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).

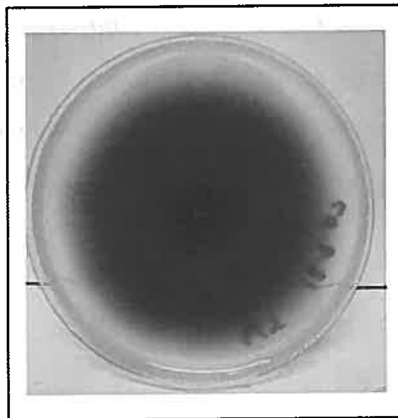

Figure 1.2. Colony Morphology of *T. basicola.*

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).

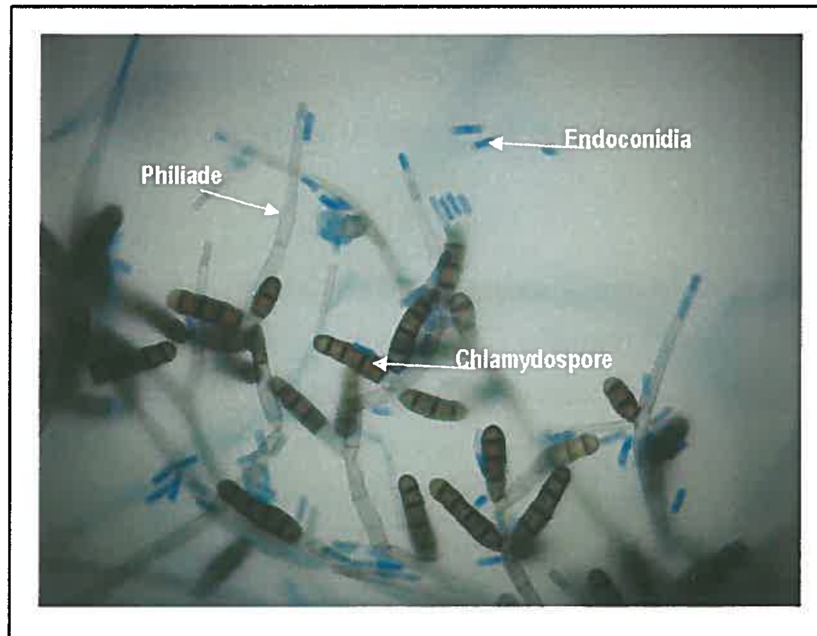

Figure 1.3. ***T. basicola*** **Spore Morphology and Formation.** Photo by Al-Jaaidi, 2007. Endoconidia are stained with cotton blue for easy visualisation.

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. ≤ 4μm) 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 *Pseudomonas 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),

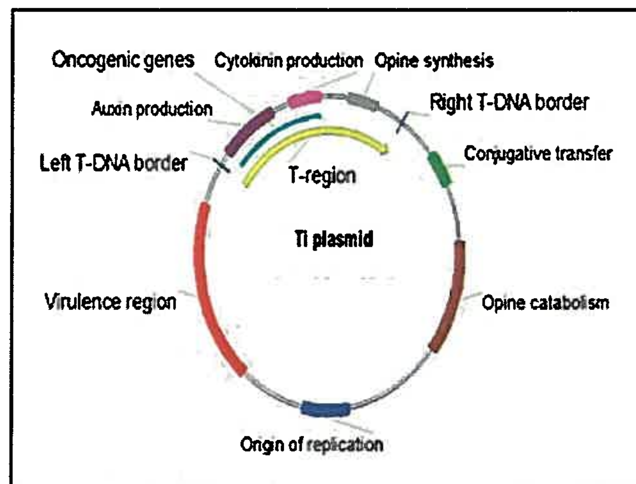

Figure 2.1. Tumour Inducing (Ti) Plasmid.

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).

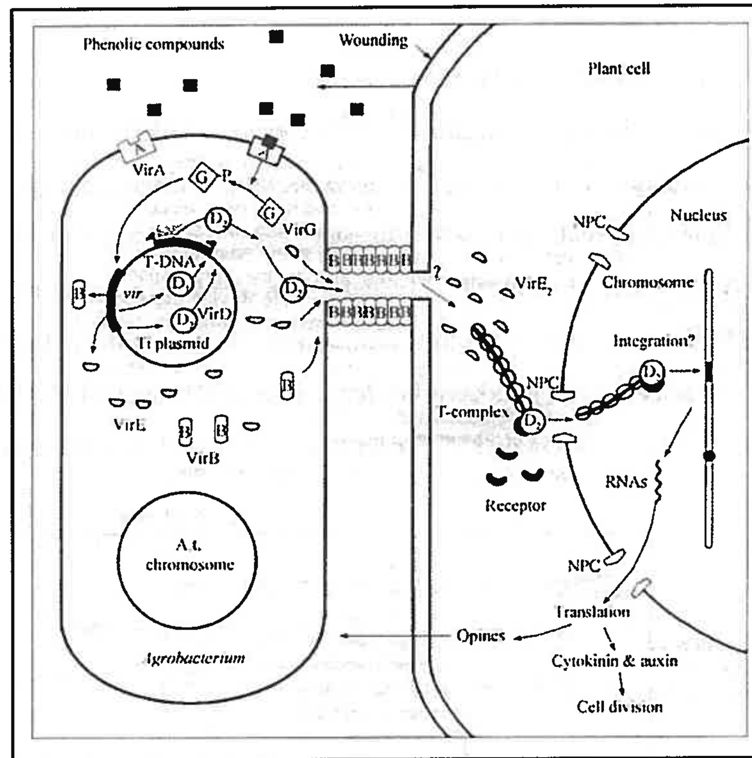

Figure 2.2. ***A. tumefaciens*-mediated Transformation.** *Figure from Wei, et al., 2000.*

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).

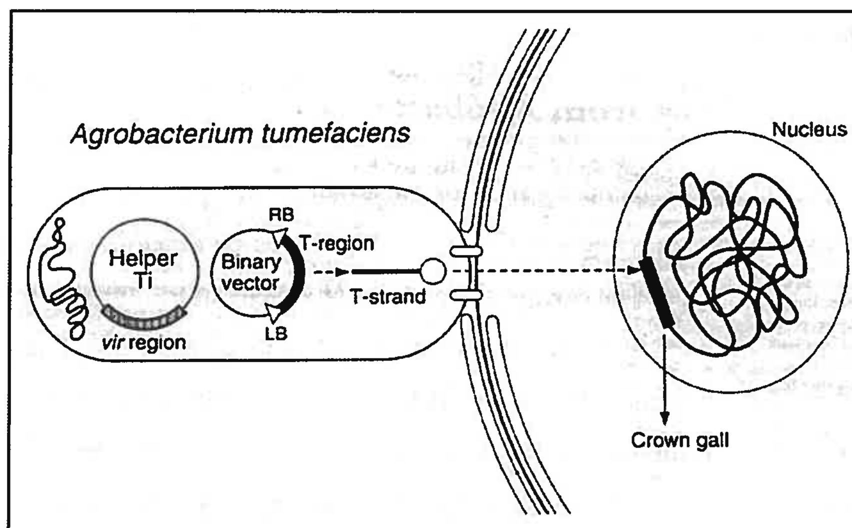

Figure 2.3. ***A. tumefaciens*** **Binary Vector System.** *Figure adapted from Bundock ,et al., 2002*

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*lac*ZΔM15, *recA*1, *endA*l, *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°C for *E.coli* and 48hrs at 28°C for *A. tumefaciens*. Cultures were stored at 4°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°C for *E.coli* and O/N (AGL1) or 2 days (LBA4404) at 250rpm, 28°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°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°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°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°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°C and successful transformants (determined as in section 2.2.4.) were stored at 4°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°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°C for 4 days with 12hr light/dark cycling and then stored at 4°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°C for ~ 2-4 weeks prior to being stored at 4°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°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°) absolute ethanol were added, inverted 3-4 times to mix, and incubated at -70°C for 30min. The sample was then spun at top speed in a benchtop microcentrifuge for 10min at 4°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°C for 10-15min. The dried pellet was resuspended in a desired volume of 10mM Tris·Cl pH 8.5 and stored at -20°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°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 $1.0x10^{0}$ 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°C for 10 days.

Selection plates from both methods were incubated at 25°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°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.

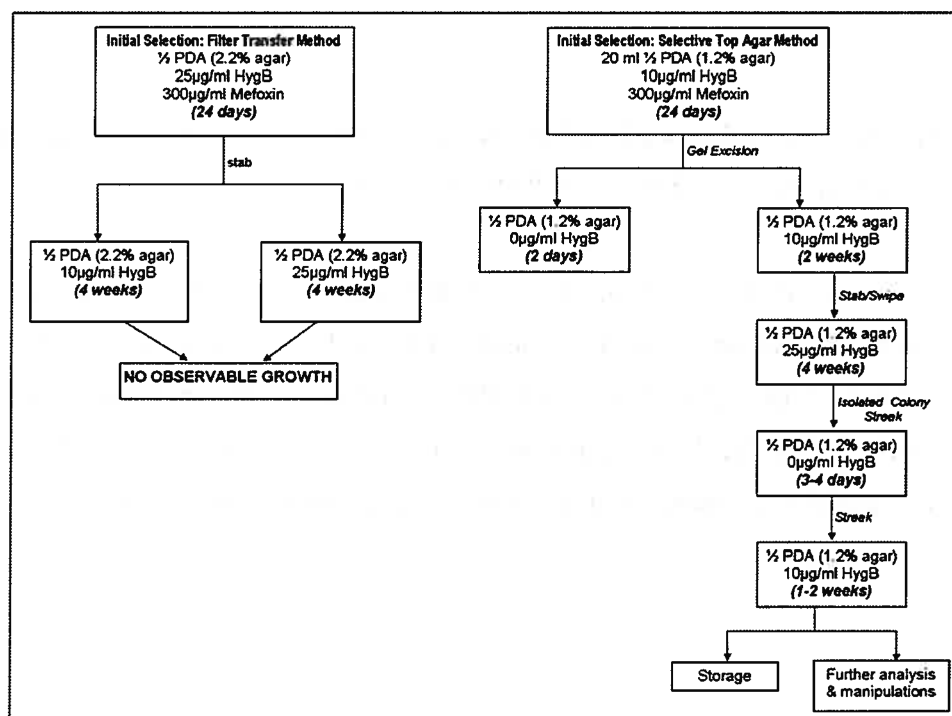

Figure 2.4. Further Antibiotic Selection and Colony Isolation Flow Chart.

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°C for 1min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec, and elongation at 72°C for 90sec. After the 30 cycles were complete, a final elongation step was performed at 72°C for 5min followed by incubation at 4°C until samples were removed (max up to 16hrs at 4°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.

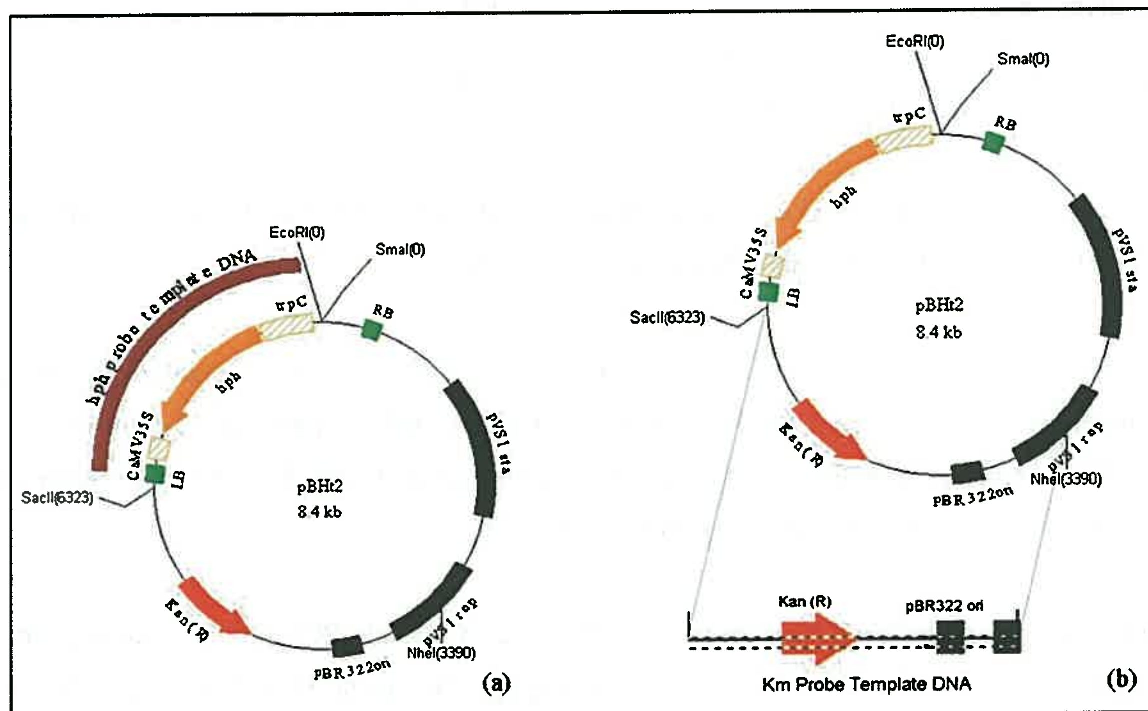

Figure 2.5. Generation of the hph and Km DIG-Labelled Probe Template DNA. (a) Generation of the 1.8kb hph probe template DNA from the plasmid pBHt2 by PCR amplification using the forward primer HYKasF1 and the reverse primer LBEcoR1. (b) Generation of the 2.9kb Km probe template DNA from the plasmid 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°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°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°C for 30min.

DNA was pelleted by centrifuging the solution at 13,000g for 15min in a table top microcentrifuge at 4°C. Ethanol was then decanted and the pellet was washed in 100µl of chilled (at -20°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°C for ~15 min. The dried DNA was resuspended in 50µl 1x TE and stored at -20°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°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°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°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°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°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°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°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°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°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°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°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°C O/N.

To remove excess unbound probe, the membrane was washed twice in pre-heated (at 65°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°C) cassette, incubated at 37°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°C) 0.2M NaOH, 0.1% SDS for 15 min at 37°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°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°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°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°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).

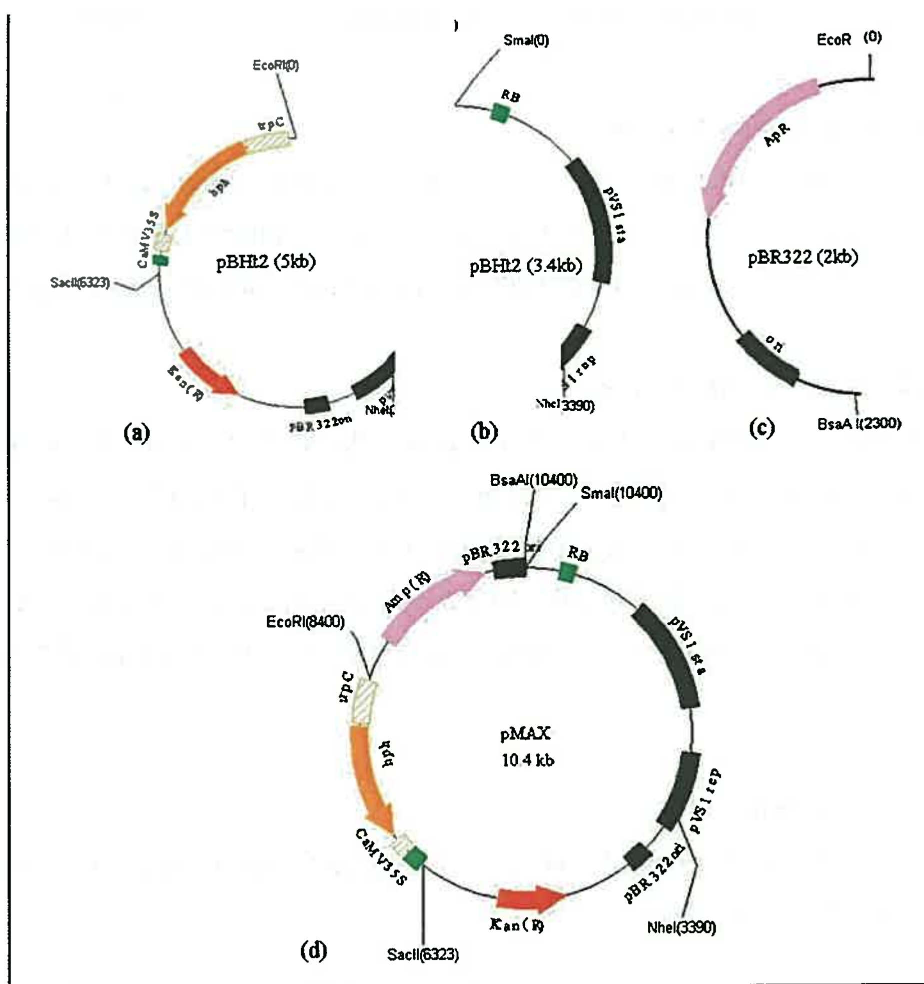

Figure 2.6. Construction of the Binary Vector pMAX. (a) The 5kb fragment generated by double digestion of the binary vector pBHt2 with the restriction enzymes *Eco*RI and *Nhe*I. (b) The 3.4kb fragment generated by double digestion of the binary vector pBHt2 with the restriction enzymes *Sma*I and *Nhe*I. (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°C, after which T4 ligase was heat inactivated at 65°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°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°C for 1min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and elongation at 72°C for 60 sec. After the 30 cycles were complete, a final elongation step was performed at 72°C for 5min followed by incubation 4°C until samples were removed (max up to 16hrs at 4°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°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°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°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°C compared to 28°C.

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

Strains	25°C	28°C
AGL1 [pBHt2]	80	40
LBA4404 [pBHt2]	70	40

AGL1 [pBHt2] and LBA4404 [pBHt2] were co-cultivated with *T. basicola* at 25°C or 28°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°C 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.

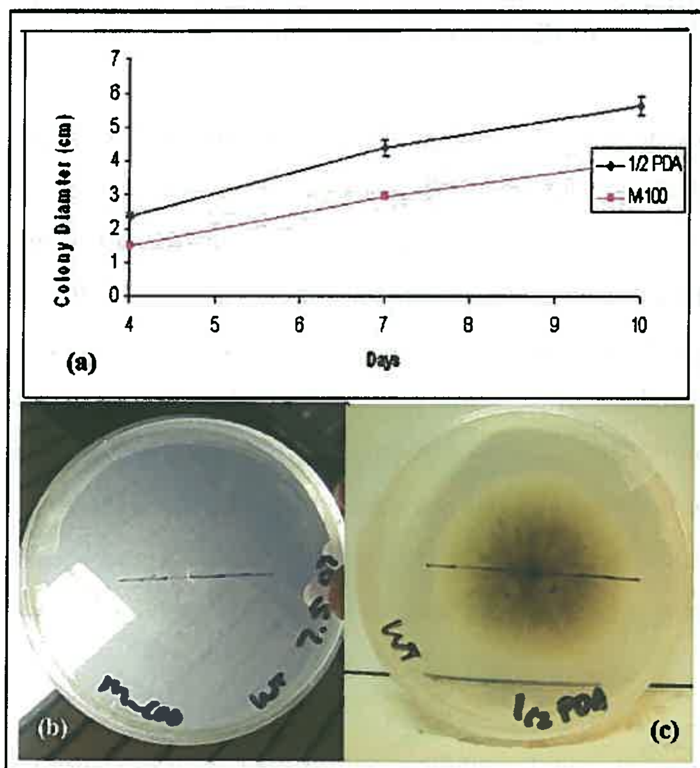

Figure 2.7. Vegetative Growth of WT *T. basicola* on M-100 (1.5% agar) vs ½ PDA (2.2% agar). Fungal cultures were stabbed into ½ PDA (2.2% agar) or M-100 (1.5% agar) and grown at 25°C for a total of 10 days. (a) Colony diameter was measured at 4, 7, and 10 days growth. (b-c) Comparison of WT *T. basicola*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

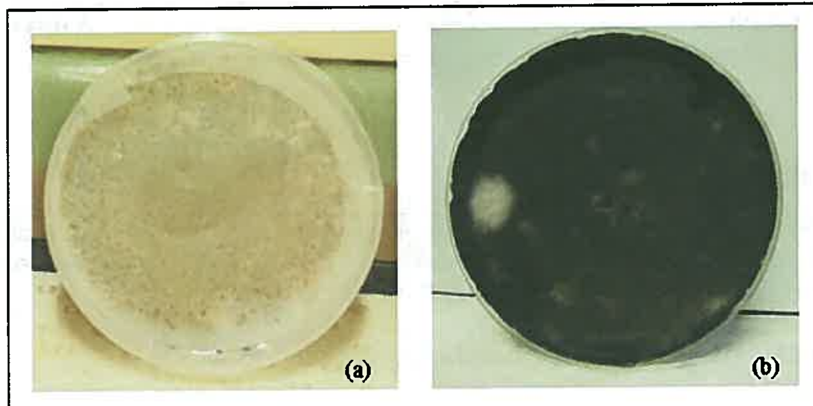

Figure 2.8. Selection by Filter Transfer. Comparison of fungal growth using (a) white and (b) black filters transferred onto ½ PDA (2.2% agar) amended with 10µg HygB/300µg Mef and incubated at 25°C for 5-10 days.

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.

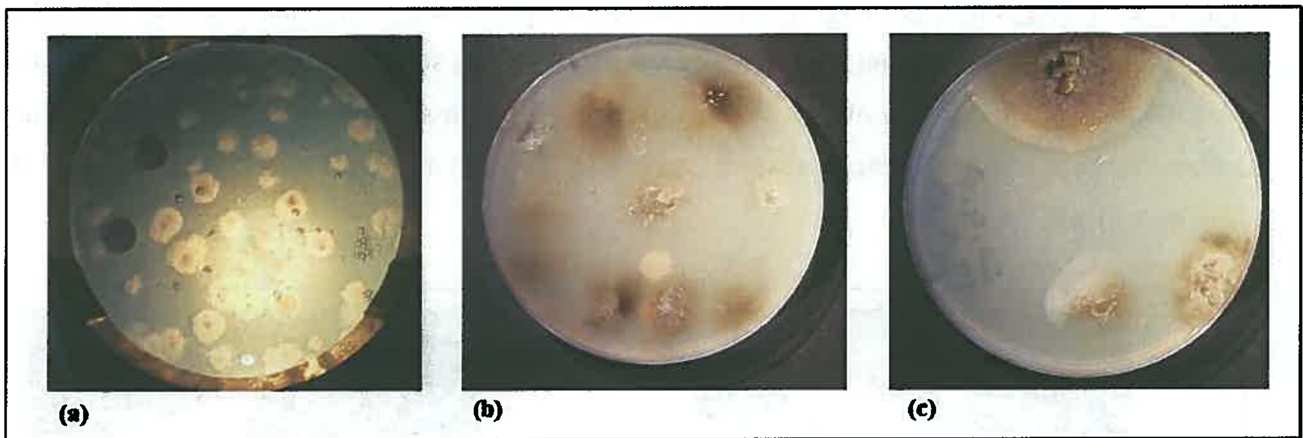

Figure 2.9. Selection of T. basicola Hyg^R Putative Transformants by Top Agar. $HygB^R$ colonies growing (a) in (b) just below surface/beginning to break through, and (c) on top agar. Twenty ml of amended top agar (10µg HygB/300µg Mef final concentration) were added to 2-3 day old co-cultivation medium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

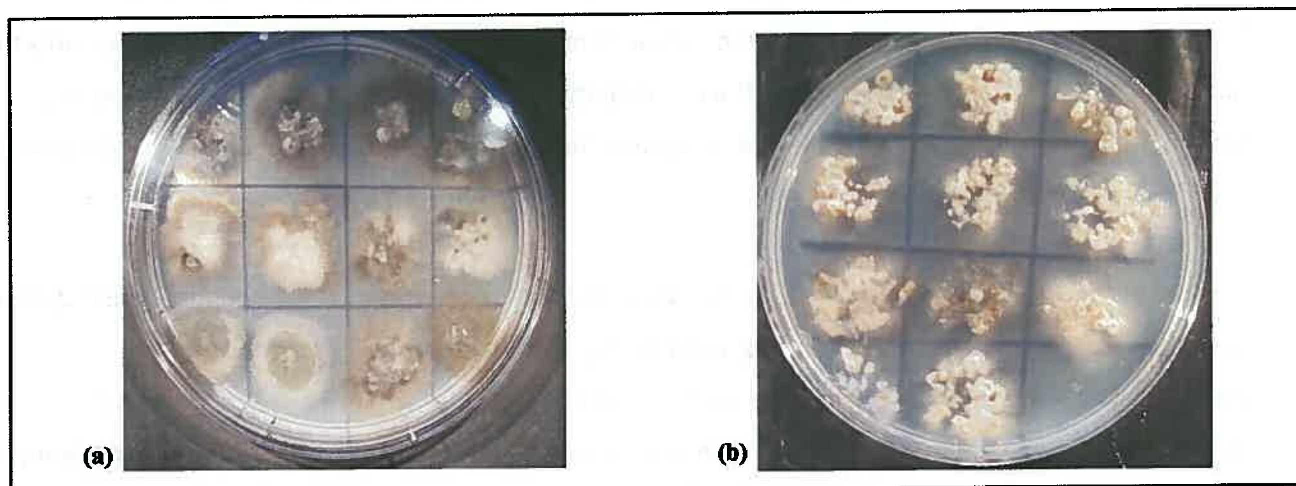

Figure 2.10. Gel Excision. Hyg^R putative transformants were transferred by gel excision from initial top agar selective medium onto ½ PDA (1.2%). (a) unamended (b)10 μg/ml HygB. Plates were incubated at 25°C for 2 days or 2 weeks respectively. WT control is in the bottom right hand corner.

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).

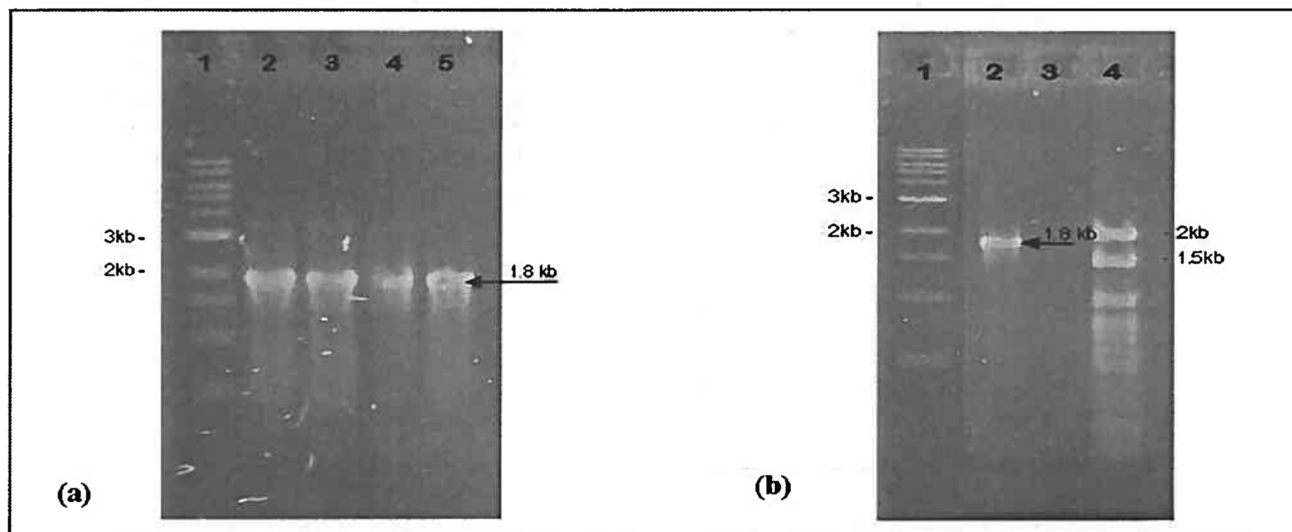

Figure 2.12. Gel Analysis of the DIG-Labelled hph Probe Template DNA. The hph probe template DNA prepared by PCR amplification of pBHt2 using the primers HYKasF1 (Forward) and LBKasR2 (Reverse).(a) lane 1: kb ladder, lanes 2-5 four amplicons of the 1.8kb hph probe template (b) lane 1: kb ladder, lane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

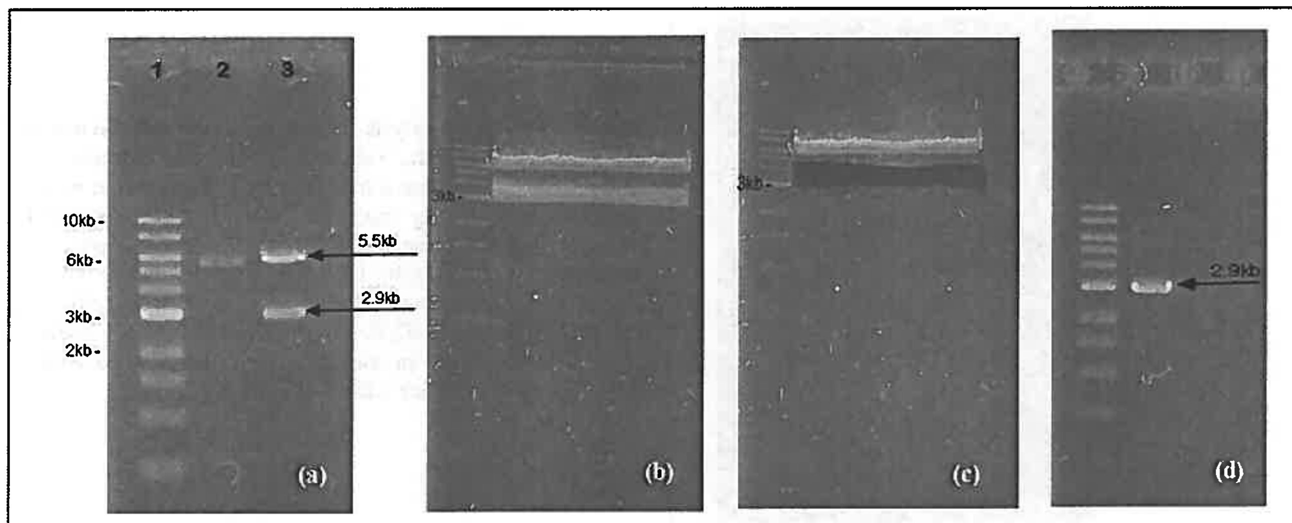

Figure 2.13. Gel Analysis and Preparation of the DIG-labelled Km Probe Template DNA. Template DNA was prepared by double digestion of pBHt2 with *Sac*II and *Nhe*I (a) Analytical gel of double digest. Lane 1: 1kb ladder, Lane2: uncut pBHt2 (supercoil) control, Lane 3: pBHt2 + *Nhe*I and *Sac*II digested 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.

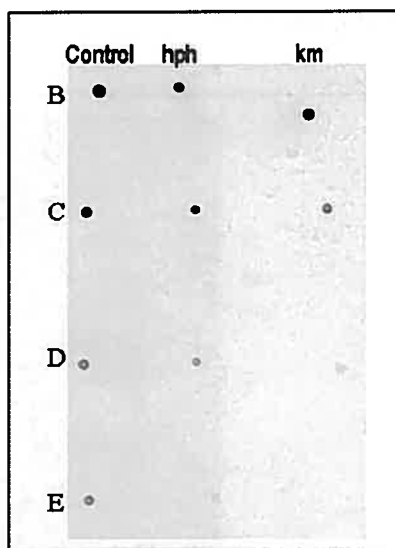

Figure 2.14. DIG-labelled Probe Check. Confirmation and concentration estimations achieved by serial dilution of the DIG labelled hph and Km probes and NBT/BCIP colour change detection. Lane 1: control DNA (Roche) dilutions, Lane 2: DIG-labelled hph probe

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).

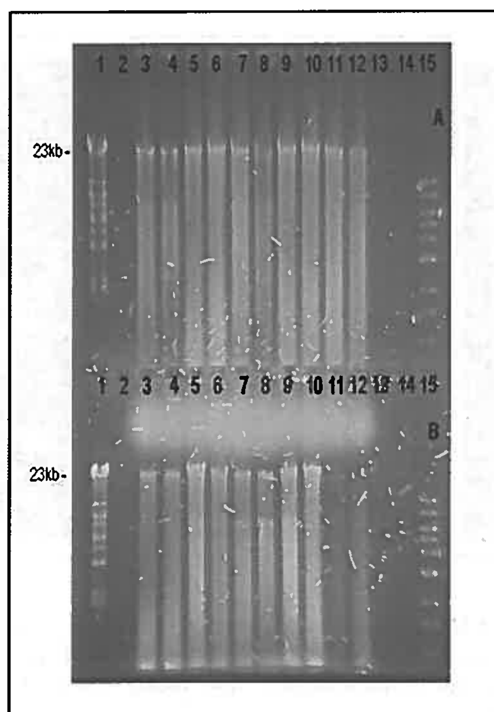

Figure 2.15. Gel Analysis of Extracted Fungal Genomic DNA digested with RNAse. Genomic DNA was extracted from 11 randomly chosen putative transformants and digested with RNAse 1hr prior to running the analytical gel (a) Lane 1: λHind III Ladder, Lanes 3-4: 43p3a, Lanes 5-6:72p1c, Lanes 7-8:66p4d, Lanes 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).

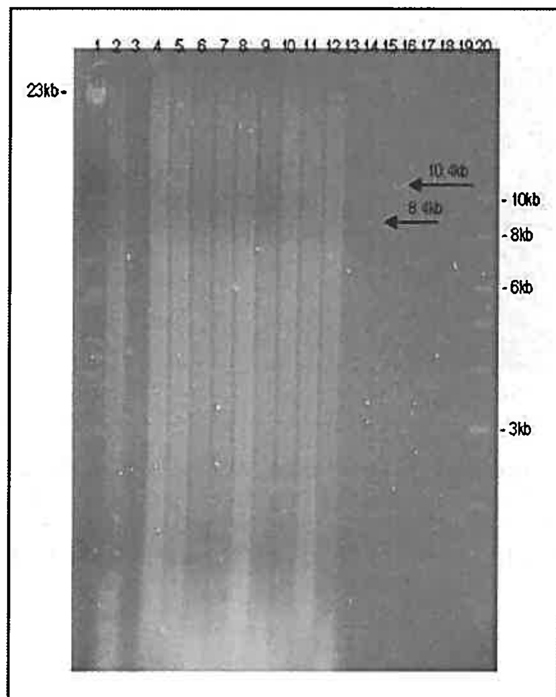

Figure 2.16. Gel Analysis of Digested Fungal Genomic DNA Prior to Southern Blot. Genomic DNA (1-1.5µg) from 9 ATMT mutants (plus WT) was digested O/N with *Nhe*I.RNase was added 1hr prior to running the samples for 16hrs on a preparative maxi gel for Southern blot analysis. Lane 1: λ *Hind* III ladder, 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.

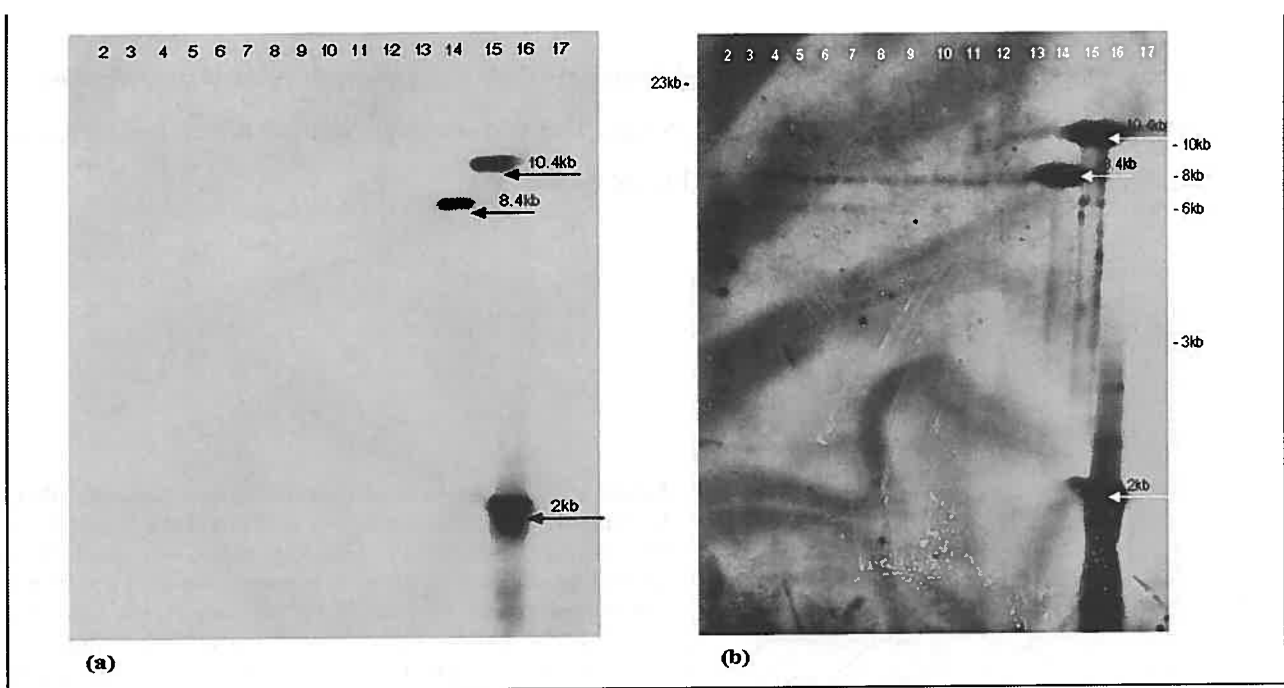

Figure 2.17 Southern Blot Analyses of Nine Putative ATMT Transformants Hybridised with the DIG-Labelled hph probe. Genomic DNA from the transformants was hybridised with the DIG-labelled hph probe under high stringency conditions. Films were exposed for (a) 30 min and (b) 2 days. Lane 2: WT, Lane 4: 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.

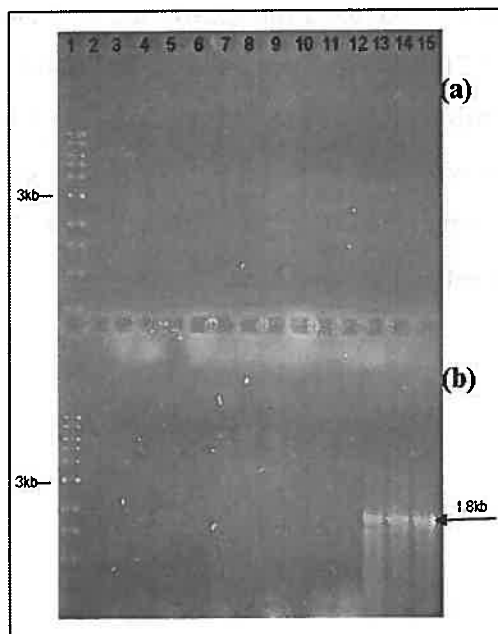

Figure 2.18. PCR Amplification of Fungal Genomic DNA of 11 ATMT Transformants. PCR amplification of fungal genomic LBKasR2, and reaction conditions as used to generate the DIG-labelled hph probe template DNA. Dilutions (1:10 and 1:100) were prepared for all DNA samples, with the exception of 64p1a and 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.

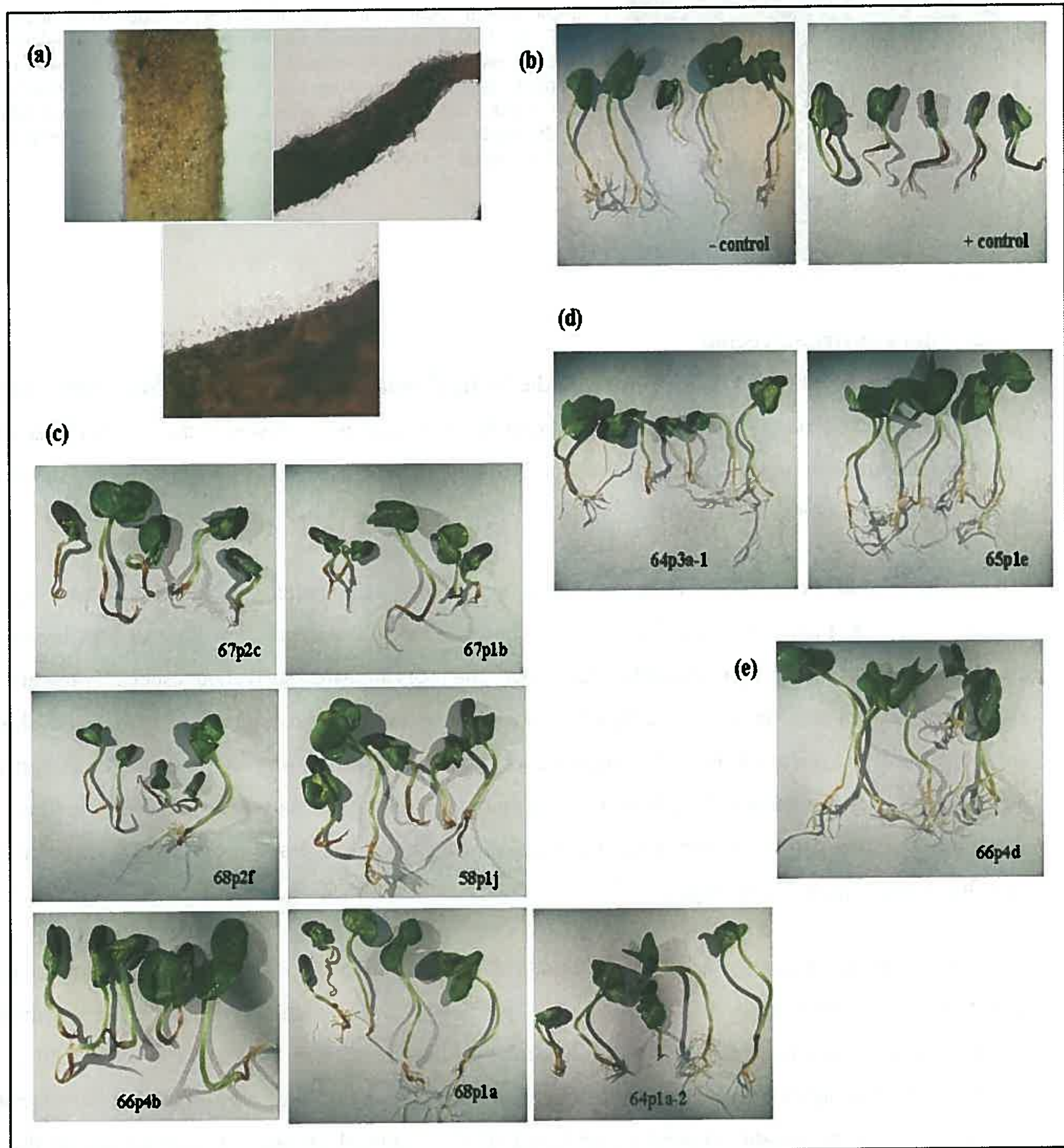

Figure 2.20. Disease Symptoms of Cotton Seedlings Infected with 10 ATMT Hyg^R Putative Transformants
(a) Microscopy of roots infected with WT (b) Controls: Positive (WT *T. basicola*) and Negative (water) (c) highly pathogenic transformants (d) weak/intermediate pathogenic transformants (e) non pathogenic 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).

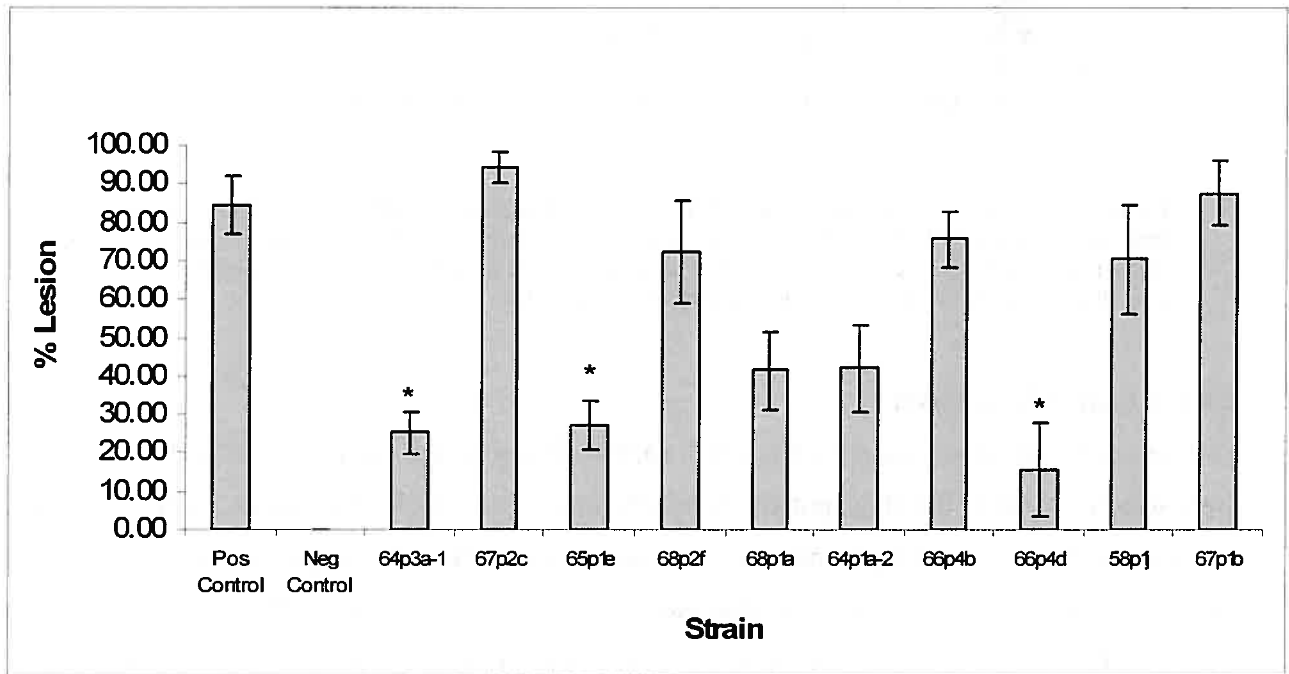

Figure 2.21. Pathogenicity Test of 10 Hyg^R Putative Transformants. Two-day old cotton seedlings were dipped in fungal suspensions and grown at 25°C for 7 days. Total root length and length of root lesions was recorded (cm) and percent root lesion (lesion/total) calculated. Values represent the means 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).

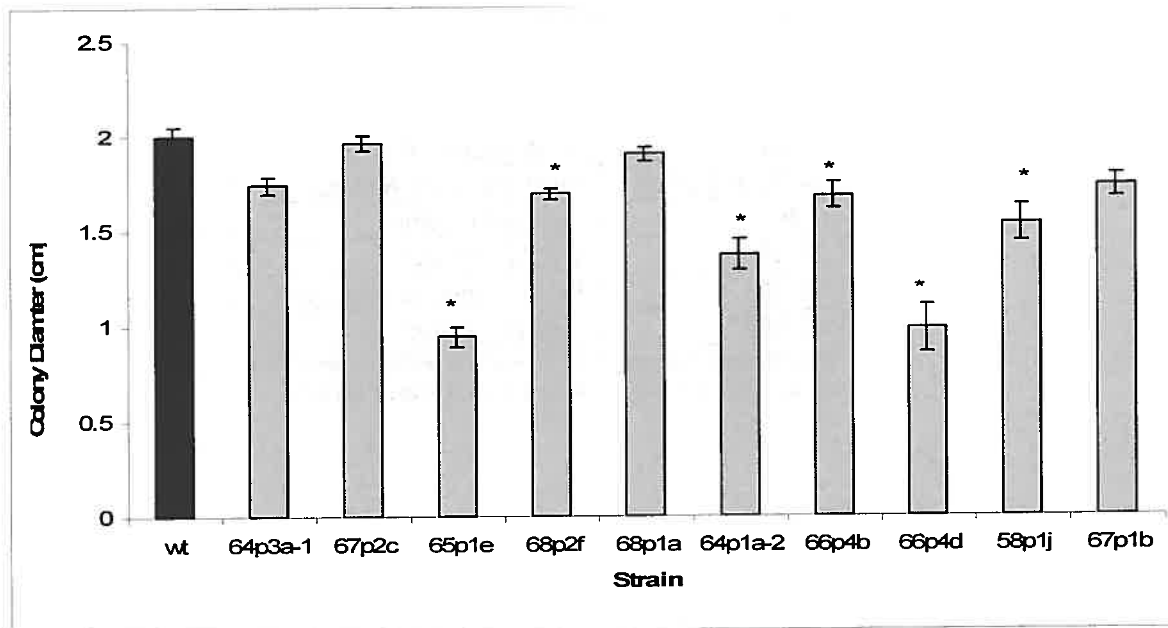

Figure 2. 22. Vegetative Growth of 10 ATMT Hyg^R putative transformants. Colony diameters measured after three day growth at 25°C. Values represent means of 2 replicates from 5 experiments. Significance was calculated using Tukey's Post Hoc Test. Values marked with an asterisk (*) show a significant (<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.

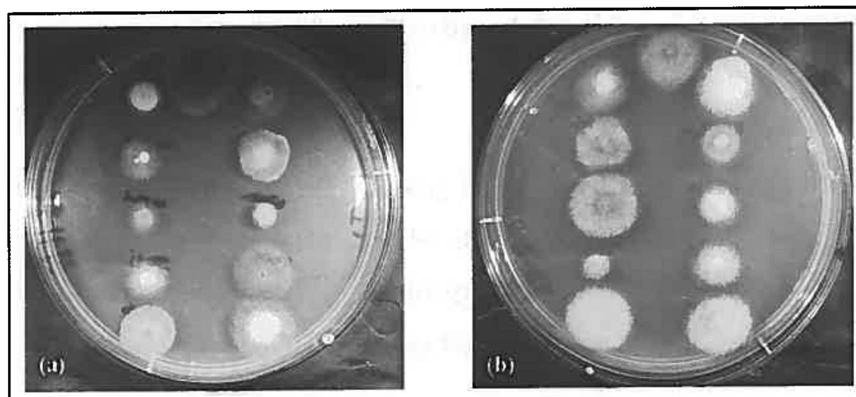

Figure 2.23. Colony Morphology of 10 ATMT Hyg^R Putative Transformants. Ten mutants were inoculated per plate with five in each of two columns, (plus the wt at the top centre of the plate). Cultures were grown at 25°C for 3-4 days. From top to bottom: (a) Column 1: 64p1a-2, 66p4b, 66p4d, 58p1j, 67p1b. 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.

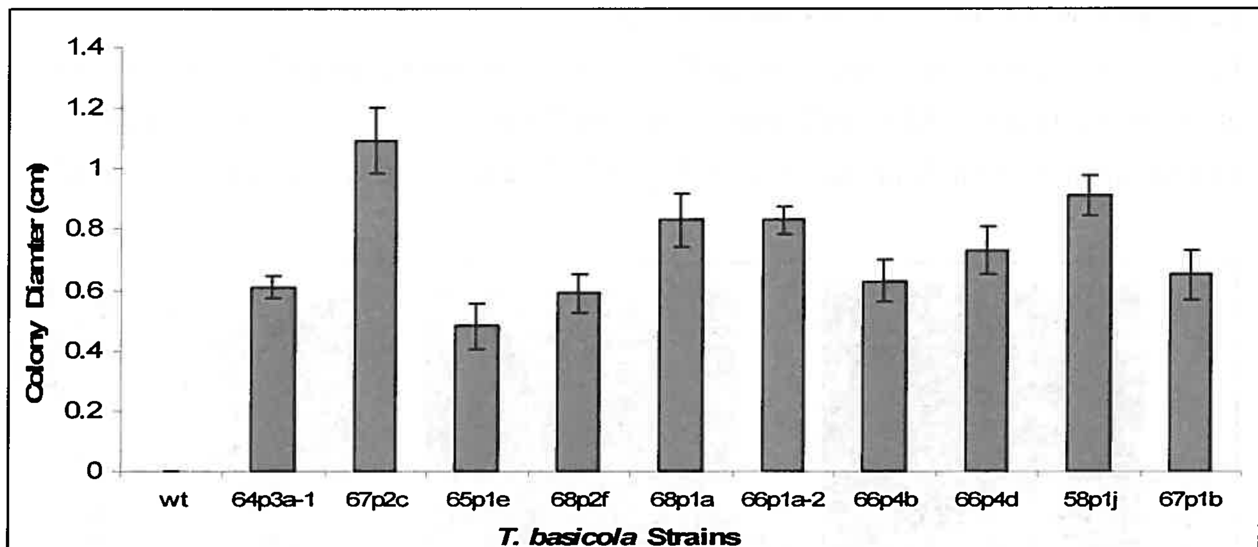

Figure 2.24.Hyg B Resistant Growth of 10 ATMT Hyg^R Putative Transformants. Hyg^R putative transformants were grown on ½ PDA (1.2% agar) medium supplemented with 10μg/ml and incubated at 25°C. Colony diameter was measured after 2 weeks growth. Values represent the means of 2 replicates from 5 experiments.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

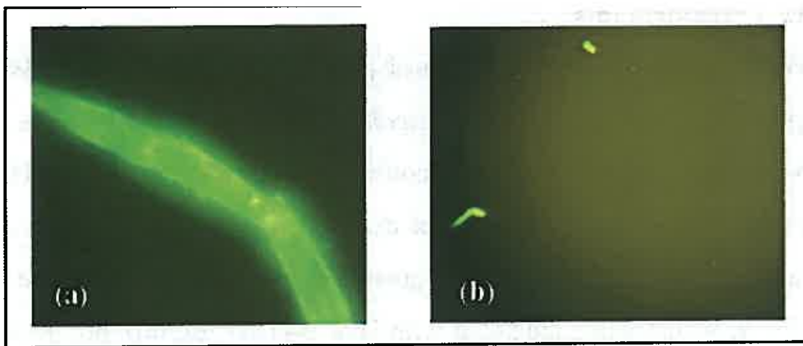

Figure 2.25. Fluorescence in *T. basicola* Transformed with pCAMgfp. Hyg^R putative transformants were first grown on ½ PDA (2.2% agar) medium at 25°C for 3-4 days and then in PDB liquid for ~5hrs prior to fluorescent microscopy. (a) Part of a hypha at x 40 objective. (b) endoconidia at x10 objective.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

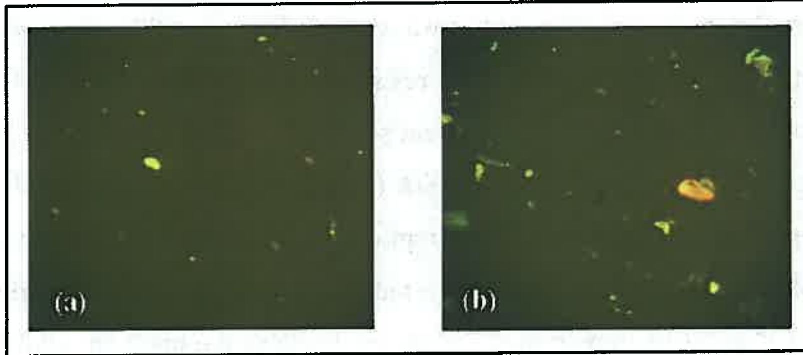

Figure 2.26. Fluorescence Surrounding *T. basicola* Transformed with pCAMgfp. Hyg^R putative transformants were first grown on ½ PDA (2.2% agar) medium at 25°C for 3-4 days and then in PDB liquid for ~5hrs prior to fluorescent microscopy. The surrounding medium for (a) one of the ten Hyg^R putative transformants 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) *EcoR*I + *Nhe*I or (2) *Sma*I + *Nhe*I. The 5kb fragment from the *EcoR*I digest and the 3.4kb fragment from the *Sma*I digest were excised (Figure 2.27c) and used in the construction of pMAX.

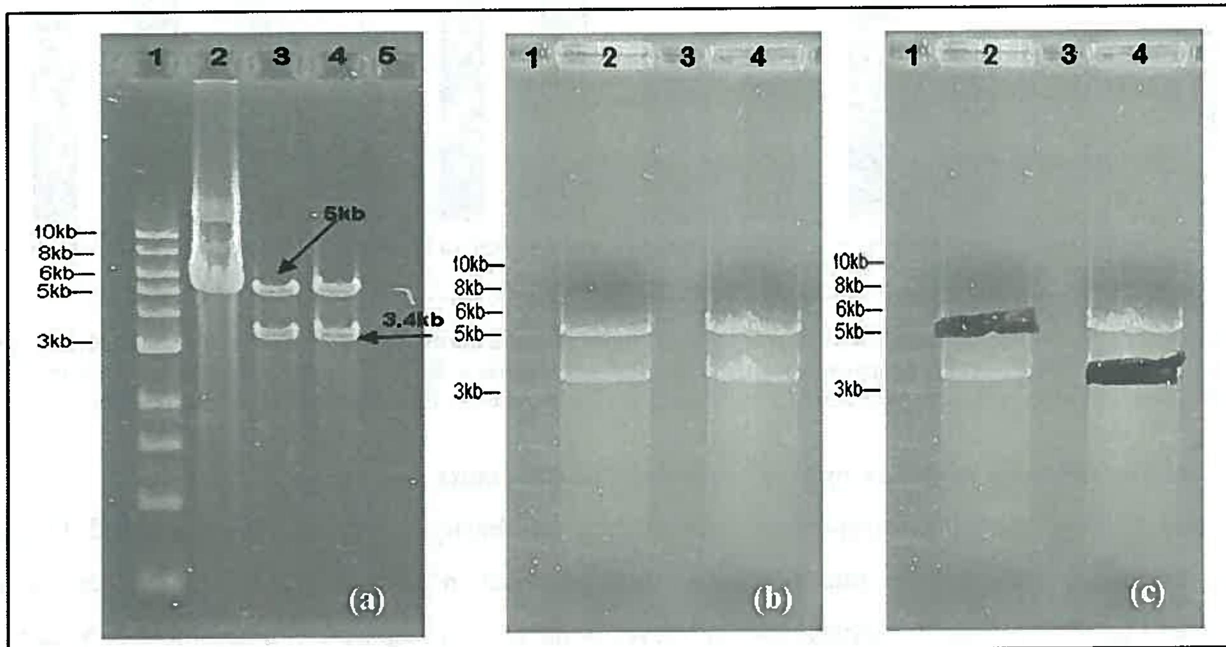

Figure 2.27. Gel Analysis and Preparation of pBHt2 Double Digestion Fragments. pBHt2 was double digested with two sets of restriction enzymes; *Eco*RI + *Nhe*I and *Sma*I + *Nhe*I. After gel analysis, the digests were run on a preparative gel and the 5kb *Eco*RI/*Nhe*I digest and the 3.4kb *Sma*I/ *Nhe*I 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.

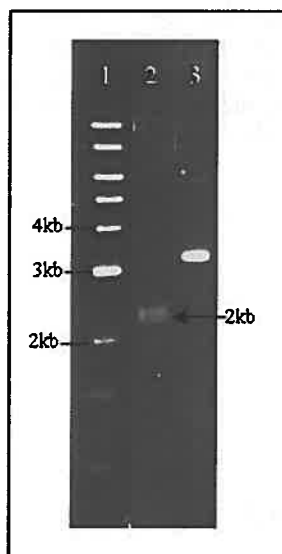

Figure 2.28. Gel Analysis of pBR322 Double Digestion. pBR322 was double digested with *Eco*RI and *Bsa*AI. After gel analysis, the digest was cleaned. Lane 1: 1kb ladder, Lane 2: pBR322 double digest, Lane 3: uncut pBR322 control.

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 *DH5a*	*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°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.

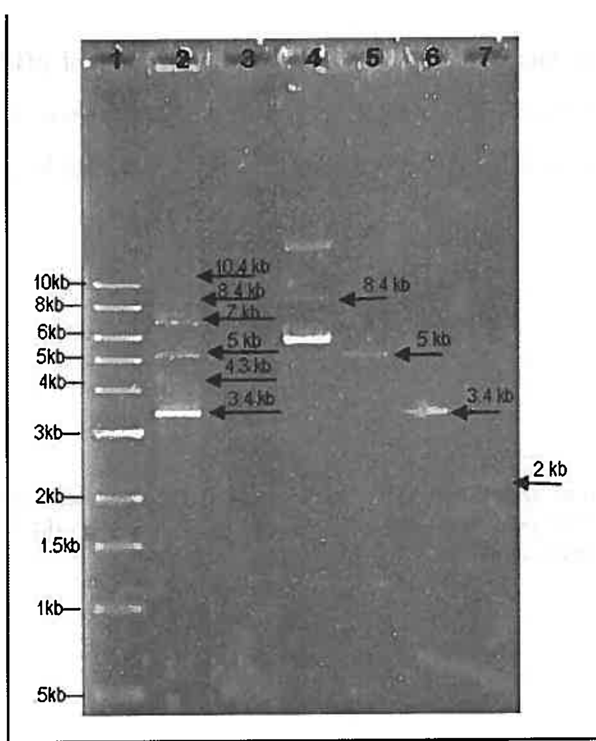

Figure 2.29 Gel Analysis of pMAX Tri-Ligation Solution. The 5kb, 3.4kb, and 2kb fragments were tri-ligated and run on an analytical gel along with uncut pBHt2 and the 3 ligation fragments as controls. Lane 1: 1kb ladder, Lane 2: Tri-ligation solution, Lane3: uncut pBHt2, Lane 4: 5kb fragment, Lane 5: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

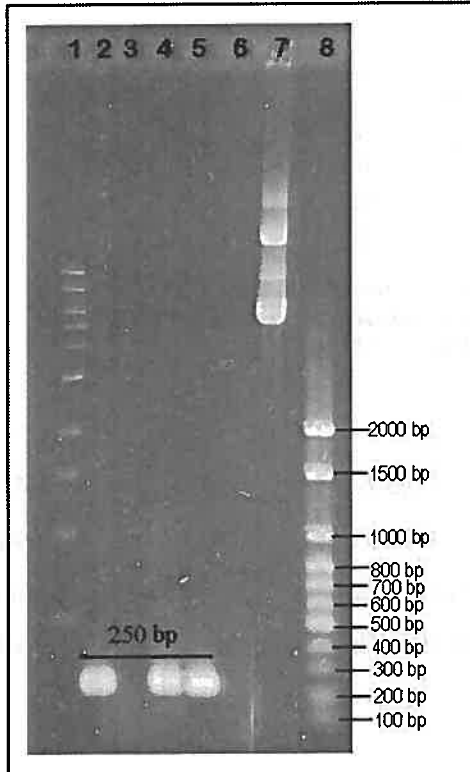

Figure 2.30 PCR amplification of the 250bp oligo for pAIM3 Construction.18.9.07. The 250bp oligo used in the construction of the plasmid pAIM3 was prepared by PCR amplification of a region within the plasmid pBHt2 using the forward primer LBEcoF1 and reverse primer LBKasR1. L1: 1kb ladder, L2, 4, and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