).

Through techniques like ATMT and TAIL-PCR, future identification of numerous pathogenicity genes for *T. basicola* seems promising. With better understanding into the nature of these pathogenicity genes, it will only be a matter of time before the mechanisms that govern the development of black root rot in cotton are elucidated and the knowledge generated used for more effective control measures to be put in place.

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Appendix 1: Media & Solutions

*Media plates are stored at 4^{o}C (max of 4 weeks for selective medium and 1-2 months for non selective medium).
*Sterilisation by autoclaving was done at 120^{o}C for 20min.
*Unless otherwise specified, solutions were stored at room temp.

Antibiotic	Stock Concentration	Final Concentration
Ampicillin	100mg/ml	100µg/ml
Kanamycin	50mg/ml	50 µg/ml
Hygromycing B	50mg/ml	5,7,10,15,20, and 25 µg/ml
Mefoxin	100mg/ml	300 µg/ml

-All stocks are prepared in sterile dH_2O. Vortex to completely dissolve powder. Filter sterilise using 0.20µm. Store 500µl aliquots at -20^{o}C.

Luria Bertani (LB)

Stock	500ml
Bacto Tryptone	5g
NaCl	2.5g
Yeast Extract	2.5g

-For solid medium, add 8g (1.6%) agar
-Make up to 500ml with dH_2O. Autoclave.

½ Potato Dextrose Agar (1/2 PDA) (2.2% or 1.2% agar)

Stock	500ml
Potato Dextrose Agar	10g
Agar	7.5g (2.2%) or 2.25g (1.2%)

Make up to 500ml with dH_2O. Autoclave

When using ½ PDA for top agar, cool to 50^{O}C before adding HygB and Mefoxin when required

Potato Dextrose Broth (PDB)

Stock	1L
Potatoes	220g
Dextrose	20g

Wash potatoes, leave unpeeled, and cut into cubes (approx 1cm). Rinse with running water and add to 1L of dH_2O. Boil potatoes until soft (20-40min). Collect the filtrate in a beaker and discard remaining potato cubes. Add the dextrose (L- Glucose) and make up final volume to 1L with dH_2O. Autoclave

Yeast Mannitol (YM) pH 7

Stock	500ml
Yeast Extract	0.2g
Mannitol	5g
NaCl	0.05g
$MgSO_4.7H_2O$	0.1g
K_2HPO_4	0.25g

- For solid medium, add 7.5g (1.5%) agar. Add 400ml dH_2O. Adjust pH to 7 with 10M HCl. Bring final volume to 500ml. Autoclave.

2.5x MM Salts

Stock	1L
KH_2PO_4	3.625g
K_2HPO_4	5.125g
NaCl	0.375g
$MgSO_4.7H_2O$	1.250g
$CaCl_2.2H_2O$	0.165g
$FeSO_4.7H_2O$	0.0062g
$(NH_4)_2SO_4$	1.250g

Dissolve each salt one at a time in 500ml sterile dH_2O, make up to 1L. Do not autoclave.

1M MES pH 5.3

Dissolve 19.52g MES hydrate in 80 ml dH_2O. Adjust pH to 5.3 with 5M KOH. Bring final volume to 100ml. Sterilise by filtration through 0.20µm filter. Store in 10ml aliquots at -20^{o}C. If salt precipitates upon thawing frozen stock, dissolve by placing tube in 65^{o}C water bath for 1-2min followed by a brief vortexing.

5M KOH

Dissolve 14.026g KOH in 25ml dH_2O. Bring final volume to 50ml.

10mM Acetosyringone (AS) (pH 8)

Dissolve 0.01962g AS in 10ml sterile dH_2O. Stir for 1hr. Adjust pH to 8 with 5M KOH. Sterilise by filtration through 0.20µm filter. Store in 5ml aliquots at -20^{o}C.

1M Glucose

Dissolve 18g glucose in 90ml of dH_2O. Make up to 100ml. Sterilise by filtration through 0.22µm filter.

Induction Medium (IM) + AS

Stock	400ml
2.5X MM Salts Stock	160ml
100% glycerol	2ml

-Add 218ml dH_2O. Autoclave. Add:

1M MES stock	16ml
.1M glucose stock	4ml

-Store in 20ml aliquots at room temperature. Add 200µM AS (400ul AS of 10mM AS stock) to liquid IM just before use.

IMAS (1.5% Agar) Plates (pH 6)

Stock	500ml
2.5X MM Salts	200ml
100% glycerol	l2.5ml
Agar (1.5%)	7.5g

-Add 262.5ml dH_2O. Autoclave. Cool to 50^{o}C. Add:

1M MES	20ml
10mM AS	10ml
1M glucose	5ml

To prepare IMAS plates at pH 4.8, add ~6.5ml 1M HCl after autoclaving (adjust dH_2O volume accordingly).

M-100 Trace Element Solution

Stock	500ml
H_3BO_3	30mg
$MnCl_2.4H_2O$	70mg
$ZnCl_2$	200mg
$Na_2MoO_4.2H_2O$	20mg
$FeCl_3.6H_2O$	50mg
$CuSO_4.5H_2O$	200mg

Make up to 500ml with sterile dH_2O. Autoclave

M-100 Salt Solution

Stock	1L
KH_2PO_4	16g
Na_2SO_4	4g
KCl	8g
$MgSO_4.7H_2O$	2g
$CaCl_2$	1g
M-100 Trace Element Solution	8ml

Make up to 1L with dH_2O. Sterilise by autoclaving.

M-100 (1.5% agar) Plates

Stock	1L
Glucose	10g
KNO_3	3g
M-100 Salt Solution	62.5ml
1.5% agar	15g

Make up to 1L with dH_2O. Sterilise by autoclaving. Cool to 50^OC before adding HygB and Mefoxin.

SOB

Stock	1L
Bacto-tryptone	20g
Bacto-Yeast Extract	5g
NaCl	0.5g

-Make up to 950ml with dH_2O. Add 10ml 250mM solution of KCl (This solution is prepared by dissolving 1.86g KCl in 100ml of dH_2O). Adjust pH to 7 with 5 M NaOH. Adjust final volume to 1L with dH_2O. Sterilise by autoclaving at 120^oC for 20min.
-Just before use, add 5ml of sterile 2M $MgCl_2$ (19g of $MgCl_2$ dissolved in 90ml dH_2O, final volume made up to 100ml. Sterilise by autoclaving at 120^oC for 20min).

SOC

Same as SOB except that SOC contains 20mM glucose. After autoclaving SOB allow media to cool to at least 60^oC. Add 20ml of sterile 1M glucose.

Surface Sterilisation Solution for Cotton Seeds

Stock	1L
Bleach	125ml
dH_2O	775ml
Ethanol	100ml

Make sure solutions are added in this order as bleach mixed directly with ethanol is explosive.

2% 6x DNA Loading Buffer (6x LB)

bromophenol blue	0.25% (w/v)
xylene cyanol	l0.25% (w/v)
sucrose	40% (w/v)

Dissolve in sterile milliQ water and store at 4°C.

5M KAc (pH 4.8)

Dissolve 14.721 g of KAc in 30ml dH_2O. Add 5.75ml of glacial acetic acid. Adjust pH to 4.8 with 10M HCl. Adjust final volume to 50ml with dH_2O. Autoclave.

0.5M EDTA

Dissolve 9.3g EDTA.$2H_2O$ in 40ml dH_2O. Adjust to desired pH with NaOH pellets (EDTA will not begin to dissolve until pH reaches close to pH 8). Make up to final volume of 50ml. Autoclave.

1M Tris-HCl

Dissolve 12.11g Tris base in 80ml dH_2O. Adjust to desired pH with HCl. Make up to final volume of 100ml. Autoclave.

10% SDS

Dissolve 5g of SDS in 50ml dH_2O, using gentle heat if necessary. Sterilise by filtration through 0.2-0.45µm membrane.

10x TE Buffer

Stock	**1L**
1M Tris-HCl	100ml
0.5M EDTA	20ml

Make up to 1L with dH_2O. Autoclave.

1x TE Buffer

Stock	**1L**
1M Tris·HCl	10ml
0.5M EDTA	2ml

-Make up to 1L with dH_2O. Autoclave..

NOTE: When preparing 10x or 1x TE/S buffers, use 1MTris-HCl and 0.5MEDTA stocks of the appropriate pH required for that TE buffer (commonly pH 8).

10x TES

Stock	1L
1MTris-HCl	100ml
0.5M EDTA	20ml
10% SDS	200ml

-Make up to 1L with dH_2O. Autoclave.

1x TAE Buffer

Stock	1L
1M Tris-acetate	40ml
0.5M EDTA	2ml

-Make up to 1L with dH_2O. Autoclave.

20x SSC

Stock	1L
NaCl	175g
Sodium Citrate	88.2g

-Dissolve in 800ml dH_2O. Adjust pH to7.0 with 10M NaOH. Make up to 1L. Autoclave.

10x Taq Buffer

Stock	10ml
1M Tris·Cl	6.7ml
Tween 20	10µl
$(NH_4)_2SO_4$	0.2114g
50mM $MgCl_2$	2.92ml

5% (w/v) Na-N-Laurylsarcosine

Dissolve 2.5g Na-N-Laurylsarcosine in 50ml dH_2O. Sterilise by filtration through 0.2-0.45µm membrane.

Blocking Stock Solution

Stock	100ml
Blocking Reagent Stock (Roche)	10g
Buffer 1	100ml

-Dissolve the blocking reagent in buffer 1 with mixing and heat; do not allow the solution to boil whilst dissolving. Autoclave. Store at 4^oC.

Buffer 1

Stock	1L
0.15M NaCl	8.76g NaCl
0.1M Maleic Acid	11.6g Maleic Acid

-Dissolve in 800ml dH_2O. Adjust pH to 7.5 with NaOH pellets. Make up to 1L. Autoclave.

Buffer 2

Stock	100ml
10% Blocking Stock Solution	10ml
Buffer 1	90ml

-Prepare fresh just prior to use.

Buffer 3

Stock	1L
Tris	12.11g
NaCl	5.84g

-Dissolve Tris in 800ml dH_2O and adjust pH to 9.5 with 10M HCl. Add NaCl. Autoclave.

Hybridization Solution

Stock	30ml
100% Formamide	15ml
20x SSC	7.5ml
10% blocking stock solution	6ml
5% Na-N-laurylsarcosine	0.6ml
10% SDS	0.06ml

Prepare fresh just prior to use

The probe/hybridisation solution is prepared by first boiling 50µl of DIG-labelled probe for 10min (in order to separate the double strand of the probe), and then cooling it immediately on ice for 5min. One ml of fresh hybridisation solution is then added to the denatured probe solution, mixed gently, and then added to 9ml of fresh hybridisation solution. After hybridisation, the probe solution can be stored in a polypropylene tube at -20^{o}C. To re-use this solution, it can be thawed and heated at 68^{o}C for 10min just prior to use.

NBT/BCIP Solution

Stock	10ml
50x NBT/BCIP Stock Solution – vial 4 (Roche DNA DIG Labelling Kit)	200µl
Buffer 3	9.8ml

-If precipitate present in NBT/BCIP stock, dissolve by briefly warming at 37^{o}C. Prepare fresh.

Anti-DIG-AP solution

Stock	50ml
5,000x Anti-DIG-AP Stock Solution-vial 3 (Roche DNA DIG Labelling Kit)	10µl
Buffer 2	49.9ml

- Centrifuge the antibody stock solution for 5min at 10,000rpm prior to each use. Carefully pipette aliquot from the surface. Can be prepared up to12hrs prior to use and stored at 4^{o}C.

CSPD Solution

Stock	1ml
100x CSPD Stock Solution	10µl
Buffer 3	990µl

-Prepare fresh just prior to use.

Washing Buffer

Stock	400ml
Tween 80	1.2ml
Buffer 1	398.8ml

-Prepare fresh. No need to sterilise Tween 80

Appendix 2 TABLE OF ATMT VARIABLES

Table Abbreviations: CC=Co-cultivation; d=days; deg=degrees; F=filters; NF=no filters; mF=mini filter; FT= filter transfer; TA=top agar; ***=see notes

ATMT #	Co-Cultivation (CC)			Selection					Agro	Agro	Fungal	T.b	Agro	T.b	Plasmid
	Type	Temp(0C)	Days	Type	Mef(ug/ml)	Hyg(ug/ml)	Temp(^{0}C)	Days	Strain	OD 660	Strain	Conc	Aliquot	Aliquot	
1	IMAS(F)	25-28	3	FT: M-100	300/300	25/0	25	6	AGL-1	0.3	T. basicola	$1.0x10^6$	100	100	pBHt2
2	IM(F)	25-28	3	FT: M-100	300/300	25/0	25	6	AGL-1	0.3	T. basicola	$1.0x10^6$	100	100	pBHt2
3	IMAS(F)	25-28	3	FT: M-100	300/300	25/0	25	6	AGL-1	0.4	T. basicola	$1.0x10^6$	100	100	pBHt2
4	IM(F)	25-28	3	FT: M-100	300/300	25/0	25	6	AGL-1	0.4	T. basicola	$1.0x10^6$	100	100	pBHt2
5	IMAS(F)	25-28	3	FT: M-100	300/300	25/0	25	6	AGL-1	0.5	T. basicola	$1.0x10^6$	100	100	pBHt2
6	IM(F)	25-28	3	FT: M-100	300/300	25/0	25	6	AGL-1	0.5	T. basicola	$1.0x10^6$	100	100	pBHt2
7	IMAS(F)	25-28	3	FT: M-100	300/300	25/0	25	6	AGL-1	0.6	T. basicola	$1.0x10^6$	100	100	pBHt2
8	IM(F)	25-28	3	FT: M-100	300/300	25/0	25	6	AGL-1	0.6	T. basicola	$1.0x10^6$	100	100	pBHt2
9	IMAS(F)	25	3	FT: M-100	300/300	25/0	25	7	AGL-1	0.75	T. basicola	$5.75x10^6$	100	100	pBHt2
10	IMAS(F)	25	3	FT: M-100	300/300	25/0	25	7	AGL-1	0.75	T. basicola	$5.75x10^6$	50	50	pBHt2
11	IMAS (mF)	25	3	FT: M-100	300/300	25/0	25	7	AGL-1	0.75	T. basicola	$5.75x10^6$	100	100	pBHt2
12	IMAS(F)4.7	25	3	FT: M-100	300/300	25/0	25	7	AGL-1	0.75	T. basicola	$5.75x10^6$	100	100	pBHt2
13	IMAS (NF)	25	3	TA:PDA25ml	300/300	25/0	25	10	AGL-1	0.64	T. basicola	$5.0x10^6$	200	200	pBHt2
14	IMAS (NF)	25	2	TA:PDA25ml	300/300	25/0	25	10	AGL-1	0.64	T. basicola	$5.0x10^6$	200	200	pBHt2
15	IMAS (NF)	27	3	TA:PDA25ml	300/300	25/0	25	10	AGL-1	0.64	T. basicola	$5.0x10^6$	200	200	pBHt2
16	IMAS (NF)	27	2	TA:PDA25ml	300/300	25/0	25	10	AGL-1	0.64	T. basicola	$5.0x10^6$	200	200	pBHt2
17	IMAS(NF)4.7	25	2.5	TA:PDA20ml	300/0	25/0	25	10	AGL-1	0.62	T. basicola	$2.7x10^5$	100	100	pBHt2
18	IMAS(F)4.7	25	2.5	TA:PDA20ml	300/0	25/0	25	10	AGL-1	0.62	T. basicola	$2.7x10^5$	100	100	pBHt2
19	PDA.2.2%*	25	2.5	TA:PDA20ml	300/0	25/0	25	10	AGL-1	0.62	T. basicola	$2.7x10^5$	300	n/a	pBHt2
20	IMAS(F)	25	3	FT: M-100	300/0	25/0	25	10	AGL-1	0.69	T. basicola	$2.7x10^6$	100	100	pBHt2
21	IMAS(NF)	25	3	TA:PDA20ml	300/0	25/0	25	10	AGL-1	0.69	T. basicola	$2.7x10^6$	100	100	pBHt2
22	IMAS(F)	25	3	FT: M-100	300/0	25/0	25	10	AGL-1	0.63	T. basicola	$2.7x10^6$	100	100	pBHt2
23	IMAS(NF)	25	3	TA:PDA20ml	300/0	25/0	25	10	AGL-1	0.63	T. basicola	$2.7x10^6$	100	100	pBHt2
24	IMAS(NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
25	IMAS(NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
26	IMAS(NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
27	IMAS(NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
28	IMAS(NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2

ATMT #	Co-Cultivation (CC)			Selection					Agro	Agro	Fungal	T.b	Agro	T.b	Plasmid
	Type	Temp(0C)	Days	Type	Mef(ug/ml)	Hyg(ug/ml)	Temp(^{0}C)	Days	Strain	OD 660	Strain	Conc	Aliquot	Aliquot	
29	IMAS(NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
30	IMAS(NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
31	IMAS(NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
32	IMAS(NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
33	IMAS(NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
34	IMAS(NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
35	IMAS(NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
36	IMAS(NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
37	IMAS(NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
38	IMAS(NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
39	IMAS(NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
40	IMAS(NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
41	IMAS(NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
42	IMAS(NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
43	IMAS(NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
44	IMAS(NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
45	IMAS(NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
46	IMAS(NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
47	IMAS(NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.79	T. basicola	$1.0x10^6$	100	100	pBHt2
48α	IMAS(NF)	25	2	TA:PDA20ml	300/0	15/0	25	10	AGL-1	0.45	T. basicola	$1.0x10^6$	100	100	pBHt2
49β	IMAS(NF)	25	2	TA:PDA20ml	300/0	15/0	25	10	AGL-1	0.45	T. basicola	$1.0x10^6$	100	100	pBHt2
50γ	IMAS(NF)	25	2	TA:PDA20ml	300/0	15/0	25	10	AGL-1	0.45	T. basicola	$1.0x10^6$	100	100	pBHt2
51δ	IMAS(NF)	25	2	TA:PDA20ml	300/0	15/0	25	10	AGL-1	0.45	T. basicola	$1.0x10^6$	100	100	pBHt2
52 (7)	IMAS(NF)	25	2	TA:PDA20ml	300/0	15/0	25	10	AGL-1	0.48	T. basicola	$1.0x10^6$	100	100	pBHt2
53 (14)	IMAS(NF)	25	2	TA:PDA20ml	300/0	15/0	25	10	AGL-1	0.48	T. basicola	$2.5x10^4$	100	100	pBHt2
54 (7)	IMAS(NF)	25	3	TA:PDA20ml	300/0	15/0	25	10	AGL-1	0.48	T. basicola	$2.5x10^4$	100	100	pBHt2
55 (14)	IMAS(NF)	25	3	TA:PDA20ml	300/0	15/0	25	10	AGL-1	0.48	T. basicola	$2.5x10^4$	100	100	pBHt2
56	IMAS(NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.53	T. basicola	$1.0x10^6$	100	100	pPK2
57	IMAS(NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.53	T. basicola	$1.0x10^6$	100	100	pPK2
58	IMAS(NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.53	T. basicola	$1.0x10^6$	100	100	pPK2
59	IMAS(NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.53	T. basicola	$1.0x10^6$	100	100	pPK2
60	AS (Agarose)I	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.64	T. basicola	$1.0x10^6$	100	100	pBHt2

ATMT #	Co-Cultivation (CC)			Selection					Agro	Agro	Fungal	T.b	Agro	T.b	Plasmid
	Type	Temp(0C)	Days	Type	Mef(ug/ml)	Hyg(ug/ml)	Temp(^{0}C)	Days	Strain	OD 660	Strain	Conc	Aliquot	Aliquot	
61	AS (Agarose)I	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.64	T. basicola	$1.0x10^6$	100	100	pBHt2
62	AS (Agarose)I	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.64	T. basicola	$1.0x10^6$	100	100	pBHt2
63	AS (Agarose)I	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL-1	0.64	T. basicola	$1.0x10^6$	100	100	pBHt2
64	S (Ag and Ags	25	2	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pBHt2
65	S (Ag and Ags	28	2	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pBHt2
66	S (Ag and Ags	25	3	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pBHt2
67	S (Ag and Ags	28	3	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pBHt2
68	S (Ag and Ags	25	2	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pPK2
69	S (Ag and Ags	28	2	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pPK2
70	S (Ag and Ags	25	3	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pPK2
71	S (Ag and Ags	28	3	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pPK2
72	S (Ag and Ags	25	7	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.64	T. basicola	$1.0x10^6$	100	100	pBHt2
73	S (Ag and Ags	28	7	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.64	T. basicola	$1.0x10^6$	100	100	pBHt2
74	IMAS (F)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.76	T. basicola	$1.0x10^6$	100	100	pBHt2
75	IMAS (F)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.76	T. basicola	$1.0x10^6$	100	100	pBHt2
76	IMAS (F)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.76	T. basicola	$1.0x10^6$	100	100	pBHt2
77	IMAS (F)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.76	T. basicola	$1.0x10^6$	100	100	pBHt2
78	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.75	oxysporum (SI	$1.0x10^6$	100	100	pBHt2
79	IMAS (NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.75	oxysporum (SI	$1.0x10^6$	100	100	pBHt2
80	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.75	oxysporum (SI	$1.0x10^6$	100	100	pBHt2
81	IMAS (NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.75	oxysporum (SI	$1.0x10^6$	100	100	pBHt2
82	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.75	oxysporum (W	$1.0x10^6$	100	100	pBHt2
83	IMAS (NF)	28	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.75	oxysporum (W	$1.0x10^6$	100	100	pBHt2
84	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.75	oxysporum (W	$1.0x10^6$	100	100	pBHt2
85	IMAS (NF)	28	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.75	oxysporum (W	$1.0x10^6$	100	100	pBHt2
86	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.44	T. basicola	$1.0x10^6$	100	100	pBHt2
87	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.44	T. basicola	$1.0x10^6$	100	100	pBHt2
88	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.7	T. basicola	$1.0x10^6$	100	100	pPK2
89	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.7	T. basicola	$1.0x10^6$	100	100	pPK2
90	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pBHt2
91	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pBHt2
92	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pPK2

ATMT #	Co-Cultivation (CC)			Selection					Agro	Agro	Fungal	T.b	Agro	T.b	Plasmid
	Type	Temp(0C)	Days	Type	Mef(ug/ml)	Hyg(ug/ml)	Temp(^{0}C)	Days	Strain	OD 660	Strain	Conc	Aliquot	Aliquot	
93	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pPK2
94	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.68	T. basicola	$1.0x10^6$	100	100	pCAMgfp
95	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.68	T. basicola	$1.0x10^6$	100	100	pCAMgfp
96	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.69	T. basicola	$1.0x10^6$	100	100	pBHt2
97	IMAS (NF)	25	2	TA:PDA20ml	300/0	10/0	25	10	AGL1	0.69	T. basicola	$1.0x10^6$	100	100	pBHt2
98	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pBHt2
99	IMAS (NF)	25	3	TA:PDA20ml	300/0	10/0	25	10	LBA4404	N/A	T. basicola	$1.0x10^6$	100	100	pBHt2

Appendix 3 Agro Pre-Cultivation and Induction

#	Strain	Method
1	LBA1119	NS*
2	AGL1	2 days at 28^{o}C in MM broth --> OD_{600} 0.15 in IMAS --> 6hrs
3	NS*	NS*
4	AGL1	NS*
5	LBA1126	NS*
6	LBA1126	3 days at 25^{o}C on LC plate --> pick single colony --> 10ml LC broth 16hrs at 28^{o}C 120rpm --> centrifuge and wash (in IM?) --> resuspend in 5ml
		IM --> adjust to OD6000.15 in 10ml IMAS --> grow 3-4 hrs
7	LBA1126	3 days on MM plates --> pick single colony --> O/N in MM broth at 28^{o}C --> 2ml aliquot washed in IM --> inoculate 5ml IMAS --> grow 6hrs at 28^{o}C
8	AGL1	same as exp 2 but use IM only
9	AGL1	2 days at 28^{o}C in LB broth --> OD_{660} 0.15 in IMAS --> 5hrs at 28^{o}C
10	LBA11…	O/N in MM broth at 29^{o}C --> OD_{660} 0.15 in IMAS --> 6hrs at 29^{o}C
11	LBA1100	O/N in LC broth at 30^{o}C unti OD_{600} 2.5 --> 5.0x10^{8} cells washed in IM --> incubate 5ml IMAS 6hrs at 30^{o}C
12	AGL1	Scrape gycerol stock and streak AB(1.4%) plate and grow 28^{o}C --> pick a single colony --> inoculate in 2ml MM --> grow 48hrs 250rpm --> dilute
		to OD6000.15 in 5ml IMAS --> 28oC 6hrs --> OD600 0.25
13	LBA4404	Grow on YM media at 28^{o}C for 2days --> pick single colony --> inoculated into LB broth --> grow 40hrs at 28^{o}C and 250rpm --> dilute to OD_{660} 0.15
		with IM --> grow 6-8hrs
14	LBA4404	Grow on YM at 28^{o}C for 2 days--> pick single isolated colony-->inoculated into 7.5ml LB broth in 50ml flask -->grow at 28^{o}C for 2 days and 1 night -->
		dilute to OD660 0.15 in 20ml IMAS in 100ml flask at 28oC and 250rpm for around 6 hours or until OD660 is close to 0.6

NS*: not specified
NOTE: all broths/mediums would contain suitable antibiotics

Co-cultivation

				Agro			*Fungus*				
#	Plates	Temp	Days	vol	strain	OD/conc	vol	Species/strain	conc	Aliguot	Membrane
1	N/A	20	9		LBA1119			*Yeast YPH250*			spread on nittrocellulose
2	IMAS	25	2	100ul	AGL1	0.15 --> 6hrs	100ul	*F. oxysporium/0685*	$1.0x10^{6}$	200ul	spread on 0.45um membrane
3	IMAS	room temp	2	100ul	NS*		100ul	*A. awamur*	$1.0x10^{6,7,8}$	200ul	spread on nittrocellulose
4	IMAS	27	2	100ul	AGL1		100ul	*Fusarium*	$1.0x10^{4-8}$	200ul	spread on 0.8um nitrocellulose or 6um filter paper
5	IMAS	NS*	2	100ul	LBA1126	$8.4x10^{6}$	100ul	*V. fungola*	$3.2x10^{4,5}$	200ul	spread on cellophane disks
6	IMAS	25	3	100ul	LBA1126	0.15-->3-4hrs	100ul	*B. bassina*	$1.0x10^{7}$	200ul	streaked onto 82mm nitrocellose
7	IMAS	28	3	50ul	LBA1126	NS*	50ul	*K. lactis*		100ul	spotted on 0.45um filters
8	IMAS	25	2	100ul	AGL1	0.15-->6hrs	100ul	*V. dimora*	$1.0x10^{6}$	200ul	spread on nitrocellulose membranes
9	IMAS	23	1-3	10-100ul	AGL1	0.15-->5hrs	50ul	*H. cylindosporum*	macerated fungal mycelia	100ul	glass microfibre disks
10	IMAS	29	3	50ul	LBA11…	$1.0x10^{7}$	50ul	*S.cerevisiae*	10x dilute of O/N culture	100ul	0.45 um cellulose nitrate filters
11	IMAS	24	2-3	100ul	LBA1100	$6x10^{6-8}$	100ul	*A. gigantus*	$1.0x10^{5-7}$	200ul	cellophane membranes
12	IMAS	28	2	100ul	AGL1	0.25 or $2.5x10^{8}$	100ul	*M. grisea*	$1.0x10^{6}$	200ul	spread on 6um black cellulose using 3mm glass beads
13	IMAS	28	2	200ul	LBA4404	0.15-->6-8hrs (approx 0.6)	200ul	*L. maculans*	$1.0x10^{5,6,7}$	400ul	spread onto black filter papers
14	IMAS	22	2	200ul	LBA4404	0.15-->6-8hrs (approx 0.6)	200ul	*L. maculans*	$1.0x10^{6-7}$	400ul	spread directly onto IMAS

*NS:not specified

Reference

(1) Bundock et al 2002: (2) Mulllins et al 2000; (3) de Groot et al 1998; (4) Covert et al 2000; (5) Amey et al 2000; (6) Leclerque 2003; (7) Bundock et al 1998;
(8) Dobinson et al 2004; (9) Combier et al 2003; (10) Bundock et al 1995; (11) Meyer et al 2003; (12) Betts et al 2007; (13) gardiner & Howlett 2004;
(14) Gardiner & Wilson revised by Elliot 2005 re-revised by Elliot 2006

NOTES

(4) 10^{3} no transform, $10^{6,7}$ too much growth to identify Hyg R, $10^{4,5}$ good

(11) germinating spores as well as endoconidia

(12) 100ul of Agro and 100ul Fungi made up to 1ml total with IM prior to plating

(13) *OD_{660} after 6-8hrs in IMAS usually never reaches 0.6 but doesn't seem to matter as long as 6-8hrs time is allowed to allow *vir* gene expression * 2day CC in 12hr light/dark cycles

(14) *OD_{660} after 6-8hrs in IMAS usually never reaches 0.6 but doesn't seem to matter as long as 6-8hrs time is allowed to allow *vir* gene expression * 2day CC in dark

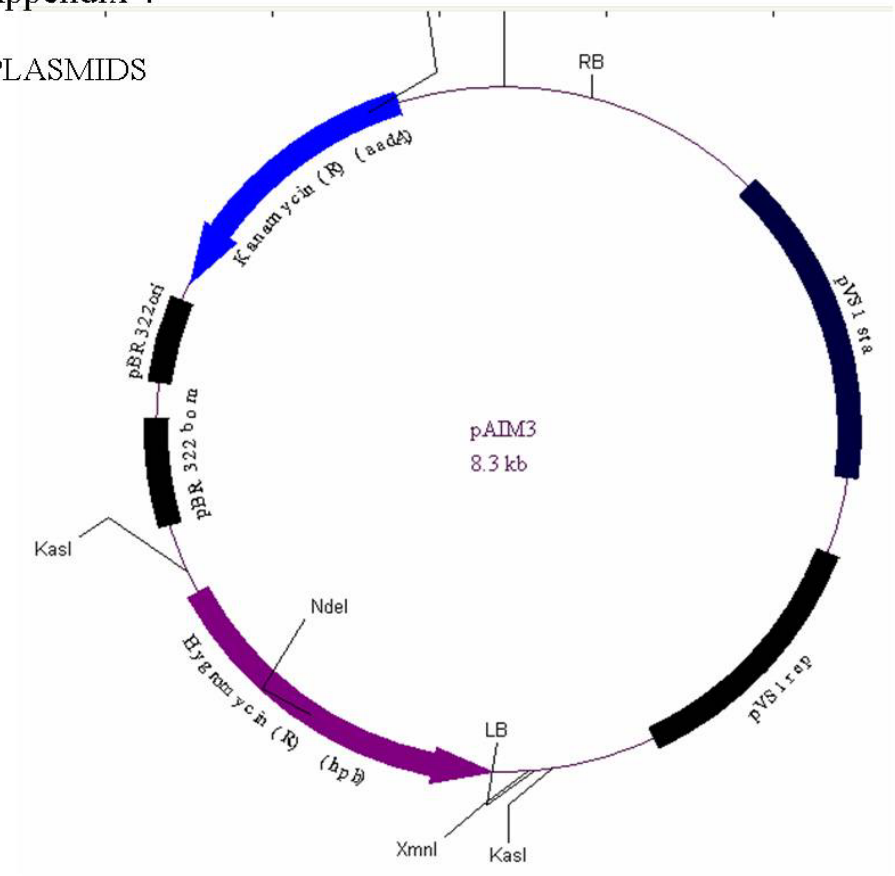
Selection

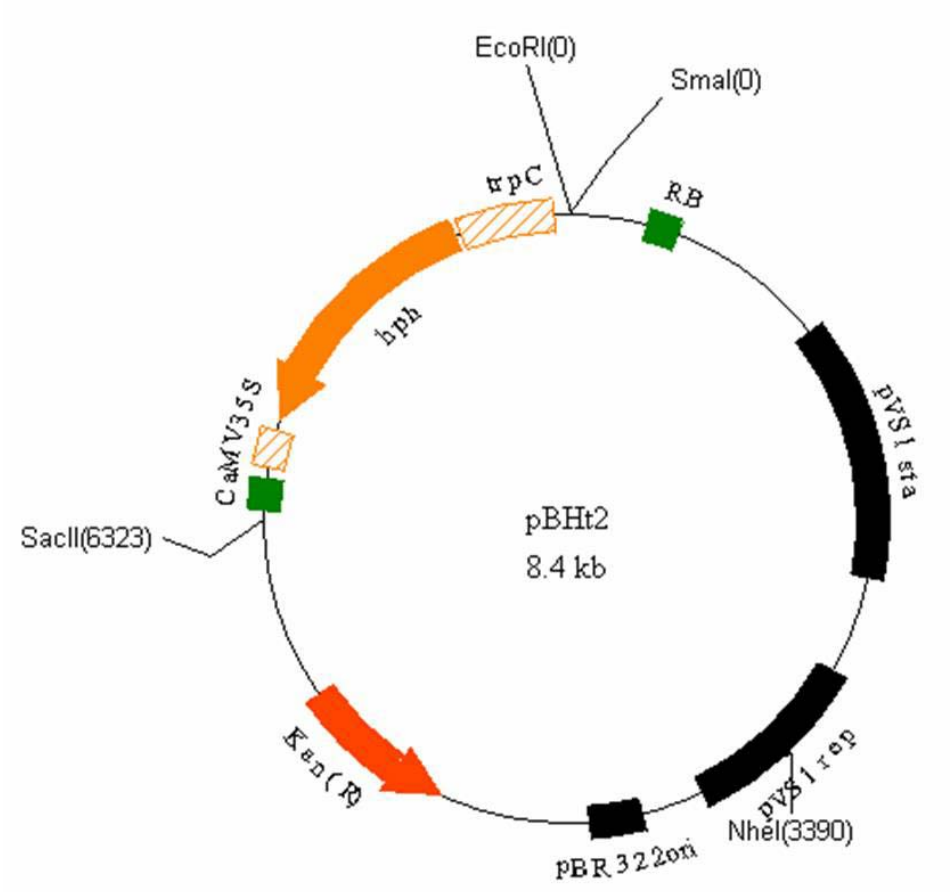
#	Plate	Method of Transfer from CC Plates to S Plates	Days
1	NS*	NS*	NS*
2	MM	filter transfer	4-5
3	MM	filter transfer	4-5
4	M-100	filter transfer	5-6
5	NS*	filter transfer	4 --> sub-culture to MM --4
6	Czapek agar	2ml spore/bacteria suspension in dH_2O rescued from CC filter --> 200ul aliquot on 10x selective plates	5
7	MY	washed cells off filters with 2ml salt solution (0.9g NaCl/L) --> 200 ul aliquots onto selective plates	NS*
8	CM	filter transfer	4-5
9	YMG	microfibre disc transfer	10
10	MY	CC mix from filters resuspended in 2ml salt solution (9gNaCl) --> 100ul aliquots onto selective plates	NS*
11	Cove agar	filter transfer	7 @ 28^oC
12	CM + 20% sucrose	filter transfer	5-6
13	10% V8 juice	filter transfer followed by 5ml top agar overlay (top and bottom agar both containing selective antibiotics	10-14 @ 22^{oC}
14	10% V8 juice	25ml top agar overlayed onto CC IMAS plate	5-10 with light

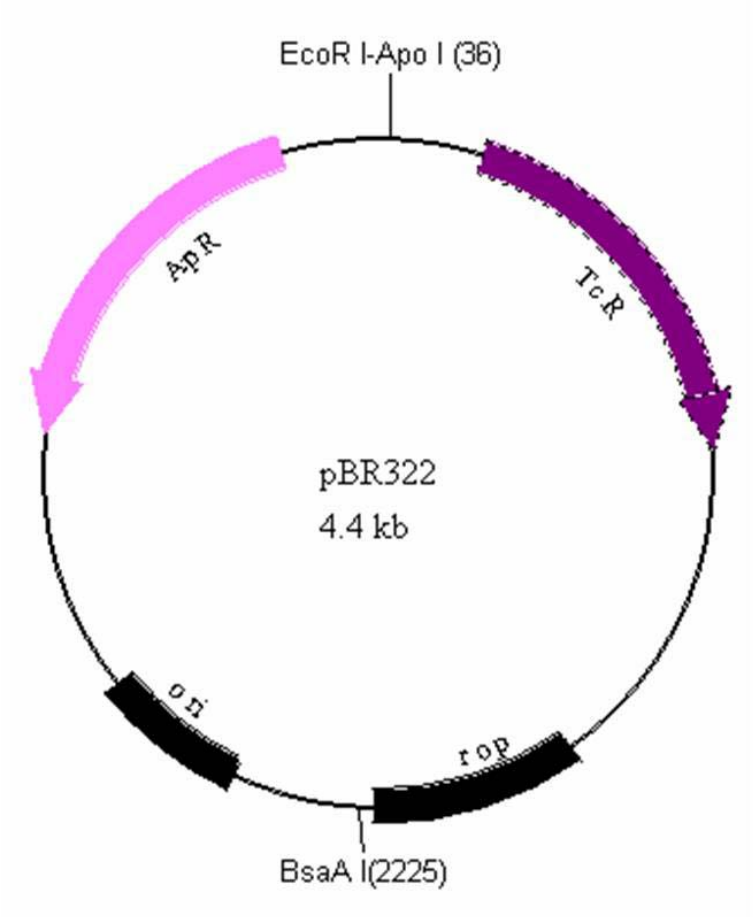
*NS: not specified
NOTE: all plates contain HygB and Cefotoxamin or Mefoxin to select for Transformed Fungi and against Agro respectively
Nota bene: unless otherwise specified S temps remain the same as CC temps

Appendix 4

PLASMIDS



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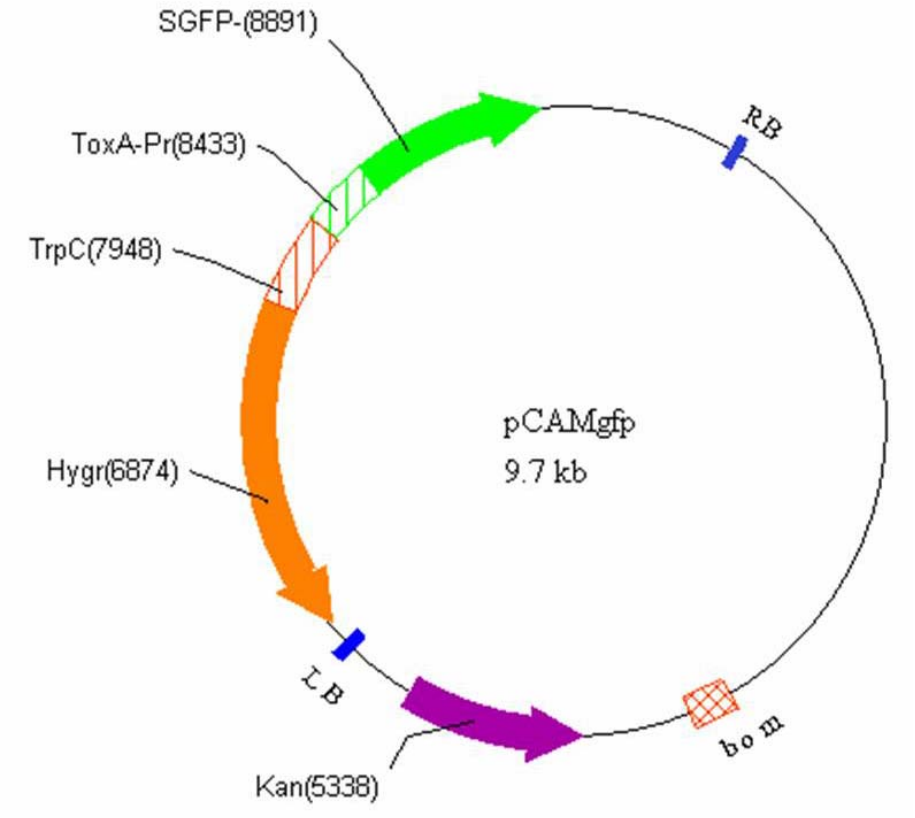
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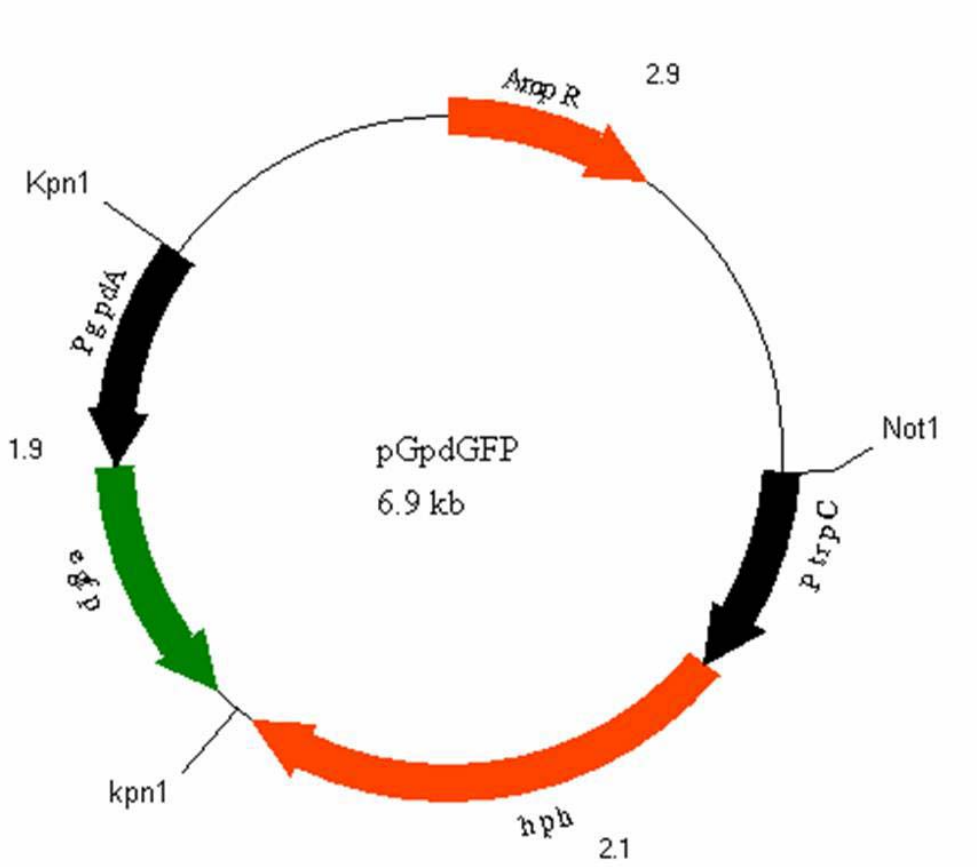
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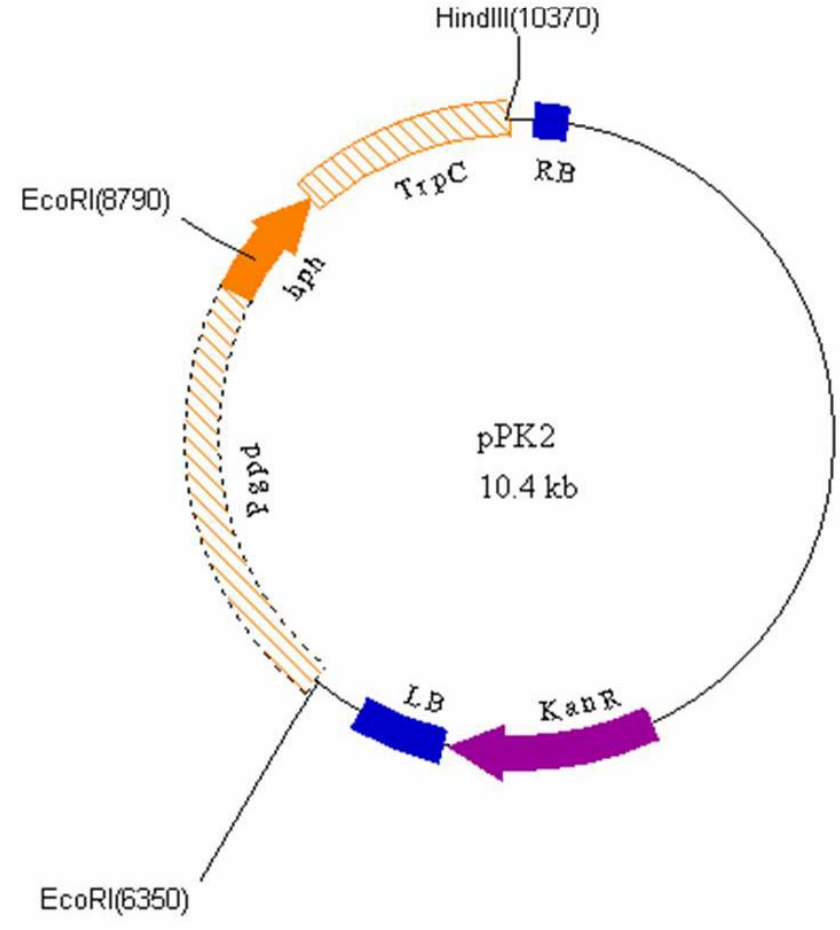
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