Using polyethylene glycol-mediated transformation when identifying pathogenicity genes in *Thielaviopsis basicola*.

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Declaration:

I declare that the substance of this thesis is my own work. I certify that this thesis has not been submitted as part of another degree and is not currently being submitted for any other degree. Any help in preparing this thesis has been acknowledged and all sources have been cited.

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Abstract

Thielaviopsis basicola is a filamentous fungus and hemibiotrophic soil-borne plant pathogen that causes the disease black root rot in over 200 commercially important plants world-wide, including cotton (*Gossypium hirsutum*). Black root rot affects the roots compromising the health and productivity of plants and has reached pandemic levels throughout Australian cotton-growing areas. To date, no pathogenicity genes have been identified in *T. basicola*. A better understanding of the genetic and molecular factors involved in the pathogenicity of *T. basicola* isolates on cotton will hopefully provide information which may result in the development of an effective control measure against black root rot. The general aim of this study was to identify pathogenicity genes in *T. basicola* cotton isolates, which did not act as housekeeping genes. In 2007, Samiya Al-Jaaidi had developed the first and only protocol for the polyethylene glycol (PEG)-mediated transformation of *T. basicola* used for random insertional mutagenesis to identify pathogenicity genes. Since its development there have been attempts by others to replicate the protocol, without any success. This study attempted to replicate this protocol and determine whether it was a valid tool for the transformation of *T. basicola*. For the first time since its development, the protocol for PEG-mediated transformation of *T. basicola* by Al-Jaaidi (2007) had been replicated with limited success after modification. Stringent execution of the original protocol failed to produce transformants, but a doubling of PEG-treatment incubation times to a total of 70 min had resulted in an average transformation frequency of 0.10 ± 0.02 *T. basicola* transformants/µg of pDNA and the generation of 31 *T. basicola* transformants. Consequently, two reduced-pathogenicity *T. basicola* transformants, G8-1 and G7-2, were identified after pathogenicity testing on cotton. This indicated that this is a viable tool for the identification of pathogenicity genes, but the original protocol may require further optimization. Aspects of the PEG-mediated transformation protocol that may require optimization were examined. During the course of this study, a modified procedure for *T. basicola* protoplast preparation was devised to improve ease and protoplast yield, and a novel plasmid named pGpdR (~ 5.0 kb) was developed as an alternative vector to be used for fungal PEG-mediated transformations.

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List of abbreviations

A_{660}	absorbance at wavelength of 660
bp	base pairs
^{o}C	degrees Celsius
cfu	colony forming units
cm	centimetres
cm^2	centimetres squared
dH_2O	distilled water
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
g	grams
× *g*	times gravity
gDNA	genomic DNA
h	hours
H_2O	water
ITS	internal transcribed spacer
kb	kilobases
L	litres
M	molar
min	minutes
mg	milligrams
mL	millilitres
mM	millimolar
µg	micrograms
µL	microlitres
NaOP	sodium orthophosphate
ng	nanograms
nt	nucleotides
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
pDNA	plasmid DNA
PEG	polyethylene glycol
pg	picograms
RNA	ribonucleic acid
rpm	revolutions per minute
s	seconds
SDS	sodium dodecyl sulfate
tPA	tissue plasminogen activator
V	volts
YMA	yeast mannitol agar
YT	yeast tryptone

Chapter 1: Introduction

1.1 General introduction

Thielaviopsis basicola (synanamorph, *Chalara elegans*) is a filamentous fungus and hemibiotrophic soil-borne plant pathogen of over 200 agricultural and ornamental plants (Otani, 1962). *T. basicola* has a cosmopolitan distribution (Nehl et al., 2004; Coumans et al., 2011), including well over 60 countries worldwide (CABI, 2006). Its exact origin remains unknown. It is prevalent in cultivated soils, but has also been identified in 'virgin' or non-cultivated areas (Yarwood, 1981). *T. basicola* invades the root system of host plants and causes the disease black root rot (King & Presley, 1942). This disease reduces the plants ability to take up nutrients and weakens plants roots, which results in compromised health and productivity of the plant. Black root rot is primarily caused in seedlings, although mature plants can also be infected. Each strain or isolate of *T. basicola* displays its own host specificity with varying degrees of pathogenicity (Keller & Shanks, 1955). Although a variety of control measures have been utilized to suppress black root rot, prevention of the disease has remained elusive.

In Australia, *T. basicola* was first recorded in Queensland in the 1930s as a pathogen of sweet pea (Simmonds, 1966). It has since been identified on a variety of commercial crops, including cotton, beans, lettuce, lucerne, lupin, peas, tobacco, and relatively recently on soybean (Simmonds, 1966; Allen, 1990; O'Brien & Davis, 1994; Mondal et al., 2004). Amongst Australian nurseries, black root rot is becoming an increasing problem with a large variety of horticultural plants being affected (Minchinton & Mebalds, 2001).

Black root rot was first reported on cotton in Arizona in 1922 (King & Presley, 1942). In Australia, black root rot was first reported on cotton (*Gossypium hirsutum*) in north-western New South Wales in 1989 (Allen, 1990) and by 2004 had resulted in a pandemic throughout cotton-growing areas (Nehl et al., 2004). Black root rot has had a significant impact on the Australian cotton industry with delayed crop maturity and yield losses as high as 1.5 bales per acre (Jhorar, 2004). A survey of 30 cotton farms in north-west New South Wales in 2003 reported black root rot among 97% of farms, with 36% of plants affected in 72% of fields within affected farms

(Nehl et al., 2004). In 2011, Australia was the world's 6th largest producer of cotton and 3rd largest exporter (National Cotton Council of America, 2011), making the industry very important to the Australian economy, and therefore it is imperative that *T. basicola* is controlled.

To date, no pathogenicity genes have been identified in *T. basicola*. The general aim of this study was to identify pathogenicity gene/s in *T. basicola*, specifically in isolates that display pathogenicity on cotton (*G. hirsutum*). A better understanding of the genetic and molecular factors involved in the pathogenicity of *T. basicola* isolates on their plant hosts will hopefully provide information which may result in the development of an effective control measure against black root rot.

This review will examine *T. basicola* and black root rot, and the control measures that are currently being utilized or developed against them. Emphasis will be placed on their relationship to cotton. It will also examine the types of pathogenicity genes that are associated with fungi in general.

1.2 *Thielaviopsis basicola*

1.2.1 Morphology and variation

As a filamentous fungus, *T. basicola* forms branching tubular filaments known as hyphae, which undergo polar growth involving apical extension and branching (Madigan et al., 2003). Integrated masses of these filaments are called mycelia. The hyphae have cell walls composed of chitin, glucans, mannans and glycoproteins, and are compartmentalised by cross-walls known as septa (Bowman & Free, 2006). The cell walls surround the plasma membrane of each individual cell.

Variation in morphology of *T. basicola* colonies has been observed in culture, and two distinct variants have been recognised: 'grey' and 'brown' (Punja & Sun, 1999). Each of these variants is capable of giving rise to the other during subculturing. The reason for this variation remains unknown. Other variants include 'albino' and 'mycelial' aberrant phenotypes (Punja & Sun, 1999). Random amplified polymorphic DNA (RAPD) analysis indicated changes in nucleotide sequence in these aberrant variants, possibly through mutation. Variation in colony morphology, colour and size has also been observed when growing *T. basicola* in the presence of different plant root extracts (Coumans et al., 2010).

T. basicola produces two types of spores. The first type of spore are conidia, also known as phialospores or endoconidia. *T. basicola* endoconidia are asexual, non-motile spores produced from phialides in a basipetal fashion. Liberated single endoconidia are, hyaline, cylindrical with rounded ends and are variable in size (Delvecchio et al., 1969). In cultures, endoconidia are produced with 24 h of incubation (Shew & Meyer, 1992). Generally, less than 1% of endoconidia survive in soil for 15 months (Schippers, 1970). The second type of spore are chlamydospores, also known as aleuriospores. *T. basicola* chlamydospores are asexual, non-motile spores produced at the tip of hyphae. A chlamydospore chain of individual spores is composed of thick-walled compartments surrounded by a distinct out wall (Delvecchio et al., 1969). The number and size of individual spores in the chain can vary according to the *T. basicola* isolate (Punja & Sun, 1999). They appear pigmented due to the presence of melanin. Their thick walls and the presence of melanin protect them from adverse conditions, such as temperature variation, lack of moisture, exposure to sunlight and microbial action, and it is these features that allow them to generally function as resting spores (Tsao & Bricker, 1966). Chlamydospores can survive in soil for several years. This provides *T. basicola* with a long-term survival strategy. In cultures, chlamydospores are produced after 3 days of incubation (Shew & Meyer, 1992).

Differences in colony appearance, colour, zonation, growth rate, production of spores, length of chains of chlamydospores and pathogenicity have been observed amongst single chlamydospores that have been cultured (Huang & Patrick, 1971). This phenomenon not only occurs amongst chlamydospores from the same culture, but also from adjacent spores in the same chlamydospore chain. The cause of this is unknown, but such variation may improve the long-term survival of *T. basicola* in adverse conditions or new environments.

The presence of double-stranded RNA (dsRNA) elements, from at least two fungal virus groups, can attribute to variation in morphology in *T. basicola* (Park et al., 2006). There is close to a 100% transmission rate of dsRNA through conidia. The absence of specific dsRNA elements can result in reduced sporulation, pigmentation and survival, but enhanced growth (Punja, 1995). Whether the presence of dsRNA elements provides an ecological advantage remains to be determined.

Sexual reproduction has not been demonstrated (Paulin-Mahady et al., 2002) and it is not known whether more than one mating type occurs in isolates (Geldenhuis et al., 2006). Until this is determined it is possible that a cryptic teleomorph of *T. basicola* could exist. In Australia, RAPD analysis revealed that migration and gene flow between populations may occur. *T. basicola* cotton isolates from three cotton growing regions were analysed. Two cotton growing regions formed distinct clusters and the isolates from the third were distributed between the other two (Pattemore & Aitken, 2000). Whether this may indicate sexual reproduction or possibly horizontal gene transfer remains unknown.

1.2.2 Host specificity and range

T. basicola has a wide host range. This includes important commercial crops, such as bean, beet, carrot, celery, citrus, cotton, lettuce, lucerne, onion, pea, peanut, sesame, soybean, sweet potato, tobacco and tomato, to name but a few (The Royal Botanic Gardens & Domain Trust, 2010). Monocotyledons are non-hosts of *T. basicola* (Wheeler & Gannaway, 2007). Its host range is appears to be increasing, although whether this is due to improved reporting of *T. basicola* infection or actual mutation of the pathogen or plant host is unknown. For example, the first-ever record of black root rot on black elderberry was only recently reported in Switzerland, where the infected plants could all be traced back to a single commercial source (Michel, 2009).

'Host specificity is a relationship in which a fungus derives its nutrition from a live host plant during some phase of its life cycle, and is restricted to a particular host or group of related species, but does not occur on other unrelated plants in the same habitat' (Zhou & Hyde, 2001). Such specificity is the result from additive effects of host and pathogen genotypes, but can also be due to specific genotype-by-genotype interactions between the plant and pathogen genomes (Lambrechts, 2010). Varying degrees of genetic specificity do exist, but the molecular mechanisms involved are not fully understood (Kirchner & Roy, 2002).

It has been observed that *T. basicola* isolates display differences in host-specificity and the degree of pathogenicity. For example, *T. basicola* isolates from poinsettia have been found to be highly pathogenic on poinsettia, moderately pathogenic on bean, but non-pathogenic on tobacco, whereas, tobacco isolates have been found to be non-pathogenic on bean and poinsettia (Keller & Shanks, 1955; Lloyd & Lockwood, 1963). *T. basicola* cotton isolates are not limited to causing black root rot on cotton, but have also been found to be highly pathogenic toward lupin, pansy and soybean, but non-pathogenic toward lettuce (Mondal et al., 2004; Al-Jaaidi, 2007). These interactions indicate that the differences in genotype between different *T. basicola* isolates results in differences in host-specificity related to pathogenicity. Given that host specificity is also dependent on the genotype of the plant host, not only can *T. basicola* host specificity occur at the species level, but also the cultivar level. For example, lettuce cultivars belonging to the loose leaf and batavia types were all resistant to lettuce black root rot, whereas cultivars of iceberg lettuce and butterhead types presented inter-varietal reactions being, some resistant and others susceptible (Sala et al., 2008). Morphological variation and RAPD analysis has revealed that different *T. basicola* isolates from the same host shared greater genetic similarity and morphology than those from a different host, and therefore host selection was likely to be determined by genotype (Punja & Sun, 1999). A recent study analysed the internal transcribed spacer sequences of various *T. basicola* isolates, indicating that isolates were grouped based on host of origin, irrespective of geographical origin (Coumans et al., 2011). Host specificity may lead to differentiation of *T. basicola* isolates into intraspecific groups. The reason for such high intraspecific variation of *T. basicola* strains may be due to a high mutation rate and/or the presence of transposable elements (Punja & Sun, 1999). The host-specificity of *T. basicola* isolates does indicate that at least some genes involved in pathogenicity are not common to all *T. basicola* isolates.

1.3 Pathogenesis

The infection process of *T. basicola* on the roots of host plants can be summarised with the following steps:

- Germination of *T. basicola* spores
- Growth of *T. basicola* toward host plant roots
- Surface contact of *T. basicola* to host roots
- Penetration of *T. basicola* into epidermal cells
- Establishment of a biotrophic phase
- Conversion to a necrotrophic phase

The nutritional environment of the rhizosphere is an important factor in triggering spore germination as dormant propagules are stimulated by soluble and volatile components present in seeds and root extracts (Nelson, 1990). In the presence of nutrients, endoconidia produce germ tubes that branch and form mycelia. In the absence of nutrients, endoconidia undergo a microcycle of conidiation producing secondary endoconidia. Secondary endoconidia are smaller in size, have thinner walls and are pyriform (pear-shaped) when compared to primary endoconidia (Punja, 1993). In the presence of nutrients, a chlamydospore chain breaks up into individual cells, each of which can germinate to produce germ tubes that branch and form mycelia (Patrick et al., 1965).

The hyphal apex of the germ tube grows toward the surface of the hosts roots (Mims et al., 2000). Hyphal directional growth appears to be determined by the composition and concentration of root exudates produced by a plant (Al-Jaaidi, 2007). These external signals may induce a directional response via *T. basicola* signalling genes involved in a signalling cascade. However, enhanced hyphal directional growth is not necessarily conducive to enhanced pathogenesis, and therefore like spore germination, is not necessarily a trait of host specificity. It has been reported in some cases, that there is strong hyphal directional growth with no pathogenicity toward the host, or conversely, weak hyphal directional growth with high pathogenicity toward a host of primary host specificity (Al-Jaaidi, 2007). Both non-host and susceptible-host root extracts influence growth, colony and hyphal morphology and the proteome of *T. basicola* (Coumans et al., 2010), and there is a positive correlation between the level of available host root extract and the diameter, length and degree of branching of hyphae (Hood & Shew, 1997a). Whether there is a correlation between hyphal morphology and either pathogenicity or directional growth is unknown.

T. basicola is a hemibiotrophic fungus (Mims et al., 2000). Fungal hemibiotrophs start their infection cycle with a biotrophic phase and then move onto a necrotrophic phase. Fungal biotrophs form infection structures such as appressoria, penetration hyphae and infection hyphae to invade the plant with minimal damage to the host cells. Each of these infection structures comes with specialised haustoria (hyphal tips) which can penetrate host cell walls and absorb nutrients (Mendgen & Hahn, 2002). Fungal necrotrophs kill host cells with cell wall degrading enzymes and toxins, then digest nutrients by employing hydrolytic and proteolytic enzymes and absorb nutrients that are able to pass through their cell wall and across their plasma membrane (Prell & Day, 2001).

Root hairs are the primary penetration sites for *T. basicola*, but penetration has also been reported through non-root hair epidermal cells (Jones, 1991; Nan et al., 1992) and through wounds (Baard & Laubsher, 1985; Punja et al., 1992). Upon contact with epidermal cells of the host root, the hyphal apex continues vegetative growth and begins to swell. Whether the swollen hyphal apex should be considered appressorium remains unclear. The swollen hyphal apex develops slender penetration hypha, which penetrate the epidermal cells protruding into the cell lumen. In vain, the plant produces callose deposits forming papilla in response to penetration. This begins the biotrophic phase of the *T. basicola* life-cycle. The tip of the penetration hypha expands to form a globulose, terminal, infection vesicle. A few hours after contact, lance or sickle-shaped infection hyphae emerge from the infection vesicles and branch intracellularly. The infection hyphae penetrate and colonize the cortical cells of the root causing necrosis. This begins the necrotrophic phase of the *T. basicola* life-cycle. Chlamydospore production is associated with the necrotrophic phase, and they are produced throughout the root cortex and on the surface of the roots and adjacent soil. The vascular tissue of roots is usually not invaded (Walker et al., 1998) allowing for the survival of the plant host.
(Hood & Shew, 1997b; Mims et al., 2000)

Although *T. basicola* is generally considered an obligate parasite (Hood & Shew, 1997a), it can also associate with hosts in a non-pathogenic manner (Yarwood, 1974) and is capable of limited saprophytic utilization of soil-borne organic matter (Gayed, 1972; Chittaranjan & Punja, 1994).

1.4 Black root rot

1.4.1 Symptoms

The below-ground symptoms in cotton seedlings start with brown to purplish-black discolouration of the affected tissues as lesions form. Chlamydospores can be observed on the root surface. Finally the cortex of the root can appear black and shrunken. This can result in the roots being so fragile that the plant host can be easily pulled from the ground. In mature cotton plants, symptoms start with abnormal swelling of the root. Externally the root appears to be healthy, but the stele of the root has begun to disintegrate forming cavities and is brown to purplish-black in colour. The above-ground symptoms in cotton begin with a reduction in growth and foliage size, and apparent foliage discoloration and withering. Sometimes, swelling of the stem above the soil surface is also evident. (King & Presley, 1942)

With other plants, such as tobacco and beans, there is evidence to show that foliar tissues are also susceptible to the disease, displaying necrosis (Hecht & Bateman, 1964; Punja, 2004). Generally, black root rot does not result in the death of seedlings or mature plants, although seedlings with extensive root damage may perish (Walker et al., 2000). The damage simply reduces the ability for the plant to absorb nutrients, thus weakening it and making it more susceptible to adverse environmental conditions or other pathogens (Rourke & Nehl, 2001, as cited in Al-Jaaidi, 2007).

1.4.2 Control of black root rot: Suppressing and promoting factors

The severity of black root rot is primarily dependent on the susceptibility of the host plant, the strain of *T. basicola* and the levels of inoculum at the time of infection. There appears to be a direct correlation between disease severity and inoculum density (Holtz & Weinhold, 1994). Although there is a level at which increased *T. basicola* inoculum density ceases to result in increased disease severity. Amongst cotton crops this was found to be around 100 cfu of *T. basicola* per gram of soil (Jhorar, 2004). Beyond these basic factors there are several other abiotic and biotic factors that can either suppress of promote *T. basicola* or black root rot that can be manipulated in attempt to control the fungus and/or the disease.

1.4.2.1 Dissemination of *Thielaviopsis basicola*

T. basicola is disseminated like most soil-borne fungi via contaminated soil, plants or soil amendments transferred either directly or passively (e.g. under foot or on vehicles and machinery). Dispersal of *T. basicola* with irrigation has also been observed, thus allowing it to spread within and among fields (Nehl et al., 2004). There have also been recent studies which indicate that it can also be spread by insect vectors (El-Hamalawi, 2008a; El-Hamalawi, 2008b). *T. basicola* has also been recovered from greenhouse air samples (Graham, 1991). Sanitation is the primary method of preventing the spread of the *T. basicola*. A 'Come Clean Go Clean' protocol was developed by the Australian Cotton Cooperative Research Centre and has been implemented by various Australian state governments to encourage farmers and those visiting farms to adopt simple measures to reduce the dispersal of pathogens such as *T. basicola*. Some of the measures include: cleaning soil and crop debris from vehicles, machinery and footwear and applying an appropriate disinfectant when coming onto or leaving a farm; correctly disposing of crop by-products, residues and trash; retaining tail-water and run-off water on farm and keeping it out of river systems; and for the farmer to maintain communication with the relevant industry and neighbours about contamination issues (Australian Cotton Cooperative Research Centre, 2000).

1.4.2.2 Temperature

Soil temperature can influence black root rot severity. The optimal temperature for *T. basicola* growth in culture is between 20 - 30^{o}C (Lucas, 1955). With soil temperatures below 24 - 26^{o}C black root rot appears to increase its severity to a variety of hosts (Lloyd & Lockwood, 1963), including cotton (Rothrock, 1992). It has been shown that increased severity of black root rot at lower temperatures was not dependent on the optimal temperature for fungal growth, but rather the optimal temperature for the plant host growth (Lloyd & Lockwood, 1963). It is thought that unfavourable conditions for the plant host result in lower resistance to infection, thus increasing disease severity. However, survival rates of *T. basicola* are greater at temperatures between 10 - 18^{o}C than at 24 - 34^{o}C (Papavizas & Lewis, 1971). None the less, the temperature at the time of planting can influence the severity of black root on any crop and can be used as a method of controlling the disease. The damage on cotton has been shown to be particularly severe when there is an extended period of cool weather in the spring or if crops are planted too early, whereas planting when temperatures are higher can reduce severity even though *T. basicola* inoculum levels may be higher (Rothrock, 1992; Jhorar, 2004). Black root rot was observed on soybean for the first time in Australia, on seedlings that were collected in August after a cold winter. Commercially, it is normally sown in December when soil temperatures are greater than 20^{o}C, which possibly explains why the disease had not been observed in commercial crops sooner (Mondal et al., 2004).

1.4.2.3 Water

Black root rot of cotton has been reported to be less severe in well-drained soils (King & Presley, 1942), although survival of *T. basicola* is lower in soils with water-holding capacities of 45% or greater than those of 15% of less (Papavizas & Lewis, 1971). Flooding as a control measure in cotton fields has been demonstrated to decrease the severity of black root rot by up to 98%, especially when performed prior to planting (Jhorar, 2004). Its use is constrained by terrain and the availability of water, and there is always the potential to disseminate the disease in any runoff.

1.4.2.4 pH and ionic concentrations

Soil pH alters the solubility of ions in the soil, thus indirectly affecting the distribution and activity of soil microorganisms (Kaufmann & Williams, 1964). Soils with a pH greater than 5.6 promote black root rot, increasing its severity. Soils with a pH of less than 5.2 suppress black root rot, decreasing its severity (Bateman, 1962; Harrison & Shew, 2001). But, pH is not the sole determining factor of disease severity in these circumstances. Alkaline soils containing high levels of calcium further promote black root rot (Oyarzun et al., 1998), but acidic soils containing high levels of calcium have also been reported to promote the disease (Meyer & Shew, 1991). Acidic soils that suppress the disease generally contain high levels of aluminium, phosphates or nitrogen, which have been reported to inhibit spore germination and hyphal growth (Meyer et al., 1994; Delgado et al., 2006). Chlamydospore production is also inhibited by high levels of aluminium at a pH less than 5.0 (Fichtner et al., 2006). Therefore, the levels of exchangeable calcium, aluminium, nitrogen and variety of other ions in these soils, coupled with pH, can affect the severity of black root rot. As a control measure for *T. basicola*, acidifying fertilizers containing nitrogen are generally recommended for burley tobacco production (Harrison & Shew, 2001). Unfortunately, pH conditions and ionic concentrations that suppress or promote black root rot often affect plants in a similar manner.

1.4.2.5 Chemical treatments

Conventional soil fumigation with fungicides can be used to control black root rot. It doesn't eradicate the fungal pathogen completely, but can reduce and suppress *T. basicola* present in the soil (Matthiessen & Kirkegaard, 2006). Thus far this has not been a viable control measure for Australian cotton farms because fumigants do not disperse and penetrate well in clay soils common to Australian cotton farms and in-furrow application of several fungicides has been shown to be ineffective (Jhorar, 2004; Matthiessen & Kirkegaard, 2006). Furthermore, some fungicides have been shown to have a phytotoxic effect on cotton, delaying emergence and slowing plant growth (Jhorar, 2004). Seeds pre-treated with fungicides, such as triazole, or host resistance-inducing chemicals, such as acibenzolar-S-methyl, can reduce the incidence of black root rot and improve seedling survival while avoiding the complications associated with soil treatment (Minton et al., 1982; Toksoz et al., 2009).

1.4.2.6 Crop rotation and biofumigation

Crop rotation with non-host crops, especially monocots, has been shown to reduce *T basicola* density in the soil and the incidence of black root rot (Holtz & Weinhold, 1994). Rotation with other host plants may contribute to the cumulative increase of inoculum from year to year, such as the use of soybean in cotton farming systems (Mondal et al., 2004). Cotton fields after two years rotation with wheat had displayed a 70% reduction in the severity of black root rot on cotton (Nehl, 2002). The addition of organic amendments to soil can suppress black root rot. In Spain, the severity of black root rot on cotton was significantly lower in plots with sugar beet as the preceding crop followed with residue incorporation and than in plots without residue incorporation. Incidence of black root rot was negatively correlated with available nitrogen when corn or sunflower was the preceding crop, and the incidence of black root rot was positively correlated to iron availability in soil when cotton was the preceding crop (Delgado et al., 2006).

The planting of a green manure crop can also release compounds that are toxic to pathogens in the soil. Biofumigation of soil with hairy vetch is capable of reducing *T. basicola* inoculum density and black root rot severity on cotton by as much as 60%, possibly due to the low amounts of ammonia released by the plant (Candole & Rothrock, 1998), but the capacity for hairy vetch biofumigation to reverse severe infestations of *T. basicola* in fields where cotton is cropped repetitively has not been demonstrated (Nehl et al., 2004). Cruciferous plants are commonly used for biofumigation because they produce isothiocyanates which inhibit *T. basicola* and other fungi (Smith & Kirkegaard, 2002). Biofumigation with mustards have displayed a reduction in black root rot in cotton by up to 70% (Matthiessen & Kirkegaard, 2006). The introduction of lucerne or corn stover to soil has displayed a decline in *T. basicola* inoculum density and suppressed black root rot, possibly because organic amendments such as these support other organisms which act as antagonists to *T. basicola* (Papavizas, 1968).

1.4.2.7 Other biotic factors

In natural soil in Switzerland, *Pseudomonas fluorescens* in the rhizosphere have been reported to suppress black root rot (Stutz et al., 1989). *Pseudomonas* species synthesize a variety of biocontrol compounds that can suppress root diseases. These include diacetylphlorogucinol (Phl), hydrogen cyanide (HCN), phenazine-1-carboxylic acid, oomycin, pyoluteorin and pyrrolnitrin. In a study by Laville et al. (1991), mutations of the *gacA* (global antibiotic and cyanide control) gene in *P. fluorescens* CHA0 blocked the production of Phl, HCN and pyoluteorin, and reduced the ability of these mutants to suppress black root rot, confirming that the antibiotics Phl and HCN individually contribute to the suppression of black root rot. The efficiency of *P. fluorescens* to suppress black root rot is dependent on its concentration in the soil, as *P. fluorescens* concentrations are higher in suppressive soils than they are in soils conducive to black root rot (Ramette et al., 2003). A recent study also implicates the influence of differences in dominant Phl biosynthetic *phl*D alleles in *P. fluorescens* (Frapolli et al., 2010).

There are a variety of other organisms that are capable of suppressing *T. basicola*. *Pseudomonas aureofaciens* strain 63-28 produces a furanone which has an antifungal activity against *T. basicola* (Paulitz et al., 2000). *Streptomyces hygroscopicus* strain TA21 can reduce the incidence of black root rot by 85.3% in greenhouse experiments by inhibiting hyphal growth and reducing spore germination (Yi et al., 2010). Coating of seeds with fungal bio-control agents is also proving to be an effective control measure against *T. basicola*. An example of this is the use of *Paenibacillus alevi* strain K-165, which is applied as a seed coat and then goes on to colonize the rhizosphere and soil, inhibiting root colonization by *T. basicola* (Schoina et al., 2011).

There are also organisms that are capable of promoting black root rot, the most notable being the root-knot nematode *Meloidogyne incognita.* Histopathological studies have revealed that cotton plants infected with both parasites have extensive necrosis of vascular tissues and contained chlamydospores inside xylem tissue (Walker et al., 2000). This is not usual for *T. basicola* infection and generally results in death of the plant.

1.4.2.8 Breeding and genetic modification of resistant host plants

There are two methods that can be used to confer resistance to black root rot to host plants. The first involves cross-breeding a plant cultivar that displays resistance with a commercially desired cultivar. The second involves using recombinant techniques to create a resistant transgenic plant. Genetic modification of host plants may be the best method of preventing *T. basicola* infection, simply because it does not require the identification of a resistant cultivar for breeding and modification of pre-existing commercially desirable traits are kept to a minimum.

Tobacco is an important commercial crop and resistance to *T. basicola* is highly desired. Host plant resistance to *T. basicola* has been observed in many cultivars of burley tobacco (*Nicotiana tabacum*), but only providing partial resistance and involving multiple genes (Wilkinson et al., 1991). A tetraploid relative of tobacco, *Nicotiana debneyi* Domin, has high resistance to a wide spectrum of *T. basicola* isolates, which is controlled by a single dominant gene (Clayton, 1969). This gene was backcrossed to burley tobacco producing the cultivar, 'Burley 49'. Although 'Burley 49' has high resistance to *T. basicola*, it is not a commercially desirable cultivar because it has small leaf size, low yields, and late maturity. Attempts to develop cultivars that overcome those issues have been made since (Wilkinson et al., 1991; Bai et al., 1996). To date, a commercially viable tobacco cultivar with complete resistance to *T. basicola* has not been developed.

Attempts to find cotton which displays resistance to *T. basicola* have been made. *Gossypium arboreum* var. "PI 1415" and *G. herbaceum* var. "A20" (PI 408778) have been identified as apparently having partial and high resistance to *T. basicola* respectively (Wheeler & Gannaway, 2007). Cross-breeding and genetic analysis in an attempt to identify quantitative trait loci of these two varieties is being examined (Niu et al., 2008). In Australia, none of the commercially available cultivars of cotton have resistance against *T. basicola* and sources of resistance for the purposes of cross-breeding are lacking (Wang & Davis, 1997; Allen, 2001).

The development of transgenic cotton varieties is also underway. A gene encoding endochitinase from *Tricoderma virens*, which degrades fungal cell walls, was engineered into cotton. Selected transgenic lines demonstrated significant levels of resistance to *T. basicola* (Howell, 2003). In Australia, there were plans to field test a genetically modified cotton line that contained the plant defensin gene, *nad1*, derived from the ornamental tobacco*, Nicotiana alata* (OGTR, 2006). This gene encodes a defensin protein, NAD1, which inhibits the growth of fungi, including *T. basicola*. Plant defensins occur naturally in many horticultural and crop plants such as dahlia, tomato, peas and wheat (OGTR, 2006). Whether this field test has been conducted is unknown.

1.5 Fungal pathogenicity genes and avirulence genes

Pathogenicity genes have been defined as those necessary for disease development, but not essential for the fungal pathogen to complete its life cycle *in vitro*, although this definition cannot apply to obligate parasites (Idnurm & Howlett, 2001). Pathogenicity genes are involved at various stages of pathogenesis.

1.5.1 Challenging plant host defences

Pathogens need to overcome the plant host defences if pathogenesis is to be successful. Internal host defences involve the production of antimicrobial secondary metabolites. Those that are produced constitutively are known as phytoanticipins, and those produced after challenge by a pathogen are known as phytoalexins (Osbourn, 1996). One method of resistance by pathogens involves the degradation of these chemical defenses with enzymes. Saponins are a class of phytoanticipins which disrupt fungal membranes. *Gaeumannomyces graminis* var. *avenae* can produce avenacinase which is capable of degrading the saponin avenacin A-1, which is localized in the epidermis of oat roots. Disruption of the avenacinase gene prevented mutants from attacking oats, but maintained full pathogenicity on wheat, which does not produce avenacin A-1 (Bowyer et al., 1995).

Pathogen-associated molecular pattern-triggered immunity in host plants involves the recognition by the host of conserved pathogen-associated molecules, such as chitin in fungal cell walls, which triggers a defense response. It is believed that most fungal avirulence genes (*Avr*) encode effectors that facilitate virulence by suppressing pathogen-associated molecular pattern-triggered immunity (De Wit et al., 2009). In *Cladosporium fulvum*, the Avr2 effector inhibits tomato cysteine proteases that are presumed to be important in basal host defense (van Esse et al., 2008), and the Avr4 effector contains a functional chitin-binding domain that protects chitinous fungi against plant chitinases (van Esse et al., 2007). Gene-for-gene interactions account for some plant hosts displaying resistance to certain pathogenic fungi (Heath, 1991). This has been observed in relation to *Avr* genes. For each dominant *Avr* gene in a pathogen there is a respective dominant resistance gene (*R*) in its plant host. There can be multiple *R* genes in the host plant acting against as many *Avr* genes in the pathogen on a one-for-one basis. *Avr* products induce effector-triggered immunity in host plants that contain respective resistance proteins, which results in the activation of host plant defence responses that stop infection by the pathogen (De Wit et al., 2009). The first fungal *Avr* gene was identified in 1991 (van Kan et al., 1991). The majority of effectors that have been identified tend to be small cystine-rich or glycine-rich proteins, such as those in *Cladosporium fulvum, Rhynchosporium secalis, Fusarium oxysporum* and *Magnaporthe oryzae* (De Wit et al., 2009). The identification of *R* genes in hosts or putative *Avr* genes in pathogens may provide valuable information and genetic resources for the control of pathogens on host plants that may not have developed resistance.

1.5.2 Fungal toxins

Some pathogens produce toxins that may disable host cellular functions or kill host cells before infection, or during a necrotrophic phase of infection. *T. basicola* may produce toxins during its necrotrophic phase. Toxins can be characterized as non-host-specific toxins (NHSTs) or host-specific toxins (HSTs).

NHSTs are capable of damaging cells of phylogenetically unrelated plants. Trichothecenes are NHSTs produced by species of *Fusarium*, *Gibberella* and at least six other genera of fungi. Disruption of *Tri5*, a gene controlling the first step of trichothecene biosynthesis, has resulted in reduced pathogenicity of *G. pulicaris* on parsnip (Desjardins et al., 1992), of *G. zeae* on some wheat cultivars (Proctor et al., 1995) and *F. graminearum* on maize (Harris et al., 1999). A pathogen with an altered toxin profile can still be pathogenic. Although disruption of *Tri5* reduced pathogenicity of *G. pulicaris* on parsnip, it had no effect on its pathogenicity toward potato (Desjardins et al., 1992). Genes that control biosynthesis of toxins are often clustered but not all genes in the cluster are involved in pathogenicity. HC-toxin biosynthesis genes in *Cochliobolus carbonum* are clustered within a locus of 600 kb. There are several of these genes which are essential for toxin biosynthesis and pathogenicity including, *HTS1*, which encodes a peptide synthase, *TOXC*, which encodes a fatty acid synthase subunit, and *TOXF,* which encodes a putative branched chain amino acid transaminase (Panaccione et al., 1992; Ahn & Walton, 1997; Cheng et al., 1999). On the other hand *TOXG* which encodes an alanine racemase protein, and the TOXEp regulatory protein, which controls the expression of *TOXC* and two other toxin biosynthesis genes, do not appear to be essential for toxin biosynthesis and pathogenicity (Ahn & Walton, 1998; Cheng & Walton, 2000).

Inverse gene-for-gene interactions account for some plant hosts displaying susceptibility to certain pathogenic fungi. This has been observed in relation to host-specific toxins (HSTs) (Friesen et al., 2010). HSTs are pathogen effectors that induce toxicity and promote disease only in a host species and only in genotypes of that host expressing a specific and often dominant susceptibility gene. HST pathogens include *Stagonospora nodorum* and species of *Cochliobolus*, *Alternaria* and *Pyrenophora* (Friesen et al., 2010). ToxA is a HST produced by *Pyrenophora tritici* f. sp. *repentis* (Cuiffetti et al., 2010) and *S. nodorum* (Friesen et al., 2006). It acts as a necrotrophic effector, and has an inverse gene-for-gene interaction with wheat lines that have the *Tsn1* susceptibility gene. ToxA was confirmed as a pathogenicity gene after testing. Transformation of *P. tritici* ToxA– isolates with the ToxA gene produced fungi that were pathogenic on wheat with *Tsn1* (Cuiffetti et al., 1997). Disruption of the gene encoding ToxA in *S. nodorum* produced fungi that were non-pathogenic on wheat with *Tsn1* (Friesen et al., 2006).

1.5.3 Fungal infection structures

For the majority of pathogens, the formation of infection structures such as appressoria, penetration hyphae and infection hyphae and their associated haustoria is imperative for successful pathogenesis.

The disruption of the *MPG1* hydrophobin gene in *Magnaporthe grisea* resulted in reduced pathogenicity on rice and was essential for appressorium formation (Talbot et al., 1993), although it also resulted in a 100-fold reduction in conidiation. The disruption of another gene in *M. grisea*, the *ACR1* (arcopetal) gene, also resulted in reduced pathogenicity, due to impaired appressorium formation (Lau & Hamer, 1998), although it also resulted in defective conidial morphogenesis.

Melanin is essential for host cell wall penetration in some fungi. Appressoria use turgor pressure to penetrate cell walls and melanin in the appressorial wall prevents the glycerol utilized to generate the pressure from leaking out. Melanin-deficient mutants of *M. grisea* are unable to generate turgor pressure and are non-pathogenic (Chumley & Valent, 1990). Melanin is also essential for the successful penetration into host plants by *Colletotrichum lagenarium* (Kubo et al., 1982), *C. lindemuthianum* (Wolkow et al., 1983) and *C. graminicola* (Rasmussen & Hanau, 1989). *In C. lagenarium*, at least three structural genes are involved in melanin biosynthesis and are essential for pathogenicity, *PKS1* (Takano et al., 1995), *SCO1* (Kubo et al., 1996) and *THR1* (Perpetua et al., 1996).

1.5.4 Degradation of plant host cell walls

To penetrate plant cells, many fungi produce enzymes that degrade cell walls and other physical barriers such as cutin and pectin. These include glucanases, xylanases, pectinases and cutinases, amongst others. Determining the involvement of these enzymes in pathogenicity can be difficult to prove because they are often encoded by multigene families or by more than one unrelated gene (Idnurm & Howlett, 2001). Therefore, if one gene is disrupted another may mask its inactivity. Cutinases degrade cutin and may be needed to penetrate the host surface during pathogenesis. The disruption of a cutinase gene in *Fusarium solani* f.sp. *pisi* resulted in fungi that were non-pathogenic on pea (Rogers et al., 1994). Pectinases degrade pectin and may be needed to penetrate cell walls or middle lamellae. In *F. solani* f. sp. *pisi,* the disruption of either the pectin-inducible *pel*A pectate lyase gene, or the plant- inducible *pel*D pectate lyase gene resulted in fungi that were still pathogenic. However, the disruption of both genes resulted in fungi with reduced pathogenicity on pea (Rogers et al., 2000). The disruption of the *pel*B pectate lyase gene in *Colletotrichum gloeosporioides* resulted in fungi with reduced pathogenicity on avocado fruits (Yakoby et al., 2001).

1.5.5 Fungal signaling genes

Signalling genes are involved in all aspects of fungal growth. Pathogen recognition of the host triggers essential signalling cascades which influence the expression of genes involved in pathogenesis. Unfortunately, due to the pleiotropic effects of these genes it is rare that housekeeping or reproductive genes are not affected in some way when a signalling gene is deleted or silenced. There are exceptions. The *Cut2* (Cutinase2) gene of *Magnaporthe oryzae* is involved in surface recognition, germling differentiation, appressorial development, host penetration and full virulence (Skamnioti & Gurr, 2007). The Cut2 protein has been proposed to be an upstream activator of cyclic AMP/protein kinase A and diacylglycerol protein kinase C signaling pathways that ultimately direct appressorium formation and infectious growth. The *SGE1* (SIX Gene Expression 1) gene of *Fusarium oxysporum* encodes the transcription factor Sge1 which is essential for pathogenicity (Michielse et al., 2009). Disruption of *SGE1* results in a loss of pathogenicity with no affect on vegetative growth. It is not required for root colonization and penetration, but is required for parasitic growth. Sge1 is also required for expression of genes encoding effectors that are secreted during infection.

1.6 Experimental aim

The general aim of this study was to identify pathogenicity genes in *Thielaviopsis basicola* cotton isolates. The specific objectives of this study were to use random insertional mutagenesis to produce *T. basicola* mutants (transformants) that displayed reduced pathogenicity or disease severity on cotton (*Gossypium hirsutum*), and to identify the inactivated gene/s in these transformants that would have originally been involved in pathogenicity but did not act as housekeeping genes.

1.7 Experimental strategy

Random insertional mutagenesis can be used to identify unknown genes in a genome by utilizing the recombinant DNA technique of insertional inactivation. Insertional inactivation involves the disruption of the expression of a gene by inserting a fragment of DNA into its coding sequence, thus inactivating it. Randomly disrupting gene expression can assist in finding genes of interest when the phenotype of interest, in this case pathogenicity, is tested in mutants.

For this to be achieved, an efficient transformation system was required to generate mutants. In 2007, Samiya Al-Jaaidi had developed the first and only protocol for the polyethylene glycol (PEG)-mediated transformation of *T. basicola*. This study attempted to use the PEG-mediated transformation protocol that had been developed. The strategy involved:

- Preparation of plasmid DNA vectors
- Preparation of *T. basicola* protoplasts
- PEG-mediated transformation of protoplasts with plasmid DNA vectors
- Southern blot analysis of transformant genomic DNA
- Pathogenicity testing of transformants
- Internal transcribed spacer sequence comparison
- Plasmid rescue and genetic analysis

1.7.1 Preparation of plasmid DNA vectors

The first requirement for transformation was a DNA vector to disrupt the *T. basicola* genome and assist in the identification of transformants with selection markers. The plasmid pGpdGFP was used by Al-Jaaidi (2007), and it was decided that the same plasmid should be used in this study given its previous success. There was also interest in the use of a plasmid vector of smaller size to determine what effect it might have on transformation frequencies, success of plasmid rescue and ease of later genetic analysis. To examine these effects the plasmid pGpdR was developed.

1.7.2 Preparation of *Thielaviopsis basicola* protoplasts

For transformation of *T. basicola* to occur protoplasts were required. Fungal protoplasts are fungal cells which have had their cell walls removed, which facilitates the passage of the DNA vector into the fungal cell for transformation. Protoplasts can be derived from mycelia or conidia. Endoconidia were utilized because they are generally uninucleate (Huang & Patrick, 1974), thus simplifying genetic purification of transformants. Preparation was achieved with cell wall degrading enzymes and conditions outlined by Al-Jaaidi (2007).

1.7.3 Polyethylene glycol (PEG)-mediated transformation

PEG-mediated transformation is defined by its use of PEG and calcium chloride. The commonly accepted effect of PEG is to cause treated protoplasts and DNA to aggregate via the volume-exclusion effect and this may facilitate the trapping of DNA (Louie & Serwer, 1994). Although, it has been proposed that the role of PEG is most likely to function after transforming DNA is incorporated into protoplasts rather than the accepted view that it functions outside of the cell (Kuwano et al., 2008). The role of calcium chloride in fungal transformations is not fully understood, but it has been shown to promote the effect of PEG (Fincham, 1989). Transformation frequency can be limited if concentrations of PEG or calcium chloride are too high or too low. Transformation frequency is also dependent on the concentrations and ratio of protoplasts to DNA, and the length of time and temperature of incubation. Finally, successful regeneration of the protoplasts is required to maintain high transformation frequencies. (Wei et al., 2010)

The protocol for PEG-mediated transformation of *T. basicola* by Al-Jaaidi (2007) had originally reported an average transformation frequency of 2.5 stable transformants/µg of plasmid DNA. Since its development there have been attempts by others to replicate the protocol, without any success. Given its original success, it was assumed that failure to replicate the protocol was possibly due to the inexperience of those attempting it. This unexpectedly resulted in an alternative experimental objective, which was to attempt to replicate the protocol for PEG-mediated transformation of *T. basicola* by Al-Jaaidi (2007) and determine whether it was a valid tool for the transformation of *T. basicola*.

1.7.4 Southern blot analysis of transformant genomic DNA

Southern blot analysis was used to confirm that transformation with the desired plasmid DNA vector had been achieved. It was also used to determine the number of integrations and how the plasmid DNA may have been integrated into the genome.

1.7.5 Pathogenicity testing of transformants

Pathogenicity testing was performed on cotton seedlings (*G. hirsutum*) to identify transformants that displayed reduced black root rot severity and therefore pathogenicity. Idnurm and Howletts (2001) definition of pathogenicity genes states that genes essential for the pathogen to complete its life cycle *in vitro* cannot be classed as pathogenicity genes, but that this definition could not be applied to obligate parasites. Although *T. basicola* is regarded as an obligate parasite, it is capable of completing its life cycle *in vitro* when incubated on or in potato dextrose media. For the purposes of this study, pathogenicity genes are defined as genes necessary for disease development, but are not identified as housekeeping genes and do not significantly influence the 'normal' growth or morphology when compared to the original organism.

1.7.6 Internal transcribed spacer (ITS) sequence comparison

The ITS region is a non-functional RNA sequence situated between structural ribosomal RNAs on a common precursor transcript. It is commonly used in molecular phylogeny because of the high degree of variation between closely related organisms. Previous ITS sequence analysis performed on *T. basicola* has indicated that all isolates from a particular host have identical ITS sequences, but they differ between isolates from different hosts (Coumans et al., 2011). ITS sequences of putative transformants were compared to the original *T. basicola* isolate to ensure that they were derived from the original isolate and not from another source.

Chapter 2: Materials and Methods

(Appendix 1. provides composition of media)

2.1 Fungal strains

The fungal strain used for fungal PEG-mediated transformations was *Thielaviopsis basicola* isolate BRIP40192, isolated from cotton (*Gossypium hirsutum*), provided by Jan Dean, Department of Primary Industries, Queensland Government, Australia.

The *T. basicola* transformants: p16; p737; p849; p888; and p954, were derived from *T. basicola* BRIP40192 by Samiya Al-Jaaidi (2007), and provided by Lily Pereg, School of Science and Technology, University of New England, Australia. These transformants have previously displayed reduced disease severity on cotton and were used for comparison with transformants from this study during pathogenicity testing.

Working cultures of *T. basicola* were incubated for 3 days or more at 25°C on ½ PDA (1.2 – 2.2% agar) plates and stored at 4°C until required. Stock cultures of *T. basicola* were preserved as mycelial agar blocks in sterile distilled water (dH_2O) at room temperature or in 10% glycerol at -70°C.

2.2 Bacterial strain and competent cell preparation

The bacterial strain used for bacterial transformations and plasmid storage was *Escherichia coli* strain DH5α. Working cultures of *E. coli* DH5α were incubated overnight at 37°C on 2 × YT (1.6% agar) plates and stored at 4°C until required.

E. coli DH5α competent cells were required for bacterial transformations. A single *E. coli* DH5α colony was used to inoculate 10 mL of YT broth and incubated overnight at 37°C and 200 rpm. An aliquot of 2 mL of *E. coli* DH5α overnight-culture was added into 100 mL of YT broth supplemented with 1 mL of 1 M KCl and 2 mL of 1 M $MgSO_4$. This was incubated at 37°C and 200 rpm to an A_{660} of 0.45 – 0.55. The culture was transferred to 50 mL sterile tubes and cooled on ice for 20 min and then centrifuged for 10 min at 3200 × *g* and 4°C. The pellets of *E. coli* DH5α cells were resuspended in ice-cold 50 mM $CaCl_2$ and incubated on ice for 15 min, and then centrifuged for 10 min at 3200 × *g* and 4^{o}C. Finally, the pellets of *E. coli* DH5α competent cells were resuspended in ice-cold 50 mM $CaCl_2$ / 15% glycerol and 100 µL aliquots were stored at -70^{o}C.

2.3 The plasmid vectors: pGpdGFP and pGpdR

The plasmid vectors used for fungal PEG-mediated transformations were pGpdGFP (6.93 kb) (Sexton & Howlett, 2001) and pGpdR (~5.0 kb) (this work).

2.3.1 The plasmid vector, pGpdGFP

The plasmid pGpdGFP (Figure 2.1) was previously used by Samiya Al-Jaaidi (2007) for the development of PEG-mediated transformations of *T. basicola*. pGpdGFP was engineered by Dr. A. Andrianopoulos, Genetics Department, University of Melbourne, Australia (Sexton & Howlett 2001), and provided by Barbara Howlett, School of Botany, University of Melbourne, Australia. It is a modified pBluescript SK (-) plasmid, therefore containing and an ampicillin resistance gene (*Amp*R) that provided a selective marker for *E. coli* transformants, and, an origin of replication for *E .coli* (ori) which facilitated plasmid cloning. Inserted into its multiple-cloning-site (MCS) is the hygromycin phosphotransferase gene (*hph*) from *E. coli*, controlled by an *Aspergillus nidulans trpC* promoter (P*trpC*) and *A. nidulans trpC* terminator (T*trpC*). The *hph* gene provided a selective marker for *T. basicola* transformants by imparting resistance to hygromycin B, to which *T. basicola* is susceptible. Also inserted into its MCS is the green fluorescent protein gene (*egfp*) from *Aequorea victoria*, controlled by an *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter (P*gpdA*) and *A. nidulans trpC* terminator (T*trpC*). The *egpf* gene was not utilised in this study.

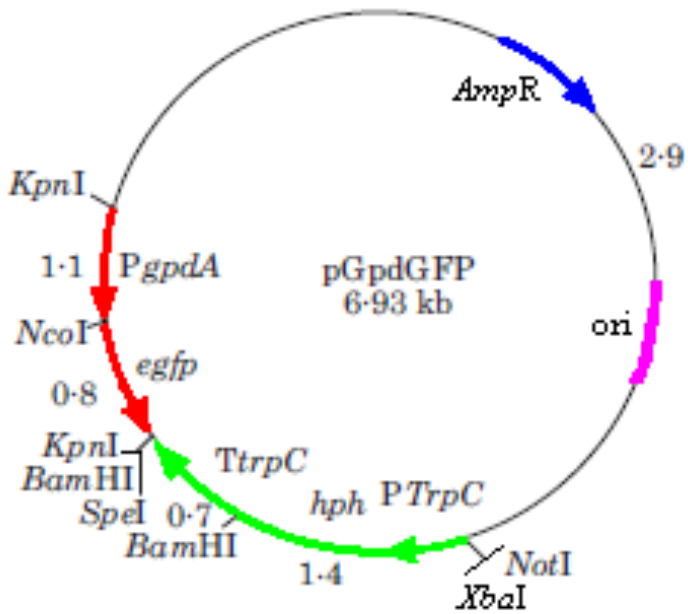


Figure 2.1) Plasmid map of pGpdGFP (6.93 kb)

Sections of interest include: the ampicillin resistance gene (*Amp*R); the origin of replication for *E. coli* (ori); the *trpC* promotor (P*trpC*); the hygromycin phosphotransferase gene (*hph*); the *trpC* terminator (T*trpC*); the green fluorescent protein gene (*egfp*); and the glyceraldehyde-3-phosphate dehydrogenase promoter (P*gpdA*). Restriction sites for some restriction endonucleases are also displayed showing approximate distances between them in kilobases.

2.3.2 Construction of the plasmid vector, pGpdR

The plasmid pGpdR was derived from pGpdGFP by removing the unutilized *egfp* gene and P*gpdA* promoter sequences (Figure 2.1: red sequences), thus producing a plasmid of approximately 5.0 kb in length that contained the *Amp*R, ori, *hph*, P*trpC* and T*trpC* sequences required for successful transformation and selection. *Kpn*I restriction sites flank the unutilized P*gpdA*/*egfp* region of pGpdGFP (Figure 2.1). The pGpdGFP plasmid was digested with the restriction endonuclease *Kpn*I (New England Biolabs). The composition of the digestion reaction followed the manufacturer's instructions and was incubated for 3 h at 37°C to ensure complete digestion, followed with heat inactivation for 20 min at 65°C. The resulting DNA fragments, of approximately 1.9 kb and 5.0 kb in length, were ligated with T4 DNA ligase (New England Biolabs). The composition of the ligation reaction followed the manufacturer's instructions and was incubated for 12 h at 16°C, followed with heat inactivation for 10 min at 65°C. The resulting solution contained a variety of plasmids which were used to transform *E. coli* DH5α (refer to section 2.4 for methods). Transformation using the unligated restriction digest solution was also performed as a negative control. Well separated single colonies of *E. coli* DH5α transformants selected on 2 × YT (1.6% agar) plates containing 100 µg/mL of ampicillin were randomly chosen and individually used to inoculate YT broths containing 100 µg/mL of ampicillin and incubated overnight at 37^{o}C and 200 rpm. Plasmid DNA (pDNA) was isolated from each culture using pDNA mini preparation (refer to section 2.5.1 for methods). A sample from each pDNA solution was digested with *Kpn*I. The compositions of the digestion reactions followed the manufacturer's instructions and were incubated for 1.5 h at 37^{o}C, followed with heat shock inactivation for 20 min at 65^{o}C. The *Kpn*I digested pDNA was analysed using agarose gel electrophoresis (refer to section 2.6 for methods). A pDNA solution that only contained the desired ~ 5.0 kb fragment corresponding to the expected size of the final plasmid was selected. The selected pDNA solution was used to transform *E. coli* DH5α (refer to section 2.4 for methods) and a well separated single colony of *E. coli* DH5α transformants was used to inoculate YT broth containing 100 µg/mL of ampicillin and incubated overnight at 37^{o}C and 200 rpm. The pDNA was isolated from the culture using pDNA midi preparation (refer to section 2.5.2 for methods). To confirm the presence of only the desired ~ 5.0 kb plasmid in the final pDNA solution, a sample was digested for analysis with *Kpn*I. The composition of the digestion reaction followed the manufacturer's instructions and was incubated for 1.5 h at 37^{o}C, followed with heat shock inactivation for 20 min at 65^{o}C. The *Kpn*I digested pDNA sample was analysed using agarose gel electrophoresis (refer to section 2.6 for methods) against pGpdGFP. pGpdGFP was linearized for analysis with the restriction endonuclease *Xba*I (New England Biolabs). The composition of the digestion reaction followed the manufacturer's instructions and was incubated for 1.5 h at 37^{o}C, followed with heat inactivation for 20 min at 65^{o}C.

2.4 Bacterial transformations

The plasmid vectors were amplified via the transformation of *E. coli* DH5α. 1 µL of plasmid solution was added to 100 µL of *E. coli* DH5α competent cells that were thawed on ice. This transformation solution was incubated for 20 min on ice, followed by 2 min of heat shock at 42^{o}C and 2 min of cooling on ice. 900 µL of YT broth was added and the solution was incubated for 30 min at 37^{o}C and 200 rpm. 100 µL of the culture was spread on 2 × YT (1.6% agar) plates containing 100 µg/mL of ampicillin to select for transformants. A negative control containing no pDNA was also performed. The plates were incubated overnight at 37°C. Plates containing colonies of *E. coli* DH5α transformed with pDNA were used immediately or stored at 4°C.

2.5 Plasmid DNA isolation

2.5.1 Mini preparation of plasmid DNA

pDNA were isolated from transformed *E. coli* DH5α cells using the miniprep protocol from the 'High Pure Plasmid Isolation Kit' (Roche Applied Science). A single recombinant *E. coli* DH5α colony transformed with either pGpdGFP or pGpdR was used to inoculate 10 mL of YT broth containing 100 μg/mL of ampicillin and incubated overnight at 37°C and 200 rpm. 4 mL of recombinant *E. coli* DH5α culture was processed according to the manufacturer's instructions. pDNA was eluted with 50 μL of elution buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C.

2.5.2 Midi preparation of plasmid DNA

Higher amounts of pDNA were isolated from transformed *E. coli* DH5α cells using the midiprep protocol from the 'Plasmid Midi Kit' (QIAGEN). A single recombinant *E. coli* DH5α colony transformed with either pGpdGFP or pGpdR was used to inoculate a 5 mL starter culture of YT broth containing 100 μg/mL of ampicillin and incubated for 8 h at 37°C and 200 rpm. 100 μL of starter culture was used to inoculate 100 mL of YT broth containing 100 μg/mL of ampicillin and incubated overnight at 37°C and 200 rpm. The recombinant *E. coli* DH5α culture was processed according to the manufacturer's instructions. pDNA was eluted with 200 μL of elution buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C.

2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments for analysis. DNA fragments expected to be greater than 1 kb in length were run against a 1 kb DNA ladder (New England Biolabs) on a 0.8 – 1.0% (w/v) agarose gel. DNA fragments expected to be less than 1 kb in length were run against a 100 bp DNA ladder (New England Biolabs) on a 1.3% (w/v) agarose gel. Genomic DNA (gDNA) was run against a λ DNA-*Hind*III digest (New England Biolabs) on a 0.8% (w/v) agarose gel. Gels were prepared using the appropriate amount of agarose in 30 mL of 1 × TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0) and heated in a microwave until the agarose had completely melted. This solution was then poured into a mini-gel mould and left to set for 20 min. DNA samples were prepared for loading onto the gel with the addition of 6 × gel loading dye (New England Biolabs). All gels were run at 80 – 100 V until gel loading dye was near the end of the gel. The gels were incubated in ethidium bromide (10 μg/mL of 1 × TAE) for 10 min and viewed and photographed under ultraviolet light with visualization equipment and the program 'Quantity One' (Bio-Rad).

2.7 Fungal protoplast preparation

Modified procedure based on protocol by Samiya Al-Jaaidi (2007).

T. basicola BRIP40192 cultures were incubated for 2 – 3 days at 25°C on fifteen ½ PDA (2.2% agar) plates. The plates were inoculated either as star plates or by using filtered endoconidial suspension to produce spread plates. The mycelia and spores of the *T. basicola* BRIP40192 wild-type cultures were scraped into 20 mL of 0.1% Tween 80 and vigorously vortexed for 1 min to free endoconidia from the mycelia and suspend them in solution. The suspension was filtered through a funnel containing a single layer of dried sterile Miracloth (Calbiochem) into 200 mL of PDB (Difco) in a 1 L flask to separate the endoconidia from mycelia and chlamydospores. The endoconidial suspension was incubated at 25°C and 120 rpm until germination of endoconidia could be viewed under a microscope (+2 h). Germination of endoconidia facilitates endoconidial cell wall degradation performed after washing. Germinating endoconidia were harvested with centrifugation for 8 min at 2500 × *g* and 4°C. The endoconidial pellet was resuspended and washed three times with ice-cold 0.6 M $MgSO_4.7H_2O$ and centrifugation for 8 min at 2500 × *g* and 4°C. The supernatant was poured off and excess removed with a pasture pipette. The wet weight of the endoconidial pellet was used to determine the amount of osmotic medium (1.2 M $MgSO_4$, 10 mM NaOP, pH 5.8) containing 5 mg/mL of Glucanex™ (Sigma-Aldrich: lysing enzymes from *Trichoderma harzianum*) required for endoconidial cell wall degradation to produce protoplasts (Table 2.1).

Table 2.1) Amount of cell wall degradation medium required according to wet weight of endoconidial pellet

Wet weight of endoconidial pellet	Amount of cell wall degradation medium required
0.10 – 0.25 g	25 mg of Glucanex / 5 mL of osmotic medium
0.30 – 0.50 g	50 mg of Glucanex / 10 mL of osmotic medium
0.60 – 1.00 g	75 mg of Glucanex / 15 mL of osmotic medium

The endoconidial pellet was resuspended in the required amount of cell wall degradation medium and incubated at 30°C and 120 rpm until protoplasts could be easily viewed under a microscope (+2 h). Occasionally the flask was manually swirled to bring endoconidia and protoplasts off its sides to improve the efficiency of the incubation. The cell wall degradation medium was then cooled on ice and equal volumes were dispensed into two sterile corex tubes. The flask was rinsed with 4 – 8 mL of osmotic medium and equal volumes were added to the two corex tubes. After this point protoplasts were kept on ice. The medium in each corex tube was gently overlayed with ice-cold trapping buffer (0.6 M sorbitol, 10 mM Tris-HCl, pH 7.0) and swing-bucket centrifuged for 30 min at 3200 × *g* and 4°C to trap protoplasts and separate them from remaining endoconidia and other debris. The protoplast bands, which formed at the interface of the trapping buffer and osmotic medium, were transferred with a sterile 5 mL pipette into a single ice-cold corex tube containing 10 mL of ice-cold 1 × STC (1.2 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5) and centrifuged for 5 min at 5000 × *g* and 4°C. The protoplast pellet was resuspended and washed two times with 10 mL of ice-cold 1 × STC and centrifugation for 5 min at 5000 × *g* and 4°C to remove contaminants such as nucleases. The final protoplast pellet was resuspended in 4 mL of ice-cold 1 × STC. The concentration of protoplasts was estimated using a Neubauer counting chamber. The protoplast concentration was adjusted to produce 150 µL aliquots containing 2×10^6 protoplasts in ice-cold 1 × STC and kept on ice for immediate use.

Preparation of true protoplasts, as opposed to spores, was initially tested by adding sterile dH_2O to protoplasts while viewing under a microscope. True protoplasts lyse in dH_2O. Secondly, protoplast aliquots were diluted 1:1000 with ice-cold 1 × STC or sterile dH_2O and spread on regeneration solid media (½ PDA (1% agar) plates containing 1 M sucrose) and incubated for 2 weeks at 25°C.

2.8 Fungal polyethylene glycol (PEG) – mediated transformations

Modified procedure based on protocol by Samiya Al-Jaaidi (2007).

Protoplasts were kept on ice. The volume of pDNA in elution buffer (10 mM Tris-HCl, pH 8.5) required to provide 10 µg of pDNA (pGpdGFP or pGpdR) was determined. The required volume of pDNA solution (10µg of pDNA) and an equal volume of ice-cold 2 × STC (2.4 M sorbitol, 20 mM Tris-HCl, 20 mM $CaCl_2$, pH 7.5) were added to 150 µL aliquots containing 2×10^6 protoplasts in ice-cold 1 × STC (1.2 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5) and adjusted to a total volume of 200 µL with ice-cold 1 × STC (Table 2.2). Negative controls lacked pDNA. These transformation solutions were incubated on ice for 5 min.

Table 2.2) Composition of transformation solutions

Transformation solution	Protoplasts (2×10^6) in 1 × STC	pDNA (10 µg)	2 × STC	1 × STC	PEG (first incubation)	PEG (second incubation)
1	150 µL	25.0 µL	25.0 µL	0 µL	100 µL	1 mL
2	150 µL	10.5 µL	10.5 µL	29 µL	100 µL	1 mL
3 (control)	150 µL	0.0 µL	0.0 µL	50 µL	100 µL	1 mL

The composition of transformation solutions and examples of volumes required. Note that 25 µL is the maximum volume of pDNA that can be used. Transformation solution 3 is the negative control which lacks the addition of pDNA.

100 µL of PEG (60% (w/v) PEG 4000, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) was added to each transformation solution, mixed gently by inverting and incubated on ice for 20 min or 40 min. An additional 1 mL of PEG was added to each transformation solution, mixed gently by inverting and incubated at room temperature for 15 min or 30 min. The transformation solutions were centrifuged for 5 min at 20,000 × *g* and the PEG supernatant was removed. The protoplast pellets were resuspended and washed two times with1 ml of ice-cold 1 × STC and centrifugation for 5 min at 20,000 × *g*. The protoplasts were resuspended in 200 µL of ice-cold 1 × STC and 1.3 mL of PDB (Difco) / 0.5 M sucrose and incubated for 4 h at 25°C and 120 rpm to regenerate them. Finally, 50 µL aliquots of the regenerated protoplasts were spread onto ½ PDA (1.2% agar) plates containing 25 µg/mL of hygromycin B for selection of *T. basicola* transformants and incubated for a minimum of 2 weeks at 25°C in the dark, to reduce degradation of the light-sensitive hygromycin B.

Large dark colonies of putative *T. basicola* transformants were transferred onto ½ PDA (1.2% agar) well plates containing 100 µg/mL of hygromycin B for selection and incubated for 3 days at 25^{o}C. Surviving putative *T. basicola* transformants were streaked onto ½ PDA (2.2% agar) plates containing 25 μg/mL of hygromycin B and incubated at 25^{o}C in order to obtain single separated colonies.

2.9 Mitotic stability testing of putative fungal transformants

Putative *T. basicola* transformants were deemed mitotically stable if they maintained hygromycin B resistance after seven generations of being subcultured on non-selective media. The putative *T. basicola* transformants, and a *T. basicola* BRIP40192 wild-type control, were subcultured onto ½ PDA (1.2% agar) plates lacking selective antibiotics and incubated for 4 – 5 days at 25^{o}C for seven generations. Each generation these plates were replicated on ½ PDA (1.2% agar) plates containing 100 μg/mL of hygromycin B to test for hygromycin B resistance conferred to the fungi by the pDNA insert. Putative transformants that lost the hygromycin B resistance phenotype were deemed mitotically unstable.

2.10 Extraction of fungal genomic DNA

The *T. basicola* BRIP40192 wild-type and putative *T. basicola* transformants were incubated for 5 days at 25^{o}C on ½ PDA (2.2% agar) plates, containing 25 μg/mL hygromycin B where necessary. Each culture was scraped into 200 mL of PDB (Difco) in a 1 L flask, containing 25 μg/mL hygromycin B where necessary, and incubated for a further 5 days at 25^{o}C and 120 rpm. Then each culture was filtered through dried sterile Miracloth (Calbiochem) and rinsed with ice-cold sterile milliQ H_2O. The mycelia remaining in the Miracloth was blot dry with absorbent paper, wrapped in aluminium foil, placed in liquid nitrogen for 5 min and freeze-dried overnight. 100 mg of dried mycelia was weighed out from each sample and ground to a fine powder with a Tissuelyzer. 1 mL of 10 × TES buffer (0.1 M Tris-HCl, 10 mM EDTA, 2% SDS, pH 8.5) was added to each ground sample, mixed gently, and incubated for 1 h at 65^{o}C, mixing occasionally to extract the genomic DNA (gDNA) into solution. The samples were centrifuged for 5 min at 14,000 rpm and the supernatant was retained. 200 μL of ice-cold 5 M potassium acetate was added to the samples, mixed gently, and incubated on ice for 1 h to precipitate proteins. The samples were centrifuged for 15 min at 14,000 rpm and the supernatant was retained. An equal volume of isopropanol was added to the samples, gently mixed, and incubated on ice for 45 min to precipitate the gDNA. The samples were centrifuged for 2 min at 14,000 rpm, the isopropanol was discarded, and the gDNA pellets were air dried. The pellets were washed twice with -20°C 70% ethanol, by gently mixing (inverting), centrifugation for 1 min at 14,000 rpm and air drying for 15 min both times. The gDNA pellets were resuspended in 400 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). 10 µL of RNAse A (10 µg/mL) was added to remove RNA. The DNA was fully dissolved with overnight incubation at 4°C. The concentration of gDNA in each sample was determined with agarose gel electrophoresis (refer to section 2.6 for methods). The dissolved gDNA samples were stored at -20°C.

2.11 Southern blot hybridization

2.11.1 Synthesis of DIG-labelled DNA probe

The DNA probe used for southern blot hybridization consisted of a DIG-labelled hygromycin phosphotransferase gene (*hph*) sequence, which would hybridise to the *hph* gene found in both pGpdGFP and pGpdR inserts, but not hybridise to other sites on the *T. basicola* genome. The probe was synthesised using the 'PCR DIG Probe Synthesis Kit' (Roche Applied Science) according to the manufacturer's instructions. 100 pg of pGpdGFP was used as template DNA for the PCR. The primers used were HYGF-1 (5' – GAT GTA GGA GGG CGT GGA TA) and HYGR-1 (5' – CGT CTG CTG CTC CAT ACA AG), which were developed and provided by Getachew Mohammed Ali, School of Science and Technology, University of New England, Australia. The expected size of the probe was 621 bp. Agarose gel electrophoresis was used to determine if the probe had been successfully synthesised (refer to section 2.6 for methods).

2.11.2 Restriction digests of fungal genomic DNA

Restriction digests consisted of 1 µg of gDNA from the *T. basicola* BRIP40192 wild-type or putative *T. basicola* transformants and the restriction endonucleases *Xba*I, *Nru*I or *Nhe*I-HF ™ (New England Biolabs). *Xba*I has a unique restriction site within both pGpdGFP and pGpdR (Figure 2.1), whereas *Nru*I and *Nhe*I do not cut within these plasmid vectors. The 25 µL digestion reactions contained double amount of endonuclease recommended by the manufacture for the digestion of 1 µg of DNA and were incubated for approximately 23 h at 37°C to ensure complete digestion, but in all other respects digestion reactions followed the manufacturer's instructions. The pGpdGFP vector was digested with *Xba*I. The composition of that digestion reaction followed the manufacturer's instructions and was incubated for 1.5 hr at 37^{o}C. All digests were inactivated with the addition of 6 × gel loading dye (New England Biolabs).

2.11.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate fragments of digested gDNA of putative *T. basicola* transformants. This was performed using 0.8% (w/v) agarose gel and run at 38 V for 16 h against a λ DNA-*Hind*III digest and 1 kb DNA ladder (New England Biolabs). Also run on the gels were the respective *T. basicola* BRIP40192 wild-type gDNA digests, acting as a negative control during hybridization, and 5 ng of linearized pGpdGFP, acting as a positive control during hybridization. The gels were incubated in ethidium bromide (10 µg/mL of 1 × TAE) for 30 min and viewed and photographed under ultraviolet light with visualization equipment and the program 'Quantity One' (Bio-Rad), to assess if gDNA had been completely digested.

2.11.4 Transfer of DNA from agarose gel to nylon membrane

Gels were pre-treated for transfer of DNA. Two washes with 0.25 M HCl, for 15 min each, depurinated the DNA fragments. Two washes with 0.5 M NaOH / 1 M NaCl, for 15 min each, denatured the DNA to facilitate hybridization of the probe. Finally, two washes with 0.5 M Tris-HCl / 1 M NaCl (pH 7.4), for 15 min each, equilibrated the pH of the gel. The gel was rinsed for 2 min in distilled H_2O after each set of washes.

The DNA was transferred from the agarose gel to a positively-charged nylon membrane (Roche Applied Science). A glass plate was placed across a glass dish. 3 MM blotting paper (Whatman), three sheets thick, was placed over the plate into the dish to be used as wick, and 20 × SSC (3 M NaCl, 300 mM trisodium citrate, pH 7.0) was placed in the dish. The gel was placed on the wet 3 MM paper wick. The nylon membrane was wet with distilled H_2O followed by 20 × SSC and placed flush on the gel. At each step bubbles were rolled out. Three sheets of 3 MM paper were placed on top the membrane and weighted paper towels on top of that. The DNA passed from the gel, via capillary action, to the membrane. The DNA couldn’t pass through the positively-charged nylon membrane but the medium could. Transfer was allowed to proceed for 4.5 h. After transfer, the nylon membrane was placed between two pieces of 3 MM paper covered in foil and baked overnight at 70 – 80^{o}C to bind the DNA to the membrane.

2.11.5 Hybridization of the DNA probe

Hybridization solution (50% formamide, 5 × SSC, 2% blocking solution, 0.1% Na-N-laurylsarcosine, 0.02% SDS) was freshly prepared. The nylon membrane was pre-hybridized in a hybridization bag with 20 mL of hybridization solution/100 cm^2 of membrane and incubated for 6 h at 37°C.

The DIG-labeled DNA probe was boiled for 10 min to denature the DNA to facilitate hybridization and immediately transferred to ice-water for a further 5 min. The pre-hybridization solution was removed from the hybridization bag. 1 μL of DIG-labeled DNA probe/1 mL of hybridization solution was used to produce a probe mixture to a total volume of 10 mL of probe mixture/100 cm^2 of membrane. The required probe mixture was added to the hybridization bag containing the membrane and incubated overnight at 37°C.

2.11.6 Washing nylon membrane and detection of DNA probe

The nylon membrane was washed at 68°C in 2 × SSC / 0.1% SDS two times for 5 min and 0.1 × SSC / 0.1% SDS two times for 15 min to remove unhybridized DNA probe. This was followed by further washing at room temperature for 1 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.5). The membrane was then incubated with gentle rocking at room temperature for 30 min in buffer 2 (1% (w/v) blocking reagent (Roche Applied Science) in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) to decrease background in the southern blot.

The membrane was incubated with gentle rocking at room temperature for 30min in 50 mL of buffer 2 containing 10 μL anti-DIG-AP conjugate (Roche Applied Science) which binds to the DIG-labeled DNA probe. The membrane was washed two times for 15 min in washing buffer, followed with incubation for 5 min in buffer 3 (0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5) to equilibrate the membrane. CSPD (Roche Applied Science) was diluted 1:100 in buffer 3. The membrane was put into a hybridization bag and 1 mL of diluted CSPD was massaged into the membrane and the excess removed. The alkaline phosphatase (AP) cleaves CSPD and this reaction produces light that can be detected by x-ray film. The sealed hybridization bag was placed in a pre-warmed film cassette and x-ray film was placed over the membrane. Film exposure time varied. Finally, the x-ray film was developed and scanned for analysis.

2.12 Pathogenicity testing of fungal transformants

2.12.1 Cotton seeds and surface sterilization

The cotton seeds used for pathogenicity testing were *Gossypium hirsutum*, cultivar SICOT 189 BR, provided by the Cotton Catchment Communities Cooperative Research Centre, Australian Cotton Research Institute, Narrabri, Australia.

G. hirsutum seeds were surface sterilized with soaking in sterile dH_2O for 5 min followed by soaking in sterilizing solution (12.5% 4%-bleach, 77.5% dH_2O, 10% ethanol) for 5 min and rinsed eight times with sterile dH_2O.

2.12.2 Inoculum production

T. basicola transformants, and *T. basicola* BRIP40192 wild-type controls, were incubated for 5 days at 25°C on ½ PDA (2.2% agar) plates lacking selective antibiotics under a 12 h light/12 h dark cycle to stimulate spore production. The mycelia and spores were scraped into sterile dH_2O and vigorously vortexed for 1 min to free endoconidia from the mycelia and suspend them in solution. The suspension was filtered through dried sterile Miracloth (Calbiochem) to separate the endoconidia from mycelia and chlamydospores. The concentration of endoconidia was estimated using a Neubauer counting chamber and adjusted to the required concentration for the final *T. basicola* endoconidial suspension (inoculum).

2.12.3 Pathogenicity testing using dipping technique

Surface sterilized *G. hirsutum* seeds were germinated on YMA plates at 25°C under a 12 h light/12 h dark cycle to a radicle (root) length of 1.5 cm. Nine seedlings were exposed to each inoculum or control. The seedling radicles were dipped into a *T. basicola* endoconidial suspension (3.5×10^5 endoconidia/mL of dH_2O) for 1 min. Inoculation with *T. basicola* BRIP40192 wild-type inoculum acted as a positive control. Inoculation with sterile dH_2O acted as a negative control. Inoculated and control seedlings were grown on water agar (1.2% agar) plates at 25°C under a 12 h light/12 h dark cycle. Roots were examined for the presence of lesions over 5 days. The presence of chlamydospores protruding from lesions was examined under a stereomicroscope, which indicated whether lesions were likely to have been caused by *T. basicola*. The length of root affected by lesions and total root length were measured. Disease severity was expressed as the percentage of the total root length affected by lesions. *T. basicola* transformants that displayed reduced disease severity, when compared to the *T. basicola* BRIP40192 wild-type, were further tested in soil.

2.12.4 Pathogenicity testing in soil

The soil used consisted of 50% black cracking clay (from Narrabri, NSW, Australia) and 50% sand that was sterilized three times by autoclaving for 20 min at 121^{o}C with 24 h between sterilizations. The sterile soil was inoculated with *T. basicola* endoconidial suspension to a final concentration of 5000 endoconidia/mL of soil and thoroughly mixed to ensure uniform dispersion of endoconidia throughout the soil. Inoculation with *T. basicola* BRIP40192 wild-type inoculum acted as a positive control. Inoculation with sterile dH_2O acted as a negative control. Ten seeds were exposed to each inoculum or control. Surface sterilized *G. hirsutum* seeds were planted near the surface of 100 mL of inoculated or control soil, watered with 10 mL of sterile dH_2O, and incubated at 25^{o}C under a 12 h light/12 h dark cycle for 13 days. The seedlings were watered every 2 days with 10 mL of sterile dH_2O. On day 13, seedlings were carefully removed from the soil and their roots were washed in sterile dH_2O. Roots were examined for the presence of lesions. The presence of chlamydospores protruding from lesions was examined under a stereomicroscope, indicating whether lesions were likely to have been caused by *T. basicola*. The length of root affected by lesions and total root length were measured. Disease severity was expressed as the percentage of the total root length affected by lesions.

2.13 Internal transcribed spacer sequence comparison

The internal transcribed spacer (ITS) sequences of *T. basicola* transformants that underwent pathogenicity testing in soil were compared to the ITS sequence of *T. basicola* BRIP40192 wild-type. PCR was used to amplify the ITS sequences. *T. basicola* gDNA stock solutions were diluted 1:10 with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 1µL was used as template DNA for the PCR. 100 pg of pGpdGFP template was used as a negative control. A second negative control contained no DNA. The primers used were ITS1 (5’ – TCC GTA GGT GAA CCT GCG G) and ITS4 (5’ – TCC TCC GCT TAT TGA TAT GC), which were provided by Lily Pereg, School of Science and Technology, University of New England, Australia. The 25 µL PCR reactions contained: 15.5 µL of PCR-grade H_2O (Sigma-Aldrich); 25 µL of 10 × Immo Buffer™ (Bioline); 7.5 µL of 50 mM $MgCl_2$; 25 µL of 10 mM dNTP mix; 12.5 µL of 10 µM ITS1; 12.5 µL of 10 µM ITS4; 1 µL of template DNA; and 2.5 µL of Immolase™ DNA polymerase (Bioline). PCR was carried out with 10 min of Immolase™ DNA polymerase activation at 95°C, followed with 30 cycles of amplification (1 min denaturation at 95°C, 45 s annealing at 58°C and 1.5 min extension at 72°C) and 10 min of final extension at 72°C. The expected size of the PCR product was approximately 600 bp. Agarose gel electrophoresis was used to determine if the sequences had been successfully amplified (refer to section 2.6 for methods). PCR products were purified using the PCR purification spin protocol from the 'MinElute PCR Purification Kit' (QIAGEN). 25 µL of PCR reaction was processed according to the manufacturer's instructions and eluted with 20 µL of PCR-grade H_2O. The purified PCR products were sequenced by the Australian Genome Research Facility Ltd. The samples for sequencing were prepared according to their instructions with the ITS1 primer included. Sequences were compared to those in GenBank with a BLASTN search, which was provided by the Australian Genome Research Facility Ltd. Sequences from the transformants were aligned and compared to that of *T. basicola* BRIP40192 wild-type using FASTA DNA:DNA sequence comparison software (FASTA Sequence Comparison at the University of Virginia – UVa FASTA Server: http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=compare).

2.14 Determining the presence of intact ampicillin resistance genes

The presence of an intact ampicillin resistance gene (*Amp*R) was tested on gDNA from *T. basicola* transformants that displayed reduced pathogenicity towards cotton seedlings in soil. PCR was used to amplify intact *Amp*R sequences. *T. basicola* gDNA stock solutions were diluted 1:10 with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 1µL was used as template DNA for the PCR. A *T. basicola* BRIP40192 gDNA template was used as a negative control. A second negative control contained no DNA. 100 pg of pGpdGFP template was used as a positive control. The primers used were AmpF1 (5' – GTG TCG CCC TTA TTC CCT TT) and AmpR1 (5' – GGC ACC TAT CTC AGC GAT CT), which were developed and provided by Getachew Mohammed Ali, School of Science and Technology, University of New England, Australia. The 25 µL PCR reactions contained: 15.5 µL of PCR-grade H_2O (Sigma-Aldrich); 25 µL of 10 × Immo Buffer™ (Bioline); 7.5 µL of 50 mM $MgCl_2$; 25 µL of 10 mM dNTP mix; 12.5 µL of 10 µM AmpF1; 12.5

μL of 10 μM AmpR1; 1 μL of template DNA; and 2.5 μL of Immolase™ DNA polymerase (Bioline). PCR was carried out with 10 min of Immolase™ DNA polymerase activation at 95^{o}C, followed with 30 cycles of amplification (1 min denaturation at 95^{o}C, 45 s annealing at 58^{o}C and 1.5 min extension at 72^{o}C) and 10 min of final extension at 72^{o}C. The expected size of the PCR product was 891 bp. Agarose gel electrophoresis was used to determine if the sequences had been successfully amplified (refer to section 2.6 for methods).

Chapter 3: Results

3.1 The plasmid vector, pGpdR

A plasmid of approximately 5.0 kb in size (Figure 3.1) was successfully derived from the digestion of pGpdGFP with the restriction endonuclease *Kpn*I. This was the expected size of the plasmid after the removal of the unutilized P*gpdA*/*egfp* region from pGpdGFP. This plasmid was named pGpdR.

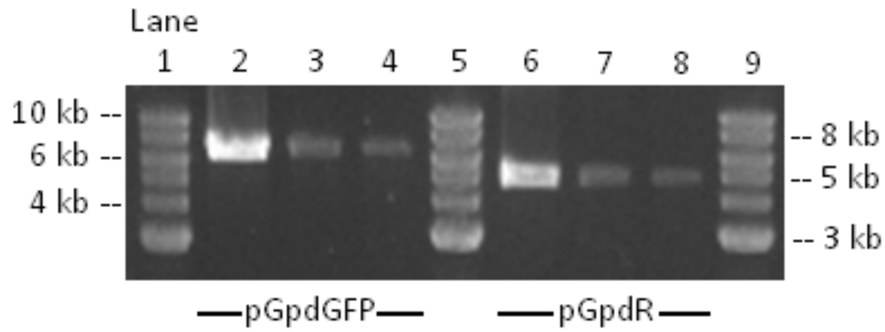


Figure 3.1) Analytical gel of the linearized pGpdGFP and pGpdR plasmids after midiprep

Lanes 2, 3 and 4 contain pGpdGFP (6.93 kb) digested with *Xba*I that displayed bands of ~ 7.0 kb in size. Lanes 6, 7 and 8 contain pGpdR digested with *Kpn*I that displayed bands of ~ 5.0 kb in size. Lanes 1, 5 and 9 contain a 1 kb DNA ladder that was run against the linearized plasmids.

3.2 *Thielaviopsis basicola* protoplast yields

T. basicola protoplast preparation was successful (Figure 3.2). There was an increase in the total number of protoplasts harvested per gram of wet endoconidial pellet when using the modified procedure compared to the original protocol for *T. basicola* protoplast preparation by Samiya Al-Jaaidi (2007). Modifications included an increase of incubation time for cell wall degradation and changes to the protoplast trapping step. When successfully completed, the original protocol yielded an average total of $12.5 \times 10^6 \pm 0.2 \times 10^6$ protoplasts/g of wet endoconidial pellet. The modified procedure yielded an average total of $42.4 \times 10^6 \pm 4.8 \times 10^6$ protoplasts/g of wet endoconidial pellet, providing a 3.4 fold increase in protoplasts/g of wet endoconidial pellet (Table 3.1).

The initial step of endoconidial filtration in the original protocol did not work due to clumping of mycelia. Data for average wet weight of endoconidial pellet using the original protocol in Table 3.1 actually involved the use of the modified procedure for endoconidial filtration. Preparations using 15 star plates of *T. basicola* cultures resulted in an average endoconidial pellet wet weight of 0.36 ± 0.01 g. In comparison, a preparation using *T. basicola* endoconidial suspension to produce 15 spread plates of *T. basicola* cultures resulted in an endoconidial pellet wet weight of 1.07 g, providing a 3 fold increase in the amount of endoconidia harvested (Table 3.1). This was translated into a 3.3 fold increase in total protoplasts harvested when comparing the $16.0 \times 10^6 \pm 1.8 \times 10^6$ protoplasts yielded using star plates with the 53.4×10^6 protoplasts yielded using spread plates (Table 3.1). The 49.9×10^6 protoplasts/g of wet endoconidial pellet yielded with spread plates was comparable to the $42.4 \times 10^6 \pm 4.8 \times 10^6$ protoplasts/g of wet endoconidial pellet yielded with star plates when using the modified procedure for protoplast preparation (Table 3.1).

Table 3.1) Results of *Thielaviopsis basicola* protoplast preparation

Procedure used	Plating technique used	Number of successful preparations performed	Average wet weight of endoconidial pellet (g)	Average total of protoplasts harvested (protoplasts)	Average total of protoplasts/gram of wet endoconidial pellet (protoplasts/g)
Original	Star	2	0.34 ± 0.02	$4.2 \times 10^6 \pm 0.1 \times 10^6$	$12.5 \times 10^6 \pm 0.2 \times 10^6$
Modified	Star	3	0.38 ± 0.01	$16.0 \times 10^6 \pm 1.8 \times 10^6$	$42.4 \times 10^6 \pm 4.8 \times 10^6$
Modified	Spread	1	1.07	53.4×10^6	49.9×10^6

Combinations of different procedures and plating techniques used and their effect on: the average wet weight of endoconidial pellet; the average total of protoplasts harvested; and the average total of protoplasts harvested per gram of wet endoconidial pellet harvested. The modified procedure included an increase of incubation time for cell wall degradation and changes to the protoplast trapping step. (Averaged data with standard error of the mean)

Protoplasts could be seen lysing under the microscope with the addition of dH_2O, indicating the preparation of true protoplasts during experimentation. Protoplasts in 1 × STC and spread on solid regeneration media produced small white colonies after 2 weeks incubation, whereas protoplasts in dH_2O and spread on solid regeneration media produced no colonies, confirming the preparation of true protoplasts capable of regeneration.

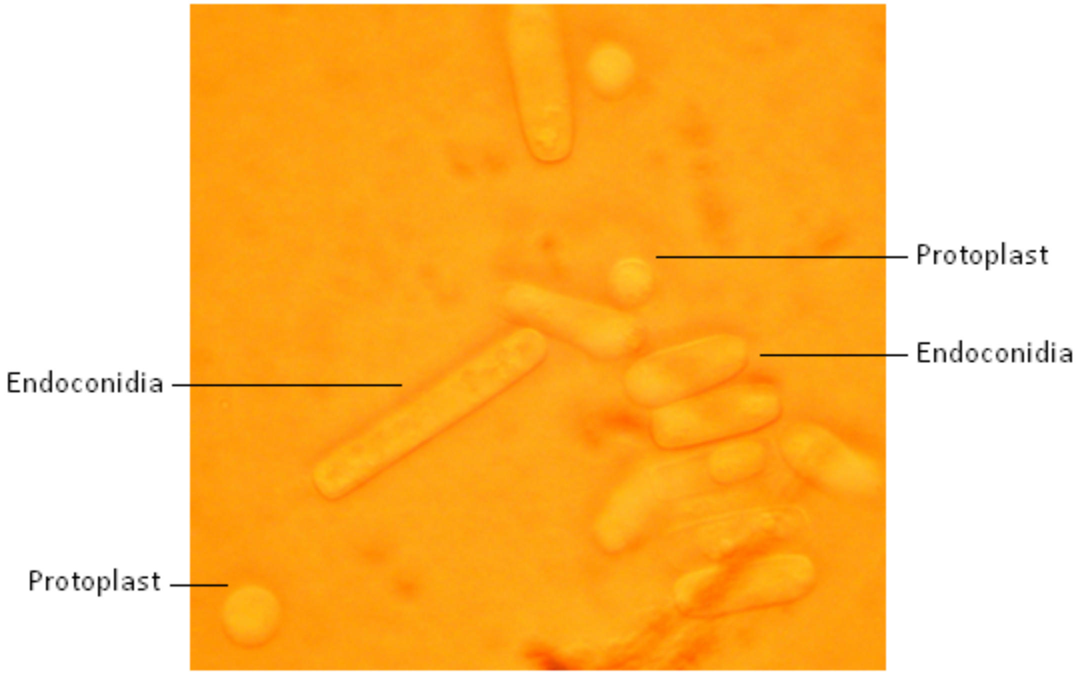


Figure 3.2) *Thielaviopsis basicola* protoplasts and endoconidia

During endoconidial cell wall degradation with 5 mg/mL of Glucanex in osmotic medium both protoplasts and endoconidia could be viewed under a microscope. The presence of protoplasts indicated that they were successfully produced and their numbers were periodically estimated in an attempt to maximise yield.

3.3 *Thielaviopsis basicola* polyethylene glycol – mediated transformations

A total of 46 *T. basicola* PEG-mediated transformations were performed, with 23 transformations using the pGpdGFP vector and 23 transformations using the pGpdR vector. Of these, 14 transformations were performed using the original protocol for *T. basicola* PEG-mediated transformation by Samiya Al-Jaaidi (2007). This protocol called for incubation times of 20 min for the first PEG incubation and 15 min for the second PEG incubation. None of these transformations produced putative transformants (Table 3.2). 32 transformations were performed using a modified procedure involving increased incubation times of 40 min for the first PEG incubation and 30 min for the second PEG incubation. 31 putative *T. basicola* transformants were produced with the modified procedure (Table 3.2). From 16 transformations using the pGpdGFP vector, 18 putative transformants were produced, resulting in an average transformation frequency of 0.11 ± 0.03 putative *T. basicola* transformants/µg of pDNA. From

16 transformations using the pGpdR vector, 13 putative transformants were produced, resulting in an average transformation frequency of 0.08 ± 0.03 putative *T. basicola* transformants/µg of pDNA. The overall average transformation frequency using the modified procedure was 0.10 ± 0.02 putative *T. basicola* transformants/µg of pDNA (Table 3.2).

Table 3.2) Results of *Thielaviopsis basicola* PEG-mediated transformations

Procedure used	Plasmid DNA used (10 µg)	N° of transformations performed	N° of putative transformants produced	Average transformation frequency (putative transformants/ µg of pDNA)
Original	pGpdGFP	7	0	0
	pGpdR	7	0	0
	Overall	14	0	0
Modified	pGpdGFP	16	18	0.11 ± 0.03
	pGpdR	16	13	0.08 ± 0.03
	Overall	32	31	0.10 ± 0.02

(Averaged data with standard error of the mean)

The 18 putative transformants produced using the pGpdGFP plasmid, were named with the prefix 'G' (e.g. G8-1). The 13 putative transformants derived using the pGpdR plasmid, were named with the prefix 'R' (e.g. R8-1).

All selective ½ PDA (1.2% agar) plates containing 25 µg/mL of hygromycin B inoculated with regenerated protoplasts displayed growth of small white colonies, excluding those inoculated with negative controls. It is proposed that these colonies may be transient *T. basicola* transformants. Selective plates inoculated with the transformation solutions prepared using the original protocol produced an average of 8.7 ± 0.9 colonies/50 µL of regenerated *T. basicola* protoplasts, whereas selective plates inoculated with the transformation solutions prepared using the modified procedure produced too many colonies to count accurately after 2 weeks of incubation. All selective plates inoculated with regenerated protoplasts from the negative controls, lacking the addition of pDNA, were completely clear and produced no colonies indicating that colonies on other plates were the result of the PEG-mediated transformation with the pDNA.

3.4 Mitotic stability of putative *Thielaviopsis basicola* transformants

After seven generations of being subcultured on non-selective media, 83.9% of putative *T. basicola* transformants continued to display hygromycin B resistance when subcultured onto ½ PDA (1.2% agar) plates containing 100 µg/mL of hygromycin B and were deemed to be mitotically stable putative transformants (Figure 3.3). There was no significant difference in mitotic stability between putative transformants derived from the pGpdGFP or pGpdR vectors. The *T. basicola* BRIP40192 wild-type control did not grow on the selective media. Mitotically unstable putative transformants maintained their hygromycin B resistant phenotype when consistently cultured on media containing 25 µg/mL of hygromycin B.

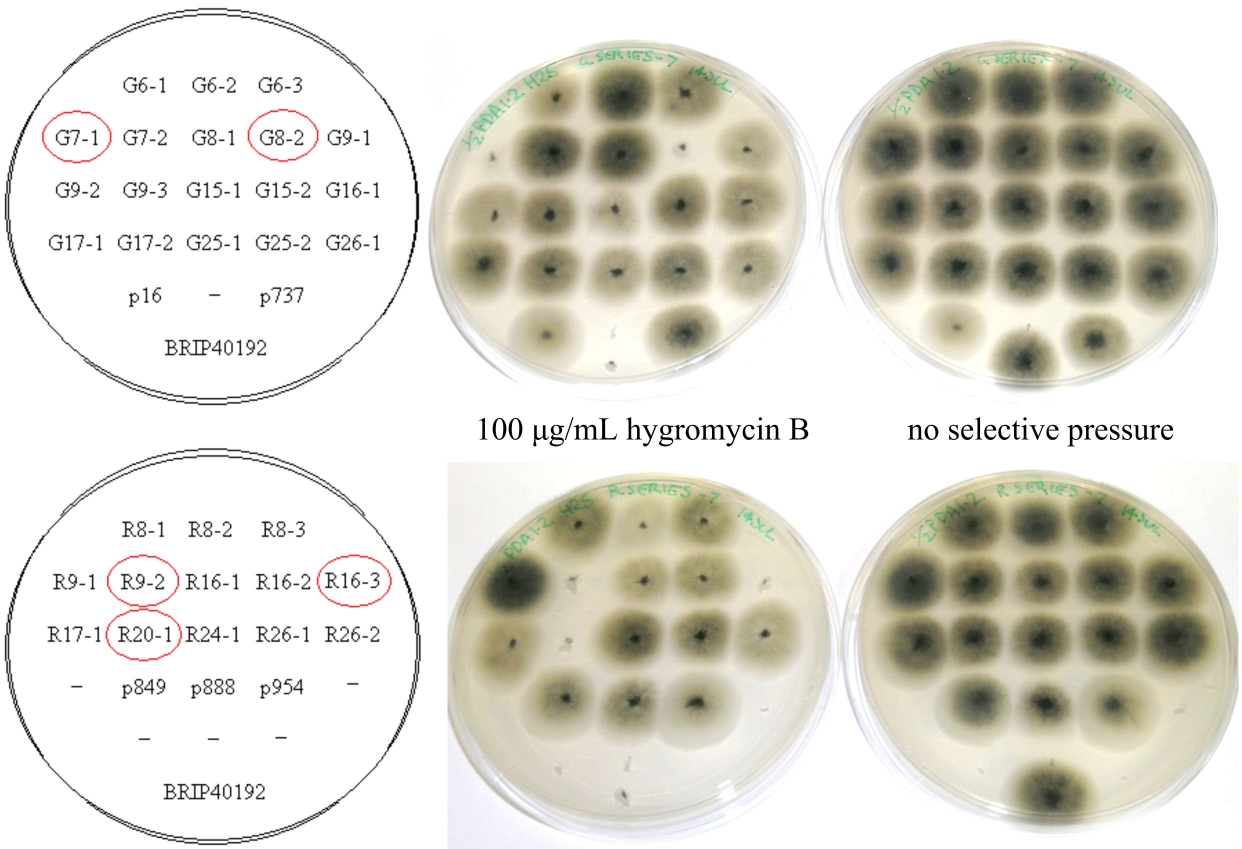


Figure 3.3) <u>Mitotic stability of putative *Thielaviopsis basicola* transformants showing growth after seven generations without selective pressure</u>

8th generation *T. basicola* subcultures grown with and without 100 µg/mL hygromycin B, after seven generations of subcultures grown without selective pressure. Putative transformants, G7-1, G8-2, R9-2, R16-3 and R20-1 lost hygromycin B resistant phenotypes and were deemed mitotically unstable. The *T. basicola* BRIP40192 wild-type control did not grow on selective plates.

3.5 Pathogenicity of *Thielaviopsis basicola* transformants on cotton

3.5.1 Results of pathogenicity testing using dipping technique

Cotton (*G. hirsutum*) seedlings were grown for 4 days, after inoculation with *T. basicola* endoconidial suspension (3.5 × 10^6 endoconidia/mL of dH_2O), before the length of root affected with lesions and total root length were measured.

Unaffected roots were off-white in colour with a high number of white root hairs. Affected roots displayed: brown-black lesions; the presence of chlamydospores on those affected areas; and relatively few or no root hairs. In seedlings with high disease severity, the root would appear shrivelled and black with high numbers of chlamydospores present (Figure 3.4).

Figure 3.4) Cotton seedlings affected and unaffected by *Thielaviopsis basicola*

A) Negative control cotton seedling, not inoculated with *T. basicola*, displaying healthy off-white roots. B) Positive control cotton seedling, inoculated with *T. basicola* BRIP40192 wild-type, with black shrivelled roots.

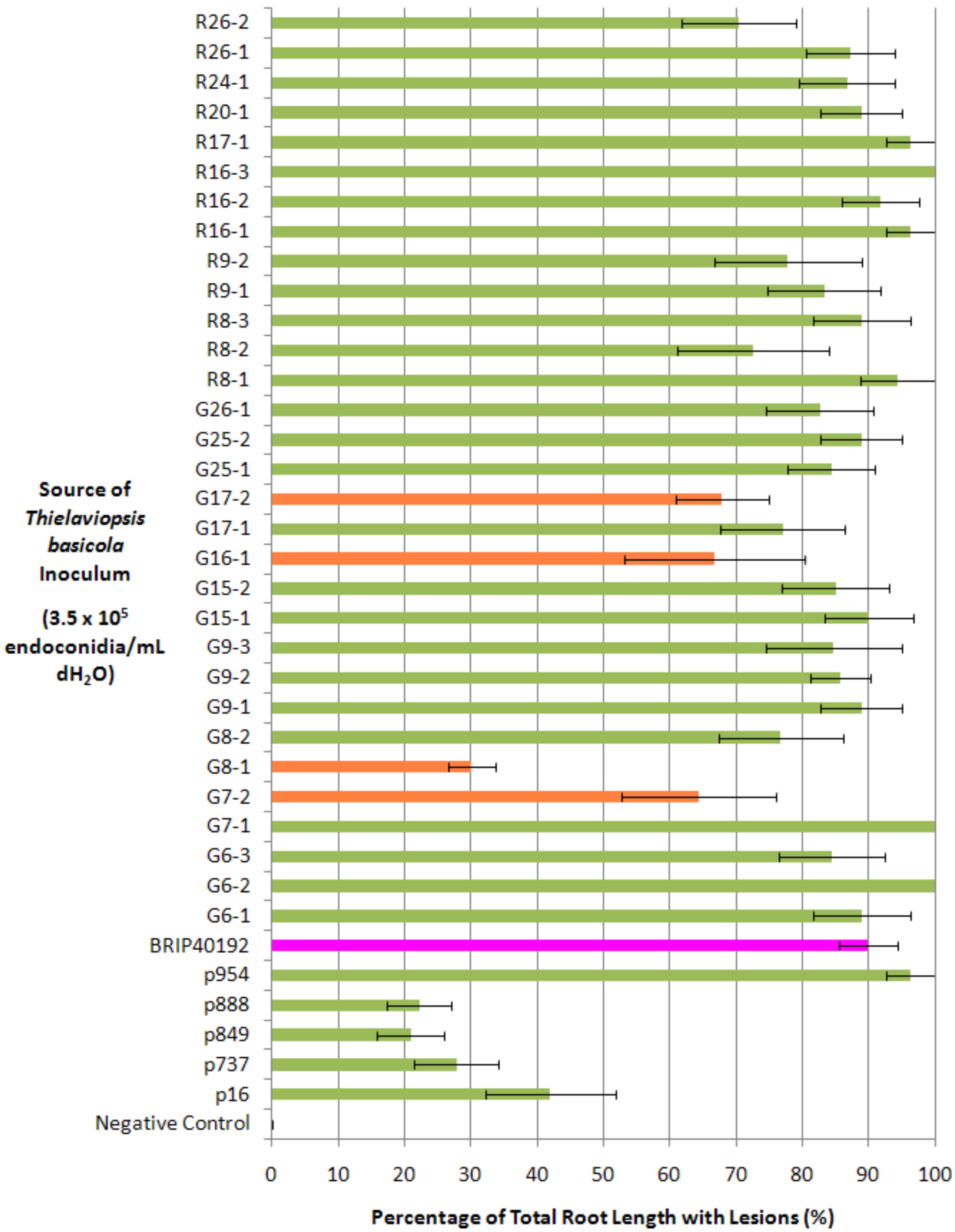


Figure 3.5) Results of pathogenicity testing using the dipping technique

The average percentage of total root length of cotton (*G. hirsutum*) seedlings affected with lesions, 4 days after inoculation with *T. basicola* endoconidial suspension. All transformants were compared to the positive control *T. basicola* BRIP40192 wild-type (90.0 ± 4.4 %) (purple). G8-1 displayed the lowest disease severity of transformants (30.1 ± 3.5 %). Four transformants that displayed the lowest disease severity were chosen for pathogenicity testing in soil (orange). (Averaged data with standard error of the mean)

Of the 31 *T. basicola* putative transformants tested for reduced pathogenicity toward cotton seedlings only G8-1 definitively displayed reduced disease severity. G8-1 inoculum resulted in an average of 30.1 ± 3.5 % of root length affected with lesions. This was significantly lower than that of the *T. basicola* BRIP40192 wild-type positive control, which resulted in an average of 90.0 ± 4.4 % of root length affected with lesions, and also that of other putative transformants (Figure 3.5). It displayed disease severity comparable to the reduced-pathogenicity transformants, p16, p737, p849 and p888, developed by Samiya Al-Jaaidi (2007), which also displayed reduced disease severity (Figure 3.5). The negative controls, not inoculated with *T. basicola*, displayed no lesions or other symptoms of *T. basicola* infection.

Four of the *T. basicola* transformants that displayed lowest disease severity were chosen for pathogenicity testing in soil. These were G8-1 (30.1 ± 3.5 %), G7-2 (64.4 ± 11.6 %), G16-1 (66.7 ± 13.6 %) and G17-2 (67.9 ± 7.1 %) (Figure 3.5).

3.5.2 Results of pathogenicity testing in soil

Unaffected roots were off-white in colour. Affected roots displayed brown-black lesions and the presence of chlamydospores on those affected areas as observed by microscope. In those with high disease severity, the formation of lesions in the form of cavities filled with chlamydospores in the main root was evident (Figure 3.6).

Of the four *T. basicola* transformants tested (chosen for having the lowest disease severity during pathogenicity testing using the dipping technique) the transformants G8-1 and G7-2 displayed reduced disease severity on cotton (*G. hirsutum*) 13 days after the planting of seeds in soil inoculated with 5000 *T. basicola* endoconidia/mL of soil. Inoculation with G8-1 resulted in an average of 16.9 ± 1.6 % of root length affected with lesions and G7-2 inoculum resulted in an average of 30.7 ± 4.2 % of root length affected with lesions (Figure 3.7). These were significantly lower than that of the *T. basicola* BRIP40192 wild-type positive control, which resulted in an average of 69.7 ± 4.9 % of root length affected with lesions. The transformants G16-1 (56.5 ±6.6 %) and G17-2 (65.1 ± 3.2 %) displayed disease severity comparable to that of the positive control (Figure 3.7). The negative controls, not inoculated with *T. basicola*, displayed no lesions or other symptoms of *T. basicola* infection.

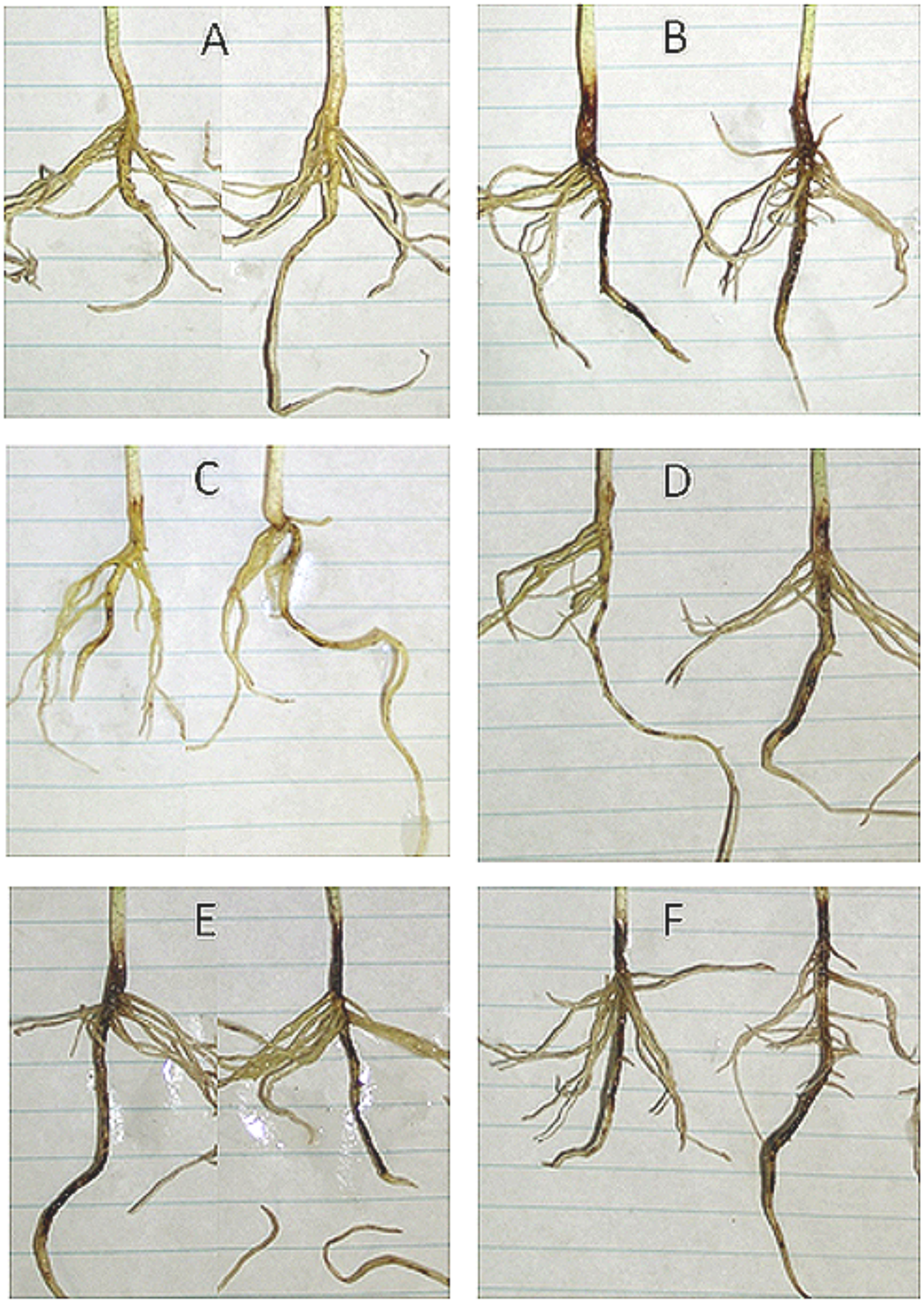


Figure 3.6) Roots of cotton seedlings from pathogenicity testing in soil

Roots of cotton (*G. hirsutum*) seedlings 13 days after planting in soil inoculated with *T. basicola* endoconidial suspension. A) Negative control – not inoculated with *T. basicola*; B) Positive control – *T. basicola* BRIP40192 wild-type; C) Transformant G8-1; D) Transformant G7-2; E) Transformant G16-1; F) Transformant G17-2. Roots affected with *T. basicola* appeared a discoloured brown-black.

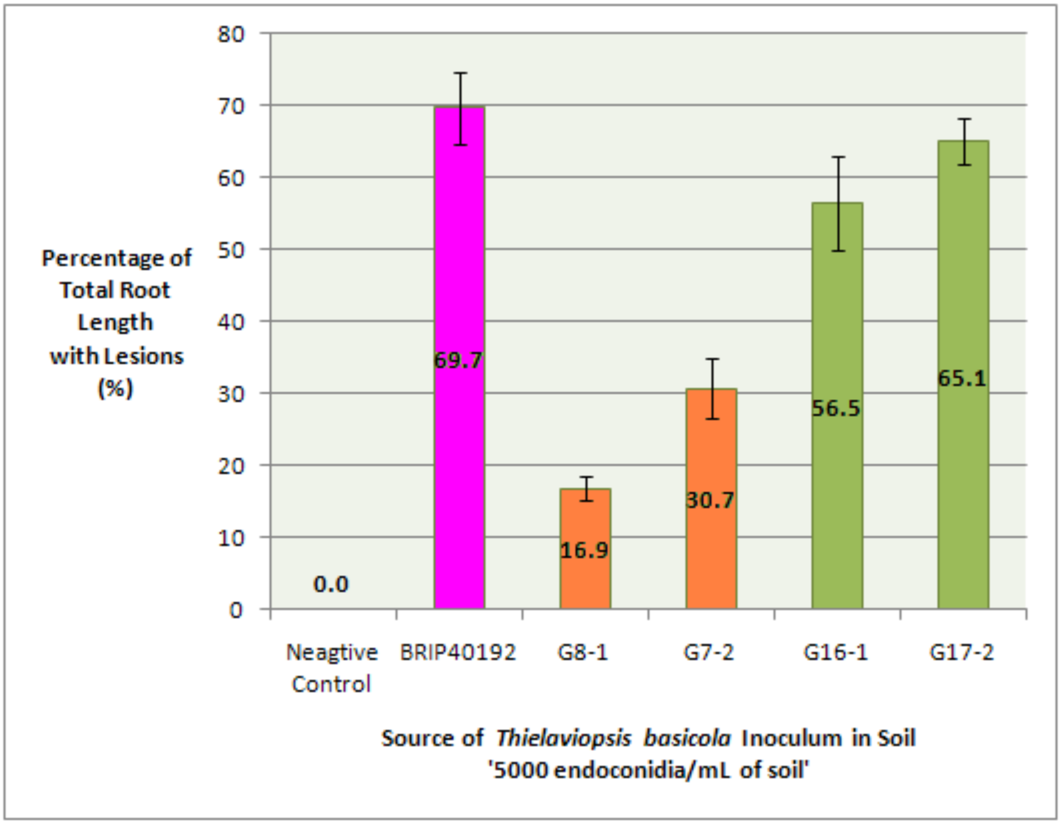


Figure 3.7) Results of pathogenicity testing in soil

The average percentage of total root length of cotton (*G. hirsutum*) seedlings affected with lesions, 13 days after planting in soil inoculated with *T. basicola* endoconidial suspension. All transformants were compared to the positive control *T. basicola* BRIP40192 wild-type (69.7 ± 4.9 %) (purple). G8-1 (16.9 ± 1.6 %) and G7-2 (30.7 ± 4.2 %) displayed significant reduction in disease severity (orange) and were deemed reduced-pathogenicity transformants. (Averaged data with standard error of the mean)

3.6 Southern blot analysis

3.6.1 DIG-labelled DNA probe

The DIG-labelled hygromycin phosphotransferase (*hph*) gene probe was successfully synthesised from the pGpdGFP template. The unlabelled positive control probe (unlabelled *hph* probe) corresponded to the 621 bp in size that was expected (Figure 3.8). The DIG-labelled *hph* probe migrated slower than the unlabelled positive control probe (Figure 3.8). The presence of DIG makes the probe run slower in the gel, indicating that the *hph* probe was successfully labelled. The presence of the DIG-labelled 'tPA' positive control probe (Roche Applied Science) of approximately 550 bp in size (Figure 3.8) indicated the overall success of the PCR DIG probe synthesis protocol.

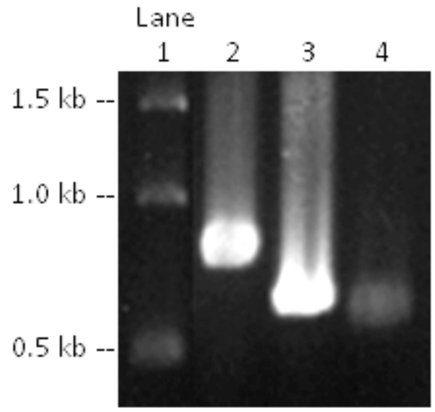


Figure 3.8) Analytical gel of DIG-labelled DNA probe after synthesis

The DIG-labelled hygromycin phosphotransferase gene probe was run in lane 2 and, due to the presence of DIG, migrated slower than the unlabelled positive control probe in lane 3, which displayed a size of ~ 600 bp. A DIG-labelled 'tPA' positive control probe was run in lane 4 and displayed an expected size of ~ 550 bp. A 1 kb DNA ladder was run against the DNA in lane 1.

3.6.2 Restriction digests of fungal genomic DNA

Three independent restriction digests were performed with the restriction endonucleases, *Xba*I, *Nru*I and *Nhe*I (Figure 3.9). Digests with *Xba*I and *Nru*I were performed on the gDNA of *T. basicola* BRIP40192 and the putative *T. basicola* transformants which underwent pathogenicity testing in soil: G8-1; G7-2; G16-1; and G17-2. Digests with *Nhe*I was performed on the afore mentioned gDNA, and the gDNA of four other randomly chosen putative *T. basicola* transformants produced using the pGpdGFP vector, and eight randomly chosen putative *T. basicola* transformants produced using the pGpdR vector. Agarose gel electrophoresis appeared to display complete digestion of the fungal gDNA by all the restriction digests performed for southern blot hybridization (Figure 3.9).

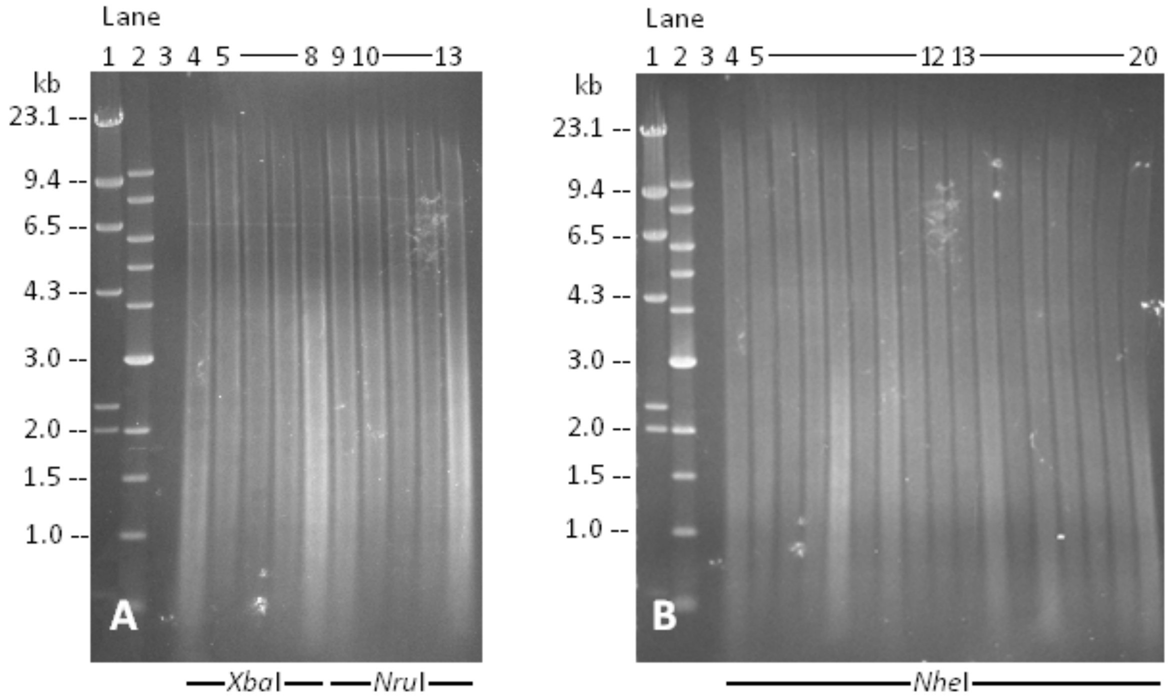


Figure 3.9) Agarose gels of digested fungal genomic DNA for southern hybridization

Gel A contained the *Xba*I restriction digests of fungal gDNA in lanes 4 to 8, and the *Nru*I restriction digests of fungal gDNA in lanes 9 to 13. Lanes 4 and 9 contained digested gDNA of *T. basicola* BRIP40192, acting as a negative control during hybridization. Lanes 5 to 8 and 10 to 13 contained digested gDNA of putative transformants produced with pGpdGFP. Gel B contained the *Nhe*I restriction digests of fungal gDNA in lanes 4 to 20. Lane 4 contained digested gDNA of *T. basicola* BRIP40192, acting as a negative control during hybridization. Lanes 5 to 12 contained digested gDNA of putative transformants produced with pGpdGFP. Lanes 13 to 20 contained digested gDNA of putative transformants produced with pGpdR. On both gels: lane 1 contained a λ DNA-*Hind*III digest; lane 2 contained a 1 kb DNA ladder; and lane 3 contained 5 ng of pGpdGFP, digested with *Xba*I, acting as a positive control during hybridization.

3.6.3 Southern blot analysis

Unfortunately, southern blot hybridization was generally unsuccessful due to degradation of the fungal gDNA. What was evident from the hybridizations was that, the *hph* probe did not hybridize to the *T. basicola* BRIP40192 negative control, which as expected displayed consistently clear lanes with no bands or darkening, but the *hph* probe did appear to hybridize to DNA belonging to transformants produced with either pGpdGFP or pGpdR vectors, which displayed dark bands or smearing in all lanes (Figure 3.10). This pattern of hybridization was evident amongst all restriction digests. If nothing else, this indicates that true transformants were likely to have been produced using both the pGpdGFP or pGpdR plasmid vectors.

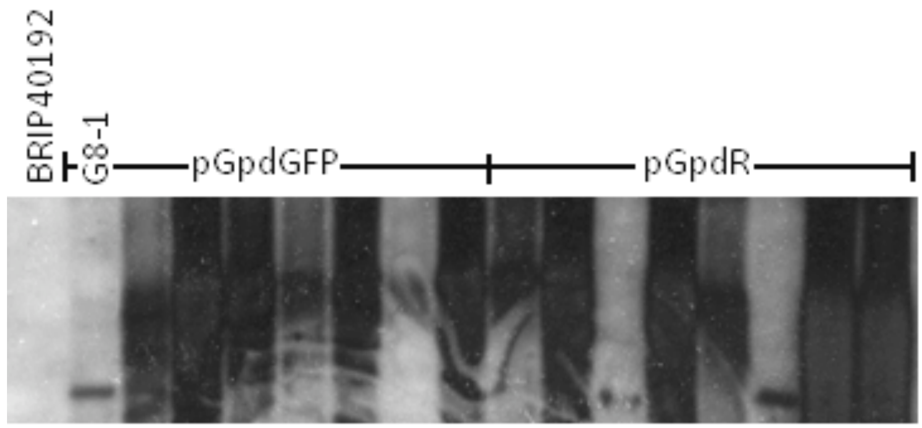


Figure 3.10) Hybridization of hygromycin phosphotransferase gene probe to various sources of digested fungal genomic DNA

Relatively unsuccessful southern blot hybridization of the *hph* probe to digested gDNA of *T. basicola* BRIP40192 and transformants produced with pGpdGFP or pGpdR vectors. *T. basicola* BRIP40192 acted as the negative control and displayed clear lanes as expected. All the lanes containing transformant gDNA appeared to display some form of hybridization with the *hph* probe.

Of all the transformants that underwent southern blot hybridization, only G8-1 provided relatively successful and relevant data. The linearized pGpdGFP positive controls on both gels produced the expected single bands of approximately 7.0 kb in size, indicating successful hybridization of the *hph* probe to the *hph* gene of the pGpdGFP vector (Figure 3.11).
The *T. basicola* BRIP40192 negative controls produced no bands as expected (Figure 3.11), and all their respective lanes were completely clear with no evidence of smearing or darkening. G8-1 gDNA digested with *Xba*I displayed two distinct bands of comparable intensity (Figure 3.11: lane 3). G8-1 gDNA digested with *Nru*I also appeared to display two bands of comparable intensity (Figure 3.11: lane 5). G8-1 gDNA digested with *Nhe*I displayed two distinct bands which may be of comparable intensity (Figure 3.11: lanes 8 and 9). Although repetition of the *Nhe*I restriction digest and southern blot hybridization of G8-1 gDNA indicated that there may also be a weak third band (Figure 3.11: lanes 8 and 9).

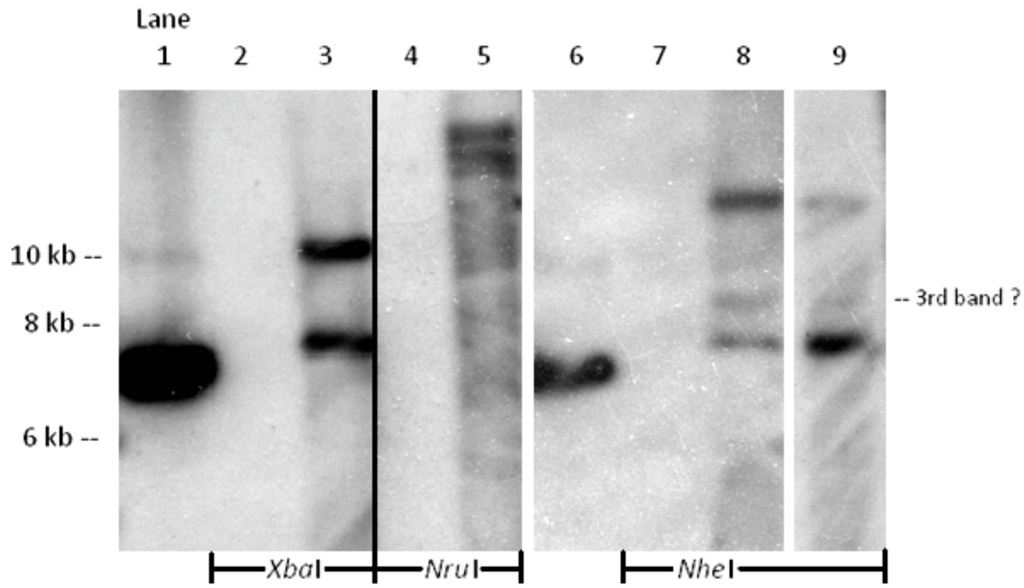


Figure 3.11) Southern blot for the *Thielaviopsis basicola* transformant G8-1

Data from three independent gels are shown. Lanes 1 and 6 contain the pGpdGFP positive controls, which displayed expected single bands of ~ 7 kb in size. Lanes 2, 4 and 7 contain *T. basicola* BRIP40192 gDNA negative controls digested with *Xba*I, *Nru*I and *Nhe*I respectively, which expectedly displayed no bands. Lane 3 contains G8-1 gDNA digested with *Xba*I, which displayed two bands. Lane 5 contains G8-1 gDNA digested with *Nru*I, which displayed two bands. Lanes 8 and 9 contain G8-1 gDNA digested with *Nhe*I from independently performed restriction digests and southern blot hybridizations, which displayed two bands with an unlikely possibility of a third weak band.

The presence of two bands of comparable intensity common to all three digests indicated that there were two single copy random integrations of the pGpdGFP vector into the G8-1 genome. These were single copy integrations because the *Xba*I digest, which cuts within the inserted pGpdGFP vector, displayed two bands of comparable intensity indicating that there were the same number of *hph* genes in both fragments, and if tandem integration had occurred it would be expected that the *Nru*I and *Nhe*I digests, which do not cut within the inserted pGpdGFP vector, would have displayed single bands. What appeared to be weak third band evident in the *Nhe*I digest (Figure 3.11: lanes 8 and 9) was not displayed in the *Xba*I and *Nru*I digests.

3.7 Results of internal transcribed spacer sequence comparison

The internal transcribed spacer (ITS) sequence of the transformants G8-1, G7-2, G16-1 and G17-2 were compared to the ITS sequence of the *T. basicola* BRIP40192 wild-type. Amplification of ITS sequences was successful (Figure 3.12). *T. basicola* BRIP40192 and all transformant gDNA templates produced comparable PCR products of the expected approximate size of 600 bp. The two negative controls, using pGpdGFP and no DNA as templates respectively, produced no PCR products as expected.

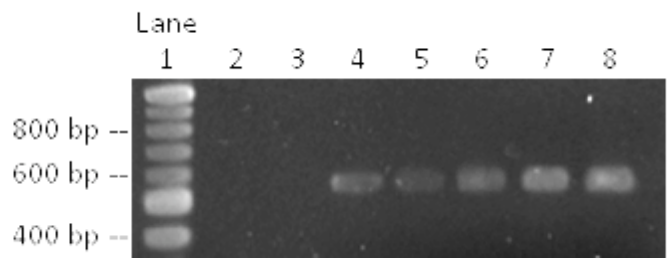


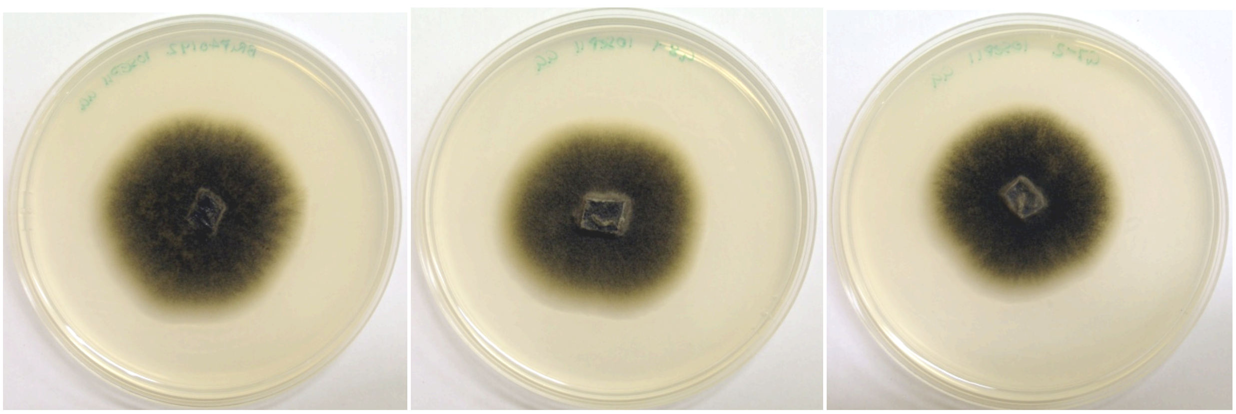
Figure 3.12) Analytical gel of PCR products using ITS1 and ITS4 primers

Fungal gDNA templates all produced comparable PCR products of ~ 600 bp in size (lane 4: *T. basicola* BRIP40192, lane 5: G8-1, lane 6: G7-2, lane 7: G16-1, lane 8: G17-2). The 'no DNA' negative control was run in lane 3 and produced no bands as expected. The 'pGpdGFP' negative control was run in lane 2 and produced no bands as expected. A 100 bp DNA ladder was run against DNA in lane 1.

When sequenced, 498 consecutive bases of the *T. basicola* BRIP40192 wild-type sequence gave a Q20 score (base call of > 99% confidence). A BLASTN search of GenBank (Appendix 2) with the *T. basicola* BRIP40192 sequence produced top significant alignments with ITS sequences from other *T. basicola* strains, indicating that the *T. basicola* BRIP40192 ITS sequence had actually been sequenced. The transformant sequences displayed BLASTN search results similar to those of the *T. basicola* BRIP40192 wild-type. FASTA: DNA:DNA comparisons (Appendix 3) of ITS sequences of the transformants, G8-1, G7-2 and G16-1 with the *T. basicola* BRIP40192 ITS sequence displayed 100% identity and similarity over a 498 nt overlap. G17-2 displayed 99.8% identity and similarity over a 498 nt overlap, because the identity of a single nucleotide in the G17-2 sequence was not be verified with sequencing. This indicated that these transformants were true *T. basicola* transformants derived from the *T. basicola* BRIP40192 wild-type.

3.8 Morphology of reduced-pathogenicity *Thielaviopsis basicola* transformants

After incubation on ½ PDA (1.2% agar) plates at 25^{o}C for 1 week, colonies of *T. basicola* BRIP40192 and the reduced-pathogenicity *T. basicola* transformants, G8-1 and G7-2, displayed similar colony morphology and growth with colony sizes of 4.8 ± 0.1 cm, 4.7 ± 0.1 cm and 4.4 ± 0.2 cm respectively (Figure 3.13). All three gave rise to 'grey' and 'brown' variants over the course of the study with circular form, raised elevation and entire margins.

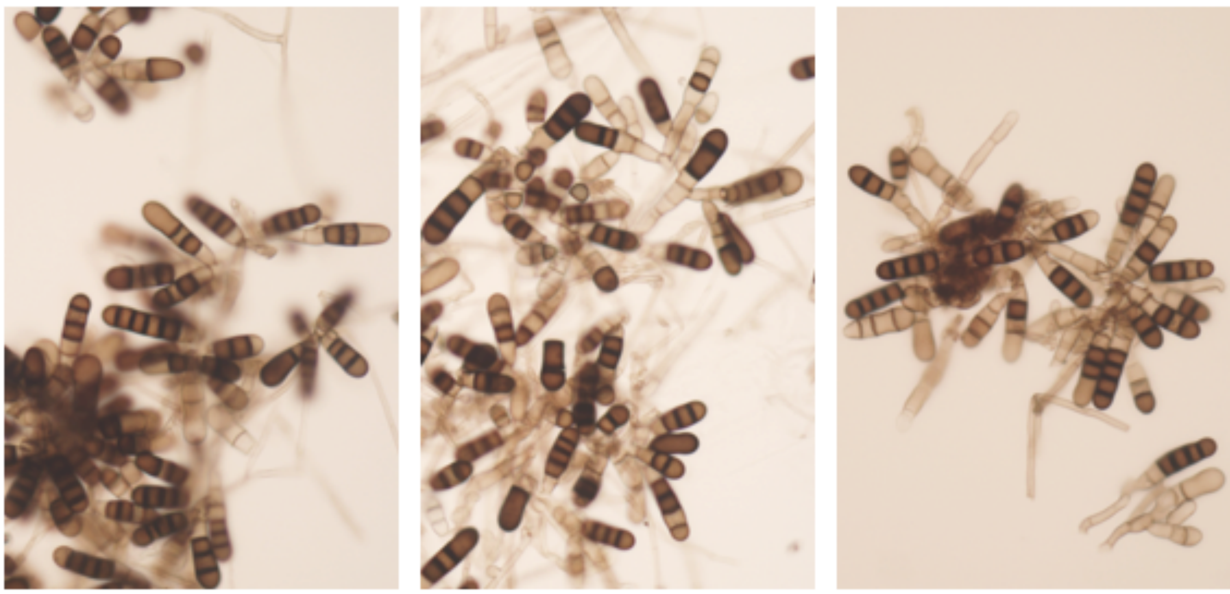


T. basicola BRIP40192 (wild-type)	*T. basicola* G8-1 (transformant)	*T. basicola* G7-2 (transformant)

Figure 3.13) Colonies of reduced-pathogenicity transformants compared to the *Thielaviopsis basicola* wild-type

Colonies from *T. basicola* BRIP40192 wild-type and the reduced-pathogenicity transformants, G8-1 and G7-2, incubated on ½ PDA (1.2% agar) plates at 25^{o}C for 1 week. All three colonies are the 'grey' variant with similar colony size, form, elevation, margins, texture and colour.

Chlamydospores from *T. basicola* BRIP40192 and the reduced-pathogenicity *T. basicola* transformants, G8-1 and G7-2, all displayed similar morphology with no obvious differences in size, shape, the number of compartments in the chain or melanisation (Figure 3.14).



T. basicola BRIP40192 (wild-type) | *T. basicola* G8-1 (transformant) | *T. basicola* G7-2 (transformant)

Figure 3.14) Chlamydospores of reduced-pathogenicity transformants compared to the *Thielaviopsis basicola* wild-type

Chlamydospores taken from colonies of the *T. basicola* BRIP40192 wild-type and the reduced-pathogenicity transformants, G8-1 and G7-2, as viewed under a microscope. There were no obvious differences in size, shape, the number of compartments in the chain or melanisation. Magnifications in of all three photos are identical.

3.9 Presence of intact ampicillin resistance genes

The presence of an intact ampicillin resistance gene was tested on the reduced-pathogenicity *T. basicola* transformants G8-1 and G7-2. Amplification of the ampicillin resistance gene was successful (Figure 3.15). The pGpdGFP template positive control and both transformant gDNA templates produced PCR products of comparable size that were approximately the expected size of 891 bp, indicating that at least one intact ampicillin resistance gene was present in the *T. basicola* transformants G8-1 and G7-2. The two negative controls, using *T. basicola* BRIP40192 gDNA and no DNA as templates respectively, produced no PCR products as expected.

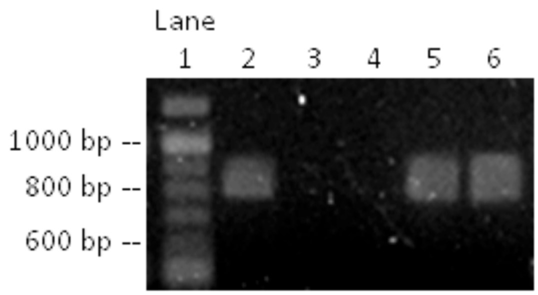


Figure 3.15) Analytical gel of PCR products using AmpF1 and AmpR1 primers

The pGpdGFP positive control and the transformant gDNA templates all produced comparable PCR products that were approximately the expected size of 891 bp (lane 2: pGpdGFP, lane 5: G8-1, lane 6: G7-2). The *T. basicola* BRIP40192 wild-type negative control was run in lane 4 and produced no band as expected. The 'no DNA' negative control was run in lane 3 and produced no band as expected. A 100 bp DNA ladder was run against the DNA in lane 1.

Chapter 4: Discussion

4.1 Modifications to improve *Thielaviopsis basicola* protoplast yield

A modified procedure for *T. basicola* protoplast preparation was devised. Protoplast harvesting can be tricky and time consuming. Although the modifications were relatively minor, they increased the number of protoplasts harvested and the ease in which the protocol was performed. The following provides recommendations on techniques and the modifications made to Al-Jaaidi's original protocol (2007):

- *T. basicola* cultures should be incubated for a maximum of 3 days. Production of endoconidia starts within 24 h, whereas chlamydospores are produced after 3 days (Shew & Meyer, 1992). To prevent excessive contamination by chlamydospores this maximum must be adhered to. Mycelial age has been reported to have an effect on protoplast yield (Wei et al., 2010; Zhou et al., 2008), but whether age is a factor when using conidia is unknown.

- If large numbers of protoplasts are required, such as during optimization of transformations, then the preparation of cultures on spread plates using endoconidial suspension will provide a greater protoplast harvest than preparing cultures using star plates. Also, the time spent on labour when producing cultures is reduced. A 3-fold increase in average wet weight of endoconidial pellet was observed when using spread plates, translating into a similar increase in the number of protoplasts harvested. This is possibly because the spread plate technique utilizes a greater area of the plate resulting in more endoconidia available.

- A maximum of fifteen culture plates in 20 mL of 0.1% Tween 80 should be used to free endoconidia for filtration. This prevents clumping of the mycelia and increases the number of endoconidia in suspension. Gentle ringing of the Miracloth is permitted to extract a greater amount of suspension, but note that contamination and damage to the Miracloth must be avoided. If clumping of mycelia continues to occur, reducing the number of culture plates used rather than using more 0.1% Tween 80 is recommended because excessive dilution of PDB can result in the arrest of endoconidial germination.

- The incubation time required for production of protoplasts during cell wall degradation is a minimum of 2 h and the number of protoplasts produced should be estimated under a microscope in the same manner as endoconidial germination. Occasionally, the flask should be manually swirled to bring endoconidia and protoplasts off its sides to improve the efficiency of the incubation.

- The flask used for cell wall degradation should be rinsed with 4 – 8 mL of osmotic medium depending on the volume of cell wall degrading medium used. This increases the physical distance between the protoplast layer and the debris at the base of the corex tube, allowing for a greater amount of medium to be extracted and therefore the potential for a greater number of protoplasts harvested. It may also reduce the possibility of endoconidial contamination from the debris.

- A swing-bucket centrifuge should be used for the trapping step as it assists in maintaining the integrity of the protoplast layer. If the interface between the osmotic medium and trapping buffer is overly disturbed, the protoplast layer cannot be retrieved and is permanently lost.

- Finally, the harvested protoplasts should be transferred to an ice-cold corex tube containing ice-cold 1 × STC, rather than adding ice-cold 1 × STC after transfer, because it reduces the possibility of protoplast degradation.

The modifications to the cell wall degradation step and protoplast trapping step provided a 3.4-fold increase in the average total number of protoplasts harvested per gram of wet endoconidial pellet when compared to the original protocol by Al-Jaaidi (2007). Possibly the most crucial modification was that of increased incubation time for cell wall degradation. Wei et al. (2010) had observed that once enzyme concentrations and incubation temperature had been determined there was an optimal incubation time to maximise protoplast yield. Protoplast yield increases with incubation time, but excessive incubation results in a reduction of protoplast yield. The optimal concentration of Glucanex (5 mg/mL) was determined by Al-Jaaidi (2007), but examination of incubation time was not reported and fixed 2 h incubation for cell wall degradation was applied. Amey et al. (2001) used the same method proposed by this study of monitoring cell wall degradation under a microscope. In this study, incubation time would vary between 3 – 3.5 h, although optimal incubation time for maximum protoplast yield may be higher. It would be advantageous to determine the optimal incubation time required for *T. basicola* cell wall degradation with fresh Glucanex to maximise protoplast yield and simplify the procedure. The age of any single batch of Glucanex may affect its efficiency to degrade cell walls, therefore independent monitoring and optimization of cell wall degradation efficiency (protoplast yield/digest time) may be required after extensive periods of storage.

4.2 *Thielaviopsis basicola* polyethylene glycol - mediated transformations

For the first time in three years and after unsuccessful attempts by others, the protocol for PEG-mediated transformation of *T. basicola* developed by Al-Jaaidi (2007) has been replicated with limited success after modification. Southern blot analysis indicated that the PEG-mediated transformation of *T. basicola* with both the pGpdGFP and pGpdR plasmid vectors was successful. This success was confirmed with the amplification of the ampicillin resistance gene from gDNA of the reduced-pathogenicity *T. basicola* transformants, G8-1 and G7-2.

The original protocol by Al-Jaaidi (2007) appears to require optimization. Stringent execution of the original protocol failed to produce transformants, with two attempts prior to modification, and one attempt after to ensure that protocol modification, rather than technique, was the cause of success. The modification to the original protocol involved the doubling of PEG-treatment incubation times to a total of 70 min, which produced transformants with each attempt. When using the modified procedure this study had only managed an overall average transformation frequency of 0.10 ± 0.02 *T. basicola* transformants/μg of pDNA, whereas Al-Jaaidi (2007) had originally reported an average transformation frequency of 2.5 stable transformants/μg of pDNA. The limited success indicates that the difficulty in performing Al-Jaaidi's protocol has not yet been identified.

There are several factors which may be influencing the transformation efficiency of this protocol, including: PEG or calcium chloride concentrations; concentrations and ratio of DNA to protoplasts; incubation time and temperature; and regeneration of protoplasts.

Concentrations between 40 – 60% PEG 4000 and 10 – 50 mM of $CaCl_2$ are commonly used for fungal transformations (Fincham, 1989). Wei et al. (2010) showed that the concentration of PEG or calcium chloride can have significant effect on transformation frequencies. In their study, a PEG 4000 concentration of 50% provided an optimal transformation frequency of ~ 2.4 transformants/μg of DNA, whereas 40% PEG 4000 only provided ~ 0.75 transformants/μg of DNA. What is interesting is that 60% PEG 4000 resulted in a drop of transformation frequency to ~ 0.1 transformants/μg of DNA. Coincidently, this drop in transformation frequency is similar to that observed between this study and that of Al-Jaaidi (2007). The affects of varying PEG or calcium chloride concentrations on transformations of *T. basicola* was not reported by Al-Jaaidi (2007), but given the initial success of the original protocol that utilises 60% PEG 4000 and 10 mM of $CaCl_2$, it would be unlikely that this is the cause of the difficulties. None the less, this may require further examination once all other avenues have been exhausted.

Generally, 10 μg of DNA are added to what sometimes appears to be an arbitrary number of protoplasts for transformation. The number of protoplast used can vary from 2×10^6 to 2×10^9 protoplasts and it has been observed that a higher number of protoplast used results in higher transformation frequency (Fincham, 1989; Malardier et al., 1989; Fitzgerald et al., 2003; Wei et al., 2010). This study only used 2×10^6 protoplasts, as stipulated by the original protocol. An increase in the number of protoplasts used should be examined as it is likely to result in increased transformation frequency.

In this study, quantification of DNA mass was achieved by performing agarose gel electrophoresis and comparing band intensity of the molecular weight standards with that of the DNA. This is a commonly used and accepted method, but molecular weight standards were not designed for precise quantification of DNA mass and can only be used to approximate the mass of DNA in comparably intense samples of similar size (New England Biolabs). This method is also subject to differences in interpretation of band intensity by different individuals. Although this method can result in inconsistency of the DNA mass used for *T. basicola* transformations, such inconsistencies were unlikely to result in no transformation success whatsoever.

As was observed in this study, incubation time can be of great importance in PEG-mediated transformations. Given the commonly accepted view that PEG's primary function is the aggregation of DNA and protoplasts (Louie & Serwer, 1994), it was decided in this study to increase PEG-treatment incubation times. But it has come to light that that the role of PEG is most likely to function after transforming DNA is incorporated into protoplasts (Kuwano et al., 2008), therefore making the uptake of DNA into protoplasts prior to the addition of PEG an important step. DNA uptake prior to the addition of PEG is generally performed for 15 – 30 min at room temperature (Fincham, 1989; Rohe et al., 1996; Fitzgerald et al., 2003; Zhou et al., 2008; Wei et al., 2010). This may be the crux of the difficulties with the original protocol by Al-Jaaidi (2007), which only calls for a 5 min incubation on ice. Although an increase in incubation time could increase transformation frequency, it may also increase the number of vector integrations into the fungal genome. Multiple integrations are not desirable because they make identifying the specific disrupted sequence/s that may be involved in affecting pathogenicity far more difficult. Given that long time PEG-treatment could make protoplasts lose the ability of regeneration (Wei et al., 2010), further increase of PEG-treatment incubation times should be avoided, and after incubation of initial DNA uptake has been optimized it may be prudent to reduce the modified PEG-treatment incubation times if it does not have a negative effect on transformation frequency.

For the regeneration of colonies from protoplasts an osmotic stabilizer in the growth medium is required until the protoplasts have regenerated their cell walls (Fincham, 1989). In this study, *T. basicola* protoplast regeneration was achieved with incubation in 200 µL of 1 × STC with 1.3 mL of PDB / 0.5 M sucrose prior to plating. The reason why both STC and regeneration broth is utilised in the original protocol is unknown. Although protoplast regeneration frequency was not determined, large numbers of transient and stable transformants were apparent on selective plates, indicating that the regeneration medium was effective. This is not likely to be the factor negatively influencing transformation frequency, but there are indications that sucrose may not be the best suited osmotic stabilizer for *T. basicola* protoplasts. *T. basicola* protoplasts incubated on regeneration plates containing 1 M sucrose displayed small white colonies that did not appear to develop greatly after 2 weeks, which is consistent with the findings of Al-Jaaidi (2007). Al-Jaaidi (2007) thoroughly examined different methods to regenerate protoplasts when using sucrose as the osmotic stabilizer in regeneration media, but the use of alternative osmotic stabilizers for the purposes of protoplast regeneration was not reported. It may be worth examining other osmotic stabilizers such as 1.0 – 1.2 M sorbitol rather than sucrose in both broth and solid media (Fincham, 1989; Zhou et al., 2008). After all, this study and Al-Jaaidi (2007) did use 1.2 M sorbitol as the osmotic stabilizer for protoplasts in 1 × STC (1.2 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5).

It should be noted that this study used manufactured PDB (Difco), whereas Al-Jaaidi (2007) produced PDB by boiling and filtering 220 g of cut potatoes, adding 20 g of D-glucose (dextrose), made up to 1 L with dH_2O. The concentration and exact composition of these PDBs are likely to vary, potentially affecting their relative protoplast regeneration efficiency, but this remains untested. Once again, large numbers of transient and stable transformants were apparent on selective plates, indicating that the use of manufactured PDB (Difco) was effective.

T. basicola transformants generally displayed mitotic stability which is consistent with the findings of Al-Jaaidi (2007). Several possibilities have been suggested for the occurrence of transient transformants (Fitzgerald et al., 2003). Transient transformants which initially appeared after transformation, but did not develop on selective media, were likely the product of plasmid DNA that did not integrate into the fungal genome and the resistance to hygromycin B conferred by non-replicating plasmids was diluted by the growing colony. Transient transformants that did develop on selective media, but did not survive selection after a few generations of being incubated on non-selective media, were likely the product of plasmid DNA that did integrate into the fungal genome, but at a site that was unstable resulting in mitotic instability. These transformants were deemed mitotically unstable rather than transient, because when colonies were consistently incubated on/in media containing 25 µg/mL hygromycin B they did not lose their hygromycin B resistance phenotype and appeared healthy.

4.3 Reduced-pathogenicity *Thielaviopsis basicola* transformants

Of the thirty-one *T. basicola* transformants produced, two displayed reduced disease severity on cotton (*G. hirsutum*). Transformant G8-1 definitely displayed reduced disease severity, and although transformant G7-2 also displayed reduced disease severity further testing could prove otherwise. Both transformants were proven to be derivatives of the *T. basicola* BRIP40192 wild-type by ITS sequence comparison.

It is impossible to predict what gene/s may have been disrupted to result in reduced disease severity. Cultures of *T. basicola* G8-1 and G7-2 were capable of completing their life-cycle *in vitro* and displayed similar visual phenotypes for morphology and growth to the *T. basicola* BRIP40192 wild-type and could therefore be classed as 'normal' in these respects. Although these factors generally indicate that a true pathogenicity gene had been disrupted and that housekeeping genes had not, this did not guarantee that a housekeeping gene was not affected and only identification of the genes and their function can give a definitive answer.

Both *T. basicola* G8-1 and G7-2 displayed mitotic stability after seven generations of cultures grown on non-selective media. Transformant G8-1 definitely contained two single copy random integrations of the pGpdGFP vector, although there may have been a remnant of a third unstable integration. Either one or more of these integrations had disrupted gene/s resulting in reducing disease severity. Which of these integrations had done so could be determined by creating gene knockouts for each of the individual sequences that were disrupted and multiple knockouts to determine if disrupted sequences had additive effects on pathogenicity. Before gene knockouts can be created the sequences that were disrupted by the random integrations need to be sequenced.

The purpose of plasmid rescue is to recover genomic sequences that flank the integrated vector. It involves the digestion of gDNA with a restriction endonuclease that does not cut within the plasmid, ligation of the DNA fragment to form plasmids and transformation into *E. coli* for amplification (Nan & Walbot, 2009). Plasmid rescue requires both the ampicillin resistance gene and origin of replication for *E. coli* to be functional so that the DNA fragment can be amplified in *E. coli* for further analysis. There was evidence for the presence of at least one intact ampicillin resistance gene in both *T. basicola* G8-1 and G7-2. Unfortunately, given that both transformants contained more than one integration, there was no indication as to whether one or more of these integrated vectors still had their ampicillin resistance gene intact. Due to time restrictions plasmid rescue was not attempted.

4.4 The plasmid vector, pGpdR

The development of the pGpdR plasmid vector provides a novel tool for random insertional mutagenesis of fungi. There was evidence that the plasmid could be amplified in *E. coli* and that the ampicillin resistance selective marker was functional. Southern blot hybridization and mitotic stability testing provided evidence that it could be successfully integrated into the *T. basicola* genome providing stable transformants, and that the hygromycin B resistance selective marker was functional.

The primary reason for developing the smaller plasmid vector was to facilitate easier genetic analysis of DNA fragments after plasmid rescue. The effect of reduced vector size on plasmid rescue itself remains to be tested. As mentioned, plasmid rescue requires both the ampicillin resistance gene and origin of replication for *E. coli* to be functional. It would be interesting to investigate whether reduced vector size increases the probability of the ampicillin resistance gene or origin of replication for *E. coli* being disrupted by the cross-over event during transformation compared to a larger vector.

Unfortunately, there was insufficient data to determine whether the reduced size of this vector had an effect on transformation frequencies in *T. basicola*, although the data presented indicates that the average transformation frequencies of transformations using either the pGpdGFP (6.93 kb) or pGpdR (~ 5.0 kb) vectors were more or less equal.

4.5 Conclusion

PEG- mediated transformation of *T. basicola* is a viable method for development of reduced or non-pathogenic transformants. Al-Jaaidi (2007) produced 4 – 5 reduced-pathogenicity mutants from 204 transformants, and this study produced 1 – 2 reduced-pathogenicity mutants from 31 transformants, which attest to the viability of this method.

This study concentrated on replicating the original protocol for PEG-mediated transformation of *T. basicola* by Al-Jaaidi (2007) and found that further optimization of this protocol may be necessary as success was only achieved after modifying the procedure. Protocol optimization should result in a transformation frequency as high, or possibly higher, than that produced by Al-Jaaidi (2007). Transformation frequency could be increased with: the use of additional protoplasts per transformation; optimized transformation incubation times; optimized PEG concentration; and/or a better suited osmotic stabilizer for protoplast regeneration. A modified procedure for *T. basicola* protoplast preparation had been developed providing improved ease and higher protoplast yield. This will make optimization experiments, which require a large number of protoplasts for comparison of different transformation conditions, easier to perform.

With further analysis, it is hoped that the novel reduced-pathogenicity mutants, *T. basicola* G8-1 and *T. basicola* G7-2, may provide information on pathogenicity genes and their function that may result in the development of strategies to control black root rot caused by *T. basicola*.

References

Ahn, J.D., & Walton, J.D. (1997). A fatty acid synthase gene in *Cochliobolus carbonum* required for production of the plant pathogenicity factor HC-toxin, cyclo (D-prolyl-L-alanyl-D-alanyl-L-2-amino-9,10-epoxy-8-oxodecanoyl). *Molecular Plant-Microbe Interactions, 10*(2), 207–214.

Ahn, J.D., & Walton, J.D. (1998). Regulation of cyclic peptide biosynthesis and pathogenicity in *Cochliobolus carbonum* by TOXEp, a novel protein with a bZIP basic DNA-binding motif and four ankyrin repeats. *Molecular and General Genetics, 260*(5), 462–469.

Al-Jaaidi, S.S. (2007). *Transformation of Thielaviopsis basicola to study host-pathogen interactions,* (PhD thesis). Armidale, Australia: University of New England.

Allen, S.J. (1990). *Thielaviopsis basicola*, a new record on cotton in Australia. *Australasian Plant Pathology, 19*(1), 24-25.

Allen, S.J. (2001) Black root rot. In T.L. Kirkpatrick & C.S. Rothrock (Eds.), *Compendium of cotton diseases* (2nd ed., pp. 16–17). St Paul, MN: APS Press.

Amey, R.C., Athey-Pollard, A., Burns, C., Mills, P.R., Bailey, A., & Foster, G.D. (2002). PEG-mediated and *Agrobacterium*-mediated transformation in the mycopathogen *Verticillium fungicola*. *Mycological Research, 106*(1), 4-11.

Australian Cotton Cooperative Research Centre. (2000). *Cotton Information Sheet: Farm Hygiene for Disease and Weed Control - March 2000 – Come Clean Go Clean*. Retrieved from http://tools/cotton.crc.org.au/Assets/PDFFiles/FrmHygi.pdf

Baard, S.W., & Laubscher, C. (1985). Histopathology of blackhull incited by *Thielaviopsis basicola* in groundnuts. *Phytophylactica, 17*, 85-88.

Bai, D., Reedler, R., Brandle J.E., & Bai, D.P. (1996). Production and characterization of tobacco addition lines carrying *Nicotiana debneyi* chromosomes with a gene for resistance to black root rot. *Crop Science 36*(4), 852-857.

Bateman, D.F. (1962). Relation of Soil pH to Development of Poinsettia Root Rots. *Phytopathology 52*, 559-566.

Bowman, S.M., & Free, S.J. (2006). The structure and synthesis of the fungal cell wall. *BioEssays, 28*(8), 799-808.

Bowyer, P., Clarke, B.R., Lunness, P., Daniels, M.J., & Osbourn, A.E. (1995). Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. *Science, 267*(5196), 371–374.

CABI (2006). *Thielaviopsis basicola.* [Distribution map]. *Distribution Maps of Plant Diseases (October): Map 218 (Edition 4)*. Retrieved from http://www.cabi.org/?loadmodule=review&page=4050&reviewed=24931&site=165

Candole, B.L., & Rothrock, C.S. (1998). Using marked strains to assess the effect of hairy fetch amendment on the inoculum densities of *Thielaviopsis basicola*, *Pythium ultimum* and *Rhizoctonia solani*. *Soil Biology & Biochemistry, 30*(4), 443-448

Cheng, Y.Q., Ahn, J.H., & Walton, J.D. (1999). A putative branch-chainamino-acid transminase gene required for HC-toxin biosynthesis and pathogenicity in *Cochliobolus carbonum*. *Microbiology, 145*(12), 3539–3546.

Cheng, Y.Q., & Walton, J.D. (2000). A eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis. *Journal of Biological Chemistry, 275*(7), 4906–4911.

Chittaranjan, S., & Punja, Z.K. (1994). Factors influencing survival of phialospores of *Chalara elegans* in organic soil. *Plant Disease, 78*(4), 411-415.

Chumley, F.G., & Valent, B. (1990). Genetic analysis of melanin-deficient, noon-pathogenic mutants of *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions, 3*(3), 135-143.

Clayton, E.E. (1969). The study of resistance to the black root rot disease of tobacco. *Tobacco Science, 13*, 30-37.

Coumans, J.V.F., Harvey, J., Backhouse, D., Poljak, A., Raftery, M.J., Nehl, D., Katz, M.E., & Pereg, L. (2011). Proteomic assessment of host-associated microevolution in the fungus *Thielaviopsis basicola*. *Environmental Microbiology, 13*(3), 576-588.

Coumans, J.V.F., Moens, P.D.J., Poljak, A., Al-Jaaidi, S., Pereg, L., & Raftery, M.J. (2010). Plant-extract-induced changes in the proteome of the soil-borne pathogenic fungus *Thielaviopsis basicola*. *Proteomics, 10*(8), 1573-1591.

Cuiffetti, L.M., Manning, V.A., Pandelova, I., Figueroa Betts, F., & Martinez, P. (2010). Host-selective toxins, Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the *Pyrenophora tritici-repentis*–wheat interaction. *New Phytologist. 187*(4), 911–919.

Cuiffetti, L.M., Tuori, R.P., & Gaventa, J.M. (1997). A single gene encodes a selective toxin causal to the development of tan spot of wheat. *The Plant Cell, 9*(2), 135–144.

De Wit, P.J.G.M., Mehrabi, R., van den Burg, H.A., & Stergiopoulos, I. (2009). Fungal effector proteins: past, present and future. *Molecular Plant Pathology, 10*(6), 735–747.

Delgado, A., Franco, G.M., Paez, J.I., Vega, J.M., Carmona, E., & Aviles M. (2006). Incidence of cotton seedling diseases caused by *Rhizoctonia solani* and *Thielaviopsis basicola* in relation to previous crop, residue management and nutrients availability in soils in SW Spain. *Journal of Phytopathology, 154*(11/12), 710-714.

Delvecchio, V.G., Corbaz, R., & Turian, G. (1969). An ultrastructural study of the hyphae, endoconidia and chlamydospores of *Thielaviopsis basicola*. *Journal of General Microbiology, 58*, 23-27.

Desjardins, A.E., Hohn, T.M., & McCormick, S.P. (1992). Effect of gene disruption of trichodiene synthase on the virulence of *Gibberella pulicaris*. *Molecular Plant-Microbe Interactions, 5*(3), 214–222.

El-Hamalawi, Z. A. (2008a). Attraction, acquisition, retention and spatiotemporal distribution of soilborne plant pathogenic fungi by shore flies. *Annals of Applied Biology 152*(2), 169-177.

El-Hamalawi, Z. A. (2008b). Acquisition, retention and dispersal of soilborne plant pathogenic fungi by fungus gnats and moth flies. *Annals of Applied Biology 153*(2), 195-203.

van Esse, H.P., Bolton, M.D., Stergiopoulos, I., De Wit, P.J.G.M., & Thomma, B.P.H.J. (2007). The chitin-binding *Cladosporium fulvum* effector protein Avr4 is a virulence factor. *Molecular Plant-Microbe Interactions, 20*(9), 1092–1101.

van Esse, H.P., van't Klooster, J.W., Bolton, M.D., Yadeta, K., Van-Baarlen, P., Boeren, S., Vervoort, J., De Wit, P.J.G., & Thomma, B.P.H.J. (2008) The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense. *The Plant Cell*, *20*(7), 1948–1963.

Fichtner, E.J., Hesterberg, D.L., Smyth, T.J., & Shew, H.D. (2006). Differential Sensitivity of *Phytophthora parasitica* var. nicotianae and *Thielaviopsis basicola* to Monomeric Aluminum Species. *Phytopathology 96*(3): 212-220.

Fincham, J.R.S. (1989). Transformation in Fungi. *Microbiological Reviews, 53*(1), 148-170.

Fitzgerald, A.M., Mudge, A.M., Gleave, A.P., & Plummer, K.M. (2003). Agrobacterium and PEG-mediated transformation of the phytopathogen *Venturia inaequalis*. *Mycological Research, 107*(7), 803-810.

Frapolli, M., Defago, G., & Moenne-Loccoz, Y. (2010). Denaturing gradient gel electrophoretic analysis of dominant 2,4-diacetylphloroglucinol biosynthetic *phlD* alleles in fluorescent *Pseudomonas* from soils suppressive or conducive to black root rot of tobacco. *Soil Biology & Biochemistry, 42*(4): 649-656.

Friesen, T.L., Faris, J.D., Solomon, P.S., & Oliver, R.P. (2010). Host-specific toxins: effectors of necrotrophic pathogenicity. *Cellular Microbiology, 10*(7), 1421–1428.

Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., Rasmussen, J.B., Solomon, P.S., McDonald, B.A., & Oliver, R.P. (2006). Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics, 38*(8), 953–956.

Gayed, S.K. (1972). Host range and persistence of *Thielaviopsis basicola* in tobacco soil. *Canadian Journal of Plant Science, 52*(6), 869-873.

Geldenhuis, M.M., Roux, J., Cilliers, A.J., Wingfield, B.D., & Wingfield, M.J. (2006). Clonality in South African isolates and evidence for a European origin of the root pathogen *Thielaviopsis basicola*. *Mycological Research, 110*(3), 306-311.

Graham, J.H. (1991). Peat-based media as a source of *Thielaviopsis basicola* causing black root rot on citrus seedlings. *Plant Disease, 75*(12), 1246-1249.

Harris, L.J., Desjardins, A.E., Plattner, R.D., Nicholson, P., Butler, G., Young, J.C., Weston, G., Proctor, R.H., & Hohn, T.C. (1999). Possible role of trichothecene mycotoxins in virulence of *Fusarium graminearum* on maize. *Plant Disease, 83*(10), 954–960.

Harrison, U.J., & Shew, H.D. (2001). Effects of soil pH and nitrogen fertility on the population dynamics of *Thielaviopsis basicola*. *Plant and Soil, 228*(2), 147-155

Heath, M.C. (1991). The role of gene-for-gene interactions in the determination of host species specificity. *Phytopathology, 81*(2), 127-129

Hecht, E.I., & Bateman, D.F. (1964). Nonspecific acquired resistance to pathogens resulting from localized infections by *Thielaviopsis basicola* or viruses in tobacco leaves. *Phytopathology, 54*, 523-530

Holtz, B.A., & Weinhold, A.R. (1994). *Thielaviopsis basicola* in San Joaquin Valley soils and the relationship between inoculum density and disease severity of cotton seedlings. *Plant Disease, 78*(10), 986–990.

Hood, M.E., & Shew, H.D. (1997a). The influence of nutrients on development, resting hyphae and aleuriospore induction of *Thielaviopsis basicola*. *Mycologia*, *89*(5), 793–800.

Hood, M.E., & Shew, H.D. (1997b). Initial cellular interactions between *Thielaviopsis basicola* and tobacco root hairs. *Phytopathology*, *87*(3), 228–235.

Howell, C.R. (2003). Mechanisms employed by *Tricoderma* species in the biological control of plant diseases: The history and evolution of current concepts. *Plant Disease, 87*(1), 4-10.

Huang, H.C., & Patrick, Z.A. (1971). Variability of *Thielaviopsis basicola* in culture. *Canadian Journal of Botany, 49*(6), 1041-1047.

Huang, H.C., & Patrick Z.A. (1974). Karyology of conidiogenesis and endoconidium germination in *Thielaviopsis basicola*. *Canadian Journal of Botany. 52*(11), 2263-2267.

Idnurm, A., & Howlett, B.J. (2001). Pathogenicity genes of phytopathogenic fungi. *Molecular Plant Pathology, 2*(4), 241-255.

Jhorar, O. (2004). *Managing Black Root Rot of Cotton,* (DAN153). Narrabri, Australia: Cotton Research and Development Corporation.

Jones, B.L. (1991). Penetration and development of *Chalara elegans* in peanut (*Arachis hypogaea*). *Phytophylactica 23*(1), 81-84.

van Kan, J.A.L., van den Ackerveken, G.F.J.M., & De Wit, P.J.G.M. (1991). Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal pathogen *Cladosporium fulvum,* causal agent of tomato leaf mold. *Molecular Plant–Microbe Interactations, 4*(1), 52–59.

Kaufmann, D.D., & Williams, L.E (1964). Effect of mineral, fertilization and soil reaction on soil fungi. *Phytopathology, 54*, 134-139.

Keller, J.R., & Shanks, J.B. (1955). Poinsettia root rot. *Phytopathology, 45,* 552-558.

King, C.J., & Presley, J.T. (1942). A root rot of cotton caused by *Thielaviopsis basicola*. *Phytopathology, 32*, 752-761

Kirchner, J., & Roy, B. (2002). Evolutionary implications of host-pathogen specificity: fitness consequences of pathogen virulence traits. *Evolutionary Ecology Research, 4*(1), 27-48.

Kubo, Y., Suzuki, K., Furusawa, I., Ishida, N., & Yamamoto, M. (1982). Relation of appressoium pigmentation and penetration of nitrocellulose membranes by *Colletotrichum lagenarium*. *Phytopathology, 72*(5), 498–501.

Kubo, Y., Takano, Y., Endo, N., Yasuda, N., Tajima, S., & Furusawa, I. (1996). Cloning and structural analysis of the melanin biosynthesis gene *SCD1* encoding scytalone dehydatase in *Colletotrichum lagenarium*. *Applied and Environmental Microbiology, 62*(12), 4340–4344.

Kuwano, T., Shirataki, C., & Itoh, Y. (2008). Comparison between polyethylene glycol- and polyethylenimine-mediated transformation of *Aspergillus nidulans*. *Current Genetics 54*(2), 95-103.

Lambrechts, L. (2010). Dissecting the genetic architecture of host-pathogen specificity. *PLoS Pathogens, 6*(8), 1-3.

Lau, G.W., & Hamer, J.E. (1998). Acropetal: a genetic locus required for conidiophore architecture and pathogenicity in the rice blast fungus. *Fungal Genetics and Biology, 24*(1-2), 228–239.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago G., & Haas D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proceedings of the National Academy of Sciences of the United States of America, 89*(5), 1562-1566.

Lloyd, A.B., & Lockwood, J.L. (1963). Effect of soil temperature, host variety and fungus strain on *Thielaviopsis* root rot of peas. *Phytopathology, 53*, 329-331.

Louie, D., & Serwer, P. (1994). Quantifcation of the effect of excluded volume on double-stranded DNA. *Journal of Molecular Biology, 242*(4), 547–558.

Lucas, G.B. (1955). The cardinal temperatures and pH response of *Thielaviopsis basicola*. *Mycologia, 47*(6), 793-798.

Madigan, M.T., Martinko, J.M., & Parker, J. (2003). *Brock Biology of Microorganisms* (10th ed), New York, NY: Prentice-Hall.

Malardier, L., Daboussi, M.J., Julien, J., Roussel, F., Scazzocchio, C., & Brygoo, Y. (1989). Cloning of the nitrate reductase gene (*niaD*) of *Aspergillius nidulans* and its use for transformation of *Fusarium oxysporum*. *Gene, 78*(1), 147-156.

Matthiessen, J., & Kirkegaard, J.A. (2006). Biofumigation and enhanced biodegradation: Opportunity and challenge in soilborne pest and disease management. *Critical Reviews in Plant Sciences, 25*(3), 235-265.

Mendgen, K., & Hahn, M. (2002). Plant infection and the establishment of fungal biotrophy. *Trends in Plant Science, 7*(8), 352-6.

Meyer, J.R., & Shew, H.D. (1991). Soils suppressive to black root rot of burley tobacco caused by *Thielaviopsis basicola*. *Phytopathology, 81*(9), 946-954.

Meyer, J.R., Shew, H.D., & Harrison, U.J. (1994). Inhibition of germination and growth of *Thielaviopsis basicola* by aluminium. *Phytopathology, 84*(6), 598-602.

Michel, V.V. (2009). First report of *Chalara elegans* on roots of black elderberry. *Plant Disease 93*(9): 963.

Michielse, C.B., van Wijk, R., Reijnen, L., Manders, E.M.M., Boas, S., Olivain, C., Alabouvette, C., & Rep, M. (2009). The nuclear protein Sge1 of *Fusarium oxysporum* is required for parasitic growth. *PLoS Pathogens, 5*(10), e1000637.

Mims, C.W., Copes, W.E., & Richardson, E.A. (2000). Ultrastructure of the penetration and infection of pansy roots by *Thielaviopsis basicola*. *Phytopathology, 90*(8), 843-850.

Minchinton, E.J., & Mebalds, M.I. (2001). *Black root rot (Chalara): A survey of Australian nurseries,* (NY00007). Sydney, Australia: Horticulture Australia Limited.

Minton, E.B., Papavizas, G.C., & Lewis, J.A. (1982). Effects of fungicide seed treatments and seed quality on seedling diseases and yield of cotton. *Plant Disease, 66*(9), 832-835.

Mondal, A.H., Nehl, D.B., & Allen, S.J. (2004). First report of *Thielaviopsis basicola* on soybean in Australia. *Australasian Plant pathology, 33*(3), 451-452.

Nan, Z. B., Long, P.G., Skipp, R.A., & Hopcroft, D.H. (1992). Microscopy of invasion of red clover roots by *Trichocladium basicola*, and effects of benomyl and procloraz. *Plant Pathology, 41*(4), 449-461.

Nan, G.L., & Walbot, V. (2009). Plasmid rescue: recovery of flanking genomic sequences from transgenic transposon insertion sites. *Methods in Molecular Biology, 526*, 101-109.

National Cotton Council of America (2011). *Rankings.* Retrieved from http://www.cotton.org/econ/cropinfo/cropdata/rankings.cfm

Nehl, D.B. (2002). *Black root rot and slow early season growth of cotton,* (DAN122C). Narrabri, Australia: Cotton Research and Development Corporation.

Nehl, D.B., Allen, S.J., Mondal, A.H., & Lonergan, P.A. (2004). Black root rot: a pandemic in Australian cotton. *Australasian Plant Pathology, 33*(1), 87-95.

Nelson, E.B. (1990). Exudate molecules initiating fungal responses to seeds and roots. *Plant and Soil*, *129*(1), 61–73.

Niu, C., Lister, H.E., Nguyen, B., Wheeler, T.A., & Wright, R.J. (2008). Resistance to *Thielaviopsis basicola* in the cultivated A genome cotton. *Theoretical and Applied Genetics, 117*(8), 1313-1323.

O'Brien, R.G., & Davis, R.D. (1994). Lettuce black root rot – a disease caused by *Chalara elegans*. *Australasian Plant pathology, 23*(3), 106-111.

Office of the Gene Technology Regulator (OGTR). (2006). *Risk Assessment and Risk Management Plan for DIR 063/2005, Limited and controlled release of fungal resistant GM cotton, Applicant: Hexima Ltd.*. Canberra, Australia: Author.

Osbourn, A.E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. *The Plant Cell, 8*(10), 1821–1831.

Otani, Y. (1962). Studies on the black root rot disease caused by *Thielaviopsis basicola* (Berk. & Br.) Ferraris. Bull. *Okayama Tobacco Experiment Station, 23*, 1-118.

Oyarzun, P.J., Gerlagh, M., & Zadocks, J.C. (1998). Factors associated with soil receptivity to some fungal root rot pathogens of peas. *Applied Soil Ecology, 10*(1-2), 151-169.

Panaccione, D.G., Scott-Craig, J.S., Pocard, J.-A., & Walton, J.D. (1992). A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. *Proceedings of the National Academy of Sciences of the U.S.A, 89*, 6590–6594.

Papavizas, G.C. (1968). Survival of root-infecting fungi in soil. VI. Effect of amendments on bean root rot caused by *Thielaviopsis basicola* and on inoculum density of the causal organism. *Phytopathology, 58*, 421-428.

Papavizas, G.C., & Lewis J.A. (1971). Survival of endoconidia and chlamydospores of *Thielaviopsis basicola* as effected by soil environmental factors. *Phytopathology, 61*(1), 108-113.

Park, Y.J., Chen, X.B., & Punja, Z.K. (2006). Diversity, complexity and transmission of double-stranded RNA elements in *Chalara elegans* (synanam. *Thielaviopsis basicola*). *Mycological Research, 110*(Pt 6), 697-704.

Patrick, Z.A., Toussoun, T.A., & Thorpe, H.J. (1965). Germination of chlamydospores of *Thielaviopsis basicola*. *Phytopathology, 55*, 466-470.

Pattemore, J., & Aitken, E.A.B (2000). Genetic variation among populations of *Thielaviopsis basicola*, the causal agent of black root rot. *10th Australian Cotton Conference, Brisbane, QLD, Australia*, 499-505.

Paulin-Mahady, A.E, Harrington, T.C., & McNew, D. (2002). Phylogenetic and taxonomic evaluation of *Chalara, Chalaropsis* and *Thielaviopsis* anamorphs associated with *Ceratocystis*. *Mycologia, 94*(1), 62-72.

Paulitz, T., Nowak-Thompson, B., Gamard, P., Tsang, E., & Loper, J. (2000). A novel antifungal furanone from *Pseudomonas aureofaciens*, a biocontrol agent of fungal plant pathogens. *Journal of Chemical Ecology, 26*(6), 1515-1524.

Perpetua, N.S., Kubo, Y., Yasuda, N., Takano, Y., & Furusawa, I. (1996). Cloning and characterization of a melanin biosynthetic THR1 reductase gene essential for appressorial penetration of *Colletotrichum lagenarium*. *Molecular Plant-Microbe Interactions, 9*(5), 323–329.

Prell, H.H., & Day, P.R. (2001). *Plant-fungal pathogen interaction: a classical and molecular view*. New York, NY: Springer.

Proctor, R.H., Hohn, T.M., & McCormick, S.P. (1995). Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Molecular Plant-Microbe Interactions. 8*(4), 593–601.

Punja, Z.K. (1993). Influence of culture conditions on mycelial growth and phialospore production and germination in *Chalara elegans*. *Canadian Journal of Botany, 71*(3), 447-456.

Punja, Z.K. (1995). Influence of double-stranded RNAs on growth, sporulation, pathogenicity, and survival of *Chalara elegans*. *Canadian Journal of Botany, 73*(7), 1001-1009.

Punja, Z.K. (2004). Virulence of *Chalara elegans* on bean leaves, and host-tissue responses to infection. *Canadian Journal of Plant Pathology, 26*(1), 52-62.

Punja, Z.K., Chittaranjan, S., & Gaye, M.M. (1992). Development of black root rot caused by *Chalara elegans* on fresh market carrots. *Canadian Journal of Plant Pathology, 14*(4), 299-309.

Punja, Z. K., & Sun, L.J. (1999). Morphological and molecular characterization of *Chalara elegans* (*Thielaviopsis basicola*), cause of black root rot on diverse plant species. *Canadian Journal of Botany, 77*(12), 1801-1812.

Ramette, A., Moenne-Loccoz, Y., & Delfago, G. (2003). Prevalence of fluorescent *pseudomonads* producing antifungal phloroglucinols and/or hydrogen cyanide in soils naturally suppressive or conducive to tobacco black root rot. *FEMS Microbiology Ecology, 44*(1), 35-43.

Rasmussen, J.B., & Hanau, R.M. (1989). Exogenous scytalone restores appressorial melanization and pathogenicity in albino mutants of *Colletotrichum graminicola*. *Canadian Journal of Plant Pathology, 11*(4), 349–352.

Rogers, L.M., Flaishman, M.A., & Kolattukudy, P.E. (1994). Cutinase gene disruption in *Fusarium solani* f. sp. pisi decreases its virulence on pea. *The Plant Cell, 6*(7), 935–945.

Rogers, L.M., Kim, Y.K., Guo, W., González-Candelas, L., Li, D., & Kolattukudy, P.E. (2000). Requirement for either a host- or pectin induced pectate lyase for infection of *Pisum sativum* by *Nectria hematococca*. *Proceedings of the National Academy of Sciences of the U.S.A, 97*, 9813–9818.

Rohe, M., Searle, J., Newton, A.C., & Knogge, W. (1996). Transformation of the plant pathogenic fungus, *Rhynchosporium secalis*. *Current Genetics, 29*(6), 587-590.

Rothrock, C.S. (1992). Infuence of soil temperature, water, and texture on *Thielaviopsis basicola* and black root rot of cotton. *Phytopathology, 82*(10), 1202–1206.

Sala, F.C., da Costa, C.P., Teixeira, L.D., Fabri, E.G., & Blat, S.F. (2008). Lettuce reaction to black root rot caused by *Thielaviospsis basicola*. *Horticultura Brasileira 26*(3), 398-400.

Schippers, B. (1970). Survival of endoconidia of *Thielaviopsis basicola* in soil. *Netherlands Journal of Plant Pathology, 76*, 206-211.

Schoina, C., Stringlis, I.A., Pantelides, I.S., Tjamos, S.E., & Paplomatas, E.J. (2011). Evaluation of application methods and biocontrol efficacy of *Paenibacillus alvei* strain K-165, against the cotton black root rot pathogen *Thielaviopsis basicola*. *Biological Control, 58*(1), 68-73.

Sexton, A.C., & Howlett, B.J. (2001). Green fluorescent protein as a reporter in the *Brassica-Leptosphaeria maculans* interaction. *Physiological and Molecular Plant Pathology, 58*, 13-21.

Shew, H.D., & Meyer, J.R. (1992). Thielaviopsis. In L.L Singleton, J.D. Mihail, & C.M. Rush (Eds.), *Methods for Research on Soilborne Pathogenic Fungi*, (pp. 171-174). St Paul, MN: APS Press.

Simmonds, J.H. (1966). *Host index of Plant Diseases in Queensland*. Brisbane, Australia: Queensland Department of Primary Industries.

Skamnioti, P., & Gurr, S.J. (2007). *Magnaporthe grisea* Cutinase2 mediates appressorium differentiation and host penetration and is required for full virulence. *Plant Cell*, *19*(8), 2674–2689.

Smith, B.J., & Kirkegaard, J.A. (2002). *In vitro* inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. *Plant Pathology, 51*(5), 585-593.

Stutz, E., Kahr, G., & Defago, G. (1989). Clays involved in suppression of tobacco black root rot by a strain of *Pseudomonas fluorescens*. *Soil Biology and Biochemistry, 21*(3), 361-366.

Takano, Y., Kubo, Y., Shimizu, K., Mise, K., Okuno, T., & Furusawa, T. (1995). Structural analysis of *PKS1*, a polyketide synthase gene involved in melanin biosynthesis in *Colletotrichum lagenarium*. *Molecular and General Genetics, 249*(2), 162–167.

Talbot, N.J., Ebbole, D.J., & Hamer, J.E. (1993). Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *The Plant Cell, 5*(11), 1575–1590.

The Royal Botanic Gardens and Domain Trust (2010). *Thielaviopsis diseases*. Retrieved from http://www.rbgsyd.nsw.gov.au/science/plant_pathology_research/Soilborne_plant_diseases/Vietnam_template3/Thielaviopsis_Diseases

Toksoz, H., Rothrock, C.S., & Kirkpatrick, T.L. (2009). Efficacy of seed treatment chemicals for black root rot, caused by *Thielaviopsis basicola*, on cotton. *Plant Disease, 93*(4), 354-362.

Tsao, P.H., & Bricker, J.L. (1966). Chlamydospores of *Thielaviopsis basicola* as surviving propagules in natural soils. *Phytopathology, 56*, 1012-1014

Walker, N.R., Kirkpatrick, T.L., & Rothrock, C.S. (1998). Interaction between *Meloidogyne incognita* and *Thielaviopsis basicola* on cotton (*Gossypium hirsutum*). *Journal of Nematology, 30*(4), 415–422.

Walker, N.R., Kirkpatrick, T.L., & Rothrock, C.S. (2000). Influence of *Meloidogyne incognita* and *Thielaviopsis basicola* populations on early-season disease development and cotton growth. *Plant Disease, 84*(4), 449–453.

Wang, H., & Davis, R.M. (1997). Susceptibility of selected cotton cultivars to seedling disease pathogens and benefits of chemical seed treatments. *Plant Disease, 81*(9), 1085–1088.

Wei, Y.M., Zhou, X.W., Liu, L., Lu, J., Wang, Z.N., Yu, G., Hu, L.C., Lin, J., Sun, X.F., & Tang, K.X. (2010). An efficient transformation system of taxol-producing endophytic fungus EFY-21 (*Ozonium* sp.). *African Journal of Biotechnology 9*(12), 1726-1733.

Wheeler, T.A., & Gannaway, J.R. (2007). *1644 Identifcation of germplasm resistant to Thielaviopsis basicola in the USDA cotton germplasm collection*. Retrieved from http://icac.org/meetings/wcrc/wcrc4/presentations/data/papers/Paper1644.pdf

Wilkinson, C.A., Rufty, R.C., & Shew, H.D. (1991). Inheritance of partial resistance to black root rot in burley tobacco. *Plant Disease, 75*(9), 889-892.

Wolkow, P.M., Sisler, H.D. and Vigil, E.L. (1983). Effect of inhibitors of melanin biosynthesis on structure and function of appressoria of *Colletotrichum lindemuthianum*. *Physiological Plant Pathology, 23*(1), 55–71.

Yakoby, N., Beno-Moualem, D., Keen, D.T., Dinoor, A., Pines, O., & Prusky, D. (2001). *Colletotrichum gloeosporioides pelB*, encoding pectate lyase, is a key gene in fungal–fruit interactions. *Molecular Plant-Microbe Interactions, 14*(8), 988–995.

Yarwood, C.E. (1974). Habitats of *Thielaviopsis* in California. *The Plant Disease Reporter, 58*, 54-56.

Yarwood, C.E. (1981). The occurrence of *Chalara elegans*. *Mycologia, 73*(3), 524-530.

Yi, L., Xiao, C., Ma, G., Wu, Q., & Dou, Y. (2010). Biocontrol effect and inhibition activity of antagonistic actinomycetes strain TA21 against *Thielaviopsis basicola*. *Chinese Journal of Biological Control, 26*(2), 186-192.

Zhou, D.Q., & Hyde, K.D. (2001). Host-specificity, host-exclusivity, and host-recurrence in saprobic fungi. *Mycological Research*, 105(12), 1449-1457.

Zhou, X., Wei, Y., Zhu, H., Wang, Z., Lin, J., Liu, L., & Tang, K. (2008). Protoplast formation, regeneration and transformation from the taxol-producing fungus *Ozonium* sp.. *African Journal of Biotechnology, 7*(12), 2017-2024.

Appendix 1: Composition of media

Osmotic Medium

(1.2 M $MgSO_4$, 10 mM NaOP, pH 5.8)

1 M NaH_2PO_4 and 1 M Na_2HPO_4 are combined to produce 1 M NaOP buffer with pH 7.0.

For 100 mL of osmotic medium:

29.57 g of $MgSO_4.7H_2O$ was dissolved in milliQ H_2O to an overall volume of approx. 80 mL.

1 mL of 1 M NaOP buffer (pH 7.0) was added.

The pH was adjusted to 5.8 with 0.2 M Na_2HPO_4.

The volume was made up to 100 mL with milliQ H_2O.

This solution was sterilized by filtration using a 0.45µm filter (Millipore).

½ Potato Dextrose Agar (½ PDA)

Potato Dextrose Agar (Oxoid)	19.5 g/L of dH_2O
Agar Bacteriological (Oxoid)	4.5 g/L of dH_2O for 1.2% agar or
	14.5 g/L of dH_2O for 2.2% agar

Immediately autoclaved for 20 min at 121^oC.

Potato Dextrose Broth (PDB)

Potato Dextrose Broth (Difco)	24 g/L of dH_2O

Immediately autoclaved for 20 min at 121^oC.

The 4 g of potato starch/L of dH_2O provided by this product 'approximates 200 g of infusion from potatoes' (Difco).

Regeneration Solid Media

(½ PDA (1% agar) containing 1 M sucrose)

Potato Dextrose Agar (Oxoid)	19.5 g/L of dH_2O
Agar Bacteriological (Oxoid)	2.5 g/L of dH_2O
Sucrose	342.3 g/L of dH_2O

Immediately autoclaved for 20 min at 121^oC.

Regeneration Broth

(PDB / 0.5 M sucrose)

Potato Dextrose Broth (Difco)	24 g/L of dH_2O
Sucrose	171.2 g/L of dH_2O

Immediately autoclaved for 20 min at 121°C.

Water Agar (1.2% agar)

Agar Bacteriological (Oxoid)	12 g/L of dH_2O

Immediately autoclaved for 20 min at 121°C.

Yeast Mannitol Agar (YMA)

Yeast Extract (Difco)	1 g/L of dH_2O
Mannitol	10 g/L of dH_2O
K_2HPO_4	0.5 g/L of dH_2O
$MgSO_4.7H_2O$	0.2 g/L of dH_2O
Agar Bacteriological (Oxoid)	15 g/L of dH_2O

Immediately autoclaved for 20 min at 121°C.

2 × Yeast Tryptone (YT) (1.6% agar)

Bacto Tryptone (Difco)	16 g/L of dH_2O
Yeast Extract (Difco)	10 g/L of dH_2O
NaCl	5 g/L of dH_2O
Agar Bacteriological (Oxoid)	16 g/L of dH_2O

Immediately autoclaved for 20 min at 121°C.

Yeast Tryptone (YT) Broth

Bacto Tryptone (Difco)	8 g/L of dH_2O
Yeast Extract (Difco)	5 g/L of dH_2O
NaCl	5 g/L of dH_2O

Immediately autoclaved for 20 min at 121°C.

Appendix 2: BLASTN results for putative ITS sequences

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BLASTN 1.8.4-Paracel [2010-10-31]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.
```

<u>*T. basicola* BRIP40192 wild-type putative ITS sequence</u>

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|67551221|gb|DQ059579.1| Thielaviopsis basicola 18S ribosomal ...   987   0.0
gi|193872640|gb|EU794001.1| Thielaviopsis basicola isolate T34 1...   967   0.0
gi|2160765|gb|U97334.1|U97334 Thielaviopsis basicola 18S ribosom...   965   0.0
gi|239737048|gb|GQ131525.1| Uncultured soil fungus isolate DGGE ...   963   0.0
gi|8778139|gb|AF275490.1| Thielaviopsis basicola strain C1602 in...   872   0.0
gi|8778131|gb|AF275482.1| Theilaviopsis basicola strain C1373 in...   872   0.0
gi|8778130|gb|AF275481.1| Theilaviopsis basicola strain C1372 in...   872   0.0
gi|8778142|gb|AF275493.1| Thielaviopsis basicola strain C185 int...   858   0.0
gi|8778143|gb|AF275494.1| Theilaviopsis basicola internal transc...   856   0.0
gi|83779372|gb|DQ318204.1| Ceratocystis fimbriata strain WIN(M) ...   779   0.0
gi|8778140|gb|AF275491.1| Thielaviopsis thielavioides strain C16...   688   0.0
gi|8778137|gb|AF275488.1| Thielaviopsis thielavioides strain C14...   688   0.0
gi|8778136|gb|AF275487.1| Thielaviopsis thielavioides strain C13...   688   0.0
gi|8778135|gb|AF275486.1| Thielaviopsis thielavioides strain C13...   688   0.0
gi|8778138|gb|AF275489.1| Thielaviopsis thielavioides strain C15...   680   0.0
gi|8778134|gb|AF275485.1| Thielaviopsis thielavioides strain C13...   680   0.0
gi|8778127|gb|AF275478.1| Thielaviopsis thielavioides internal t...   680   0.0
gi|8778129|gb|AF275480.1| Thielaviopsis populi strain C1369 inte...   672   0.0
gi|8778128|gb|AF275479.1| Thielaviopsis populi strain C1368 inte...   672   0.0
gi|37695609|gb|AY423551.1| Thielaviopsis populi strain C2049 int...   672   0.0
gi|8778133|gb|AF275484.1| Thielaviopsis ovoidea strain C1376 int...   648   0.0
gi|8778132|gb|AF275483.1| Thielaviopsis ovoidea strain C1375 int...   648   0.0
gi|83779373|gb|DQ318205.1| Ceratocystis resinifera strain WIN(M)...   634   e-178
gi|83779365|gb|DQ318197.1| Ceratocystis coerulescens strain WIN(...   634   e-178
gi|83779371|gb|DQ318203.1| Ceratocystis paradoxa strain WIN(M) 9...   575   e-161
gi|2267207|gb|U75621.1|CRU75621 Ceratocystis rufipenni internal ...   567   e-158
gi|2267206|gb|U75620.1|CRU75620 Ceratocystis rufipenni internal ...   567   e-158
gi|2267205|gb|U75619.1|CRU75619 Ceratocystis rufipenni internal ...   567   e-158
gi|2865507|gb|AF043606.1|AF043606 Ceratocystis adiposa internal ...   561   e-156
gi|2865508|gb|AF043607.1|AF043607 Ceratocystis paradoxa internal...   551   e-153



>gi|67551221|gb|DQ059579.1| Thielaviopsis basicola 18S ribosomal RNA
           gene, partial sequence; internal transcribed spacer 1,
           5.8S ribosomal RNA gene, and internal transcribed spacer
           2, complete sequence; and 28S ribosomal RNA gene,
           partial sequence
          Length = 554

 Score =  987 bits (498), Expect = 0.0
 Identities = 498/498 (100%)
 Strand = Plus / Plus
```

```
>gi|193872640|gb|EU794001.1| Thielaviopsis basicola isolate T34 18S
           ribosomal RNA gene, partial sequence; internal
           transcribed spacer 1, 5.8S ribosomal RNA gene, and
           internal transcribed spacer 2, complete sequence; and
           28S ribosomal RNA gene, partial sequence
          Length = 517

 Score =  967 bits (488), Expect = 0.0
 Identities = 488/488 (100%)
 Strand = Plus / Plus

>gi|2160765|gb|U97334.1|U97334 Thielaviopsis basicola 18S ribosomal
           RNA gene, partial sequence, internal transcribed spacer
           1, 5.8S ribosomal RNA gene, complete sequence, internal
           transcribed spacer 2 and 25S ribosomal RNA gene, partial
           sequence
          Length = 557

 Score =  965 bits (487), Expect = 0.0
 Identities = 494/495 (99%), Gaps = 1/495 (0%)
 Strand = Plus / Plus

>gi|239737048|gb|GQ131525.1| Uncultured soil fungus isolate DGGE gel
           band 12a 18S ribosomal RNA gene, partial sequence;
           internal transcribed spacer 1, 5.8S ribosomal RNA gene,
           and internal transcribed spacer 2, complete sequence;
           and 28S ribosomal RNA gene, partial sequence
          Length = 562

 Score =  963 bits (486), Expect = 0.0
 Identities = 492/494 (99%)
 Strand = Plus / Plus

>gi|8778139|gb|AF275490.1| Thielaviopsis basicola strain C1602
           internal transcribed spacer 1, partial sequence; 5.8S
           ribosomal RNA gene, complete sequence; and internal
           transcribed spacer 2, partial sequence
          Length = 466

 Score =  872 bits (440), Expect = 0.0
 Identities = 446/448 (99%)
 Strand = Plus / Plus
```

<u>Transformant G7-2 putative ITS sequence</u>

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                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|67551221|gb|DQ059579.1| Thielaviopsis basicola 18S ribosomal ...  1003   0.0
gi|2160765|gb|U97334.1|U97334 Thielaviopsis basicola 18S ribosom...   981   0.0
gi|239737048|gb|GQ131525.1| Uncultured soil fungus isolate DGGE ...   977   0.0
gi|193872640|gb|EU794001.1| Thielaviopsis basicola isolate T34 1...   967   0.0
gi|8778139|gb|AF275490.1| Thielaviopsis basicola strain C1602 in...   872   0.0
gi|8778131|gb|AF275482.1| Theilaviopsis basicola strain C1373 in...   872   0.0
gi|8778130|gb|AF275481.1| Theilaviopsis basicola strain C1372 in...   872   0.0
gi|8778142|gb|AF275493.1| Thielaviopsis basicola strain C185 int...   858   0.0
gi|8778143|gb|AF275494.1| Theilaviopsis basicola internal transc...   856   0.0
gi|83779372|gb|DQ318204.1| Ceratocystis fimbriata strain WIN(M) ...   797   0.0
gi|8778140|gb|AF275491.1| Thielaviopsis thielavioides strain C16...   688   0.0
gi|8778137|gb|AF275488.1| Thielaviopsis thielavioides strain C14...   688   0.0
gi|8778136|gb|AF275487.1| Thielaviopsis thielavioides strain C13...   688   0.0
gi|8778135|gb|AF275486.1| Thielaviopsis thielavioides strain C13...   688   0.0
gi|8778138|gb|AF275489.1| Thielaviopsis thielavioides strain C15...   680   0.0
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gi|8778134|gb|AF275485.1| Thielaviopsis thielavioides strain C13...   680   0.0
gi|8778127|gb|AF275478.1| Thielaviopsis thielavioides internal t...   680   0.0
gi|8778129|gb|AF275480.1| Thielaviopsis populi strain C1369 inte...   672   0.0
gi|8778128|gb|AF275479.1| Thielaviopsis populi strain C1368 inte...   672   0.0
gi|37695609|gb|AY423551.1| Thielaviopsis populi strain C2049 int...   672   0.0
gi|83779373|gb|DQ318205.1| Ceratocystis resinifera strain WIN(M)...   652   0.0
gi|83779365|gb|DQ318197.1| Ceratocystis coerulescens strain WIN(...   652   0.0
gi|8778133|gb|AF275484.1| Thielaviopsis ovoidea strain C1376 int...   648   0.0
gi|8778132|gb|AF275483.1| Thielaviopsis ovoidea strain C1375 int...   648   0.0
gi|83779371|gb|DQ318203.1| Ceratocystis paradoxa strain WIN(M) 9...   593   e-166
gi|2865507|gb|AF043606.1|AF043606 Ceratocystis adiposa internal ...   573   e-160
gi|2267207|gb|U75621.1|CRU75621 Ceratocystis rufipenni internal ...   567   e-158
gi|2267206|gb|U75620.1|CRU75620 Ceratocystis rufipenni internal ...   567   e-158
gi|2267205|gb|U75619.1|CRU75619 Ceratocystis rufipenni internal ...   567   e-158
gi|2865508|gb|AF043607.1|AF043607 Ceratocystis paradoxa internal...   563   e-157
```

Transformant G8-1 putative ITS sequence

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|67551221|gb|DQ059579.1| Thielaviopsis basicola 18S ribosomal ...  1009   0.0
gi|2160765|gb|U97334.1|U97334 Thielaviopsis basicola 18S ribosom...   987   0.0
gi|239737048|gb|GQ131525.1| Uncultured soil fungus isolate DGGE ...   977   0.0
gi|193872640|gb|EU794001.1| Thielaviopsis basicola isolate T34 1...   967   0.0
gi|8778139|gb|AF275490.1| Thielaviopsis basicola strain C1602 in...   872   0.0
gi|8778131|gb|AF275482.1| Theilaviopsis basicola strain C1373 in...   872   0.0
gi|8778130|gb|AF275481.1| Theilaviopsis basicola strain C1372 in...   872   0.0
gi|8778142|gb|AF275493.1| Thielaviopsis basicola strain C185 int...   858   0.0
gi|8778143|gb|AF275494.1| Theilaviopsis basicola internal transc...   856   0.0
gi|83779372|gb|DQ318204.1| Ceratocystis fimbriata strain WIN(M) ...   801   0.0
gi|8778140|gb|AF275491.1| Thielaviopsis thielavioides strain C16...   688   0.0
gi|8778137|gb|AF275488.1| Thielaviopsis thielavioides strain C14...   688   0.0
gi|8778136|gb|AF275487.1| Thielaviopsis thielavioides strain C13...   688   0.0
gi|8778135|gb|AF275486.1| Thielaviopsis thielavioides strain C13...   688   0.0
gi|8778138|gb|AF275489.1| Thielaviopsis thielavioides strain C15...   680   0.0
gi|8778134|gb|AF275485.1| Thielaviopsis thielavioides strain C13...   680   0.0
gi|8778127|gb|AF275478.1| Thielaviopsis thielavioides internal t...   680   0.0
gi|8778129|gb|AF275480.1| Thielaviopsis populi strain C1369 inte...   672   0.0
gi|8778128|gb|AF275479.1| Thielaviopsis populi strain C1368 inte...   672   0.0
gi|37695609|gb|AY423551.1| Thielaviopsis populi strain C2049 int...   672   0.0
gi|83779373|gb|DQ318205.1| Ceratocystis resinifera strain WIN(M)...   656   0.0
gi|83779365|gb|DQ318197.1| Ceratocystis coerulescens strain WIN(...   656   0.0
gi|8778133|gb|AF275484.1| Thielaviopsis ovoidea strain C1376 int...   648   0.0
gi|8778132|gb|AF275483.1| Thielaviopsis ovoidea strain C1375 int...   648   0.0
gi|83779371|gb|DQ318203.1| Ceratocystis paradoxa strain WIN(M) 9...   597   e-167
gi|2865507|gb|AF043606.1|AF043606 Ceratocystis adiposa internal ...   573   e-160
gi|2267207|gb|U75621.1|CRU75621 Ceratocystis rufipenni internal ...   567   e-158
gi|2267206|gb|U75620.1|CRU75620 Ceratocystis rufipenni internal ...   567   e-158
gi|2267205|gb|U75619.1|CRU75619 Ceratocystis rufipenni internal ...   567   e-158
gi|2865508|gb|AF043607.1|AF043607 Ceratocystis paradoxa internal...   563   e-157
```

Transformant G16-1 putative ITS sequence

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|67551221|gb|DQ059579.1| Thielaviopsis basicola 18S ribosomal ...  1003   0.0
gi|2160765|gb|U97334.1|U97334 Thielaviopsis basicola 18S ribosom...   981   0.0
gi|239737048|gb|GQ131525.1| Uncultured soil fungus isolate DGGE ...   977   0.0
gi|193872640|gb|EU794001.1| Thielaviopsis basicola isolate T34 1...   967   0.0
gi|8778139|gb|AF275490.1| Thielaviopsis basicola strain C1602 in...   872   0.0
gi|8778131|gb|AF275482.1| Theilaviopsis basicola strain C1373 in...   872   0.0
gi|8778130|gb|AF275481.1| Theilaviopsis basicola strain C1372 in...   872   0.0
```

```
gi|8778142|gb|AF275493.1| Thielaviopsis basicola strain C185 int...   858   0.0
gi|8778143|gb|AF275494.1| Theilaviopsis basicola internal transc...   856   0.0
gi|83779372|gb|DQ318204.1| Ceratocystis fimbriata strain WIN(M) ...   795   0.0
gi|8778140|gb|AF275491.1| Thielaviopsis thielavioides strain C16...   688   0.0
gi|8778137|gb|AF275488.1| Thielaviopsis thielavioides strain C14...   688   0.0
gi|8778136|gb|AF275487.1| Thielaviopsis thielavioides strain C13...   688   0.0
gi|8778135|gb|AF275486.1| Thielaviopsis thielavioides strain C13...   688   0.0
gi|8778138|gb|AF275489.1| Thielaviopsis thielavioides strain C15...   680   0.0
gi|8778134|gb|AF275485.1| Thielaviopsis thielavioides strain C13...   680   0.0
gi|8778127|gb|AF275478.1| Thielaviopsis thielavioides internal t...   680   0.0
gi|8778129|gb|AF275480.1| Thielaviopsis populi strain C1369 inte...   672   0.0
gi|8778128|gb|AF275479.1| Thielaviopsis populi strain C1368 inte...   672   0.0
gi|37695609|gb|AY423551.1| Thielaviopsis populi strain C2049 int...   672   0.0
gi|83779373|gb|DQ318205.1| Ceratocystis resinifera strain WIN(M)...   650   0.0
gi|83779365|gb|DQ318197.1| Ceratocystis coerulescens strain WIN(...   650   0.0
gi|8778133|gb|AF275484.1| Thielaviopsis ovoidea strain C1376 int...   648   0.0
gi|8778132|gb|AF275483.1| Thielaviopsis ovoidea strain C1375 int...   648   0.0
gi|83779371|gb|DQ318203.1| Ceratocystis paradoxa strain WIN(M) 9...   591   e-165
gi|2865507|gb|AF043606.1|AF043606 Ceratocystis adiposa internal ...   573   e-160
gi|2267207|gb|U75621.1|CRU75621 Ceratocystis rufipenni internal ...   567   e-158
gi|2267206|gb|U75620.1|CRU75620 Ceratocystis rufipenni internal ...   567   e-158
gi|2267205|gb|U75619.1|CRU75619 Ceratocystis rufipenni internal ...   567   e-158
gi|2865508|gb|AF043607.1|AF043607 Ceratocystis paradoxa internal...   563   e-157
```

<u>Transformant G17-2 putative ITS sequence</u>

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|67551221|gb|DQ059579.1| Thielaviopsis basicola 18S ribosomal ...   987   0.0
gi|2160765|gb|U97334.1|U97334 Thielaviopsis basicola 18S ribosom...   965   0.0
gi|239737048|gb|GQ131525.1| Uncultured soil fungus isolate DGGE ...   961   0.0
gi|193872640|gb|EU794001.1| Thielaviopsis basicola isolate T34 1...   952   0.0
gi|8778139|gb|AF275490.1| Thielaviopsis basicola strain C1602 in...   856   0.0
gi|8778131|gb|AF275482.1| Theilaviopsis basicola strain C1373 in...   856   0.0
gi|8778130|gb|AF275481.1| Theilaviopsis basicola strain C1372 in...   856   0.0
gi|8778142|gb|AF275493.1| Thielaviopsis basicola strain C185 int...   842   0.0
gi|8778143|gb|AF275494.1| Theilaviopsis basicola internal transc...   841   0.0
gi|83779372|gb|DQ318204.1| Ceratocystis fimbriata strain WIN(M) ...   779   0.0
gi|8778140|gb|AF275491.1| Thielaviopsis thielavioides strain C16...   672   0.0
gi|8778137|gb|AF275488.1| Thielaviopsis thielavioides strain C14...   672   0.0
gi|8778136|gb|AF275487.1| Thielaviopsis thielavioides strain C13...   672   0.0
gi|8778135|gb|AF275486.1| Thielaviopsis thielavioides strain C13...   672   0.0
gi|8778138|gb|AF275489.1| Thielaviopsis thielavioides strain C15...   664   0.0
gi|8778134|gb|AF275485.1| Thielaviopsis thielavioides strain C13...   664   0.0
gi|8778127|gb|AF275478.1| Thielaviopsis thielavioides internal t...   664   0.0
gi|8778129|gb|AF275480.1| Thielaviopsis populi strain C1369 inte...   656   0.0
gi|8778128|gb|AF275479.1| Thielaviopsis populi strain C1368 inte...   656   0.0
gi|37695609|gb|AY423551.1| Thielaviopsis populi strain C2049 int...   656   0.0
gi|83779373|gb|DQ318205.1| Ceratocystis resinifera strain WIN(M)...   650   0.0
gi|83779365|gb|DQ318197.1| Ceratocystis coerulescens strain WIN(...   650   0.0
gi|8778133|gb|AF275484.1| Thielaviopsis ovoidea strain C1376 int...   632   e-178
gi|8778132|gb|AF275483.1| Thielaviopsis ovoidea strain C1375 int...   632   e-178
gi|83779371|gb|DQ318203.1| Ceratocystis paradoxa strain WIN(M) 9...   587   e-164
gi|2865507|gb|AF043606.1|AF043606 Ceratocystis adiposa internal ...   573   e-160
gi|2267207|gb|U75621.1|CRU75621 Ceratocystis rufipenni internal ...   567   e-158
gi|2267206|gb|U75620.1|CRU75620 Ceratocystis rufipenni internal ...   567   e-158
gi|2267205|gb|U75619.1|CRU75619 Ceratocystis rufipenni internal ...   567   e-158
gi|2865508|gb|AF043607.1|AF043607 Ceratocystis paradoxa internal...   563   e-157
```

Appendix 3: FASTA: DNA:DNA comparison of ITS sequences

fasta36 -n -q -w 80 -m 6 -z 11 -Z 10000 -f -12 -g -4 -r +5/-4 TMP.q TMP.q2 6

```
FASTA searches a protein or DNA sequence data bank
 version 36.3.5a Jun, 2011(preload8)
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448
```

<u>*T. basicola* BRIP40192 wild-type (WT_H08) and transformant G7-2 (G7_H10)</u>

```
>>G7_H10                                                                   (515 nt)
 initn: 2490 init1: 2490 opt: 2490  Z-score: 2470.9  bits: 466.8 E(10000): 8e-136
banded Smith-Waterman score: 2490; 100.0% identity (100.0% similar) in 498 nt overlap (1-498:1-
498)

               10        20        30        40        50        60        70        80
WT_H08 TCTTAACCATATGTGAACGTACCTTTTCTAGCTGCTTTGGCAGGTGCCTCTCGGGGCTTCTGCCGGTAGCATTTATAAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G7_H10 TCTTAACCATATGTGAACGTACCTTTTCTAGCTGCTTTGGCAGGTGCCTCTCGGGGCTTCTGCCGGTAGCATTTATAAAC
               10        20        30        40        50        60        70        80


               90       100       110       120       130       140       150       160
WT_H08 TCTTTATATTTCTATAGAATTATTCATTGCTGAGTGGCATTAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G7_H10 TCTTTATATTTCTATAGAATTATTCATTGCTGAGTGGCATTAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGG
               90       100       110       120       130       140       150       160

              170       180       190       200       210       220       230       240
WT_H08 CTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G7_H10 CTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
              170       180       190       200       210       220       230       240

              250       260       270       280       290       300       310       320
WT_H08 CGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCTCTGCTTGGTGTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G7_H10 CGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCTCTGCTTGGTGTTGG
              250       260       270       280       290       300       310       320

              330       340       350       360       370       380       390       400
WT_H08 AGGACCCGCGTTTAGTCGCGGGCCGCCGAAATGCATCGGCTGTTGTATATACAGCTTCCCTGTGTAGTAAATGCTTAGCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G7_H10 AGGACCCGCGTTTAGTCGCGGGCCGCCGAAATGCATCGGCTGTTGTATATACAGCTTCCCTGTGTAGTAAATGCTTAGCT
              330       340       350       360       370       380       390       400

              410       420       430       440       450       460       470       480
WT_H08 TTACACTTTGAAACTTTTATATAACATGCCGAAAAACCCTCAACTTTTGAAAGGTTGACCTCGGATCAGGTAGGAATACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G7_H10 TTACACTTTGAAACTTTTATATAACATGCCGAAAAACCCTCAACTTTTGAAAGGTTGACCTCGGATCAGGTAGGAATACC
              410       420       430       440       450       460       470       480

              490
WT_H08 CGCTGAACTTAAGCATAT
       ::::::::::::::::::
G7_H10 CGCTGAACTTAAGCATATCAATAAGCCGGAGGAAA
              490       500       510
```

<u>*T. basicola* BRIP40192 wild-type (WT_H08) and transformant G8-1 (G8_H09)</u>

```
>>G8_H09                                                                   (509 nt)
 initn: 2490 init1: 2490 opt: 2490  Z-score: 3408.9  bits: 640.3 E(10000): 4.6e-188
banded Smith-Waterman score: 2490; 100.0% identity (100.0% similar) in 498 nt overlap (1-498:1-
498)

              10        20        30        40        50        60        70        80
WT_H08 TCTTAACCATATGTGAACGTACCTTTTCTAGCTGCTTTGGCAGGTGCCTCTCGGGGCTTCTGCCGGTAGCATTTATAAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G8_H09 TCTTAACCATATGTGAACGTACCTTTTCTAGCTGCTTTGGCAGGTGCCTCTCGGGGCTTCTGCCGGTAGCATTTATAAAC
              10        20        30        40        50        60        70        80

              90       100       110       120       130       140       150       160
WT_H08 TCTTTATATTTCTATAGAATTATTCATTGCTGAGTGGCATTAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G8_H09 TCTTTATATTTCTATAGAATTATTCATTGCTGAGTGGCATTAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGG
              90       100       110       120       130       140       150       160

             170       180       190       200       210       220       230       240
WT_H08 CTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G8_H09 CTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
             170       180       190       200       210       220       230       240

             250       260       270       280       290       300       310       320
WT_H08 CGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCTCTGCTTGGTGTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G8_H09 CGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCTCTGCTTGGTGTTGG
             250       260       270       280       290       300       310       320


             330       340       350       360       370       380       390       400
WT_H08 AGGACCCGCGTTTAGTCGCGGGCCGCCGAAATGCATCGGCTGTTGTATATACAGCTTCCCTGTGTAGTAAATGCTTAGCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G8_H09 AGGACCCGCGTTTAGTCGCGGGCCGCCGAAATGCATCGGCTGTTGTATATACAGCTTCCCTGTGTAGTAAATGCTTAGCT
             330       340       350       360       370       380       390       400

             410       420       430       440       450       460       470       480
WT_H08 TTACACTTTGAAACTTTTATATAACATGCCGAAAAACCCTCAACTTTTGAAAGGTTGACCTCGGATCAGGTAGGAATACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G8_H09 TTACACTTTGAAACTTTTATATAACATGCCGAAAAACCCTCAACTTTTGAAAGGTTGACCTCGGATCAGGTAGGAATACC
             410       420       430       440       450       460       470       480

             490
WT_H08 CGCTGAACTTAAGCATAT
       ::::::::::::::::::
G8_H09 CGCTGAACTTAAGCATATCAATAAGCGGA
             490       500
```

<u>*T. basicola* BRIP40192 wild-type (WT_H08) and transformant G16-1 (G16_H11)</u>

```
>>G16_H11                                                               (514 nt)
 initn: 2490 init1: 2490 opt: 2490  Z-score: 2258.0  bits: 427.4 E(10000): 5.8e-124
banded Smith-Waterman score: 2490; 100.0% identity (100.0% similar) in 498 nt overlap (1-498:1-
498)

               10        20        30        40        50        60        70        80
WT_H08 TCTTAACCATATGTGAACGTACCTTTTCTAGCTGCTTTGGCAGGTGCCTCTCGGGGCTTCTGCCGGTAGCATTTATAAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G16_H1 TCTTAACCATATGTGAACGTACCTTTTCTAGCTGCTTTGGCAGGTGCCTCTCGGGGCTTCTGCCGGTAGCATTTATAAAC
               10        20        30        40        50        60        70        80

               90       100       110       120       130       140       150       160
WT_H08 TCTTTATATTTCTATAGAATTATTCATTGCTGAGTGGCATTAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G16_H1 TCTTTATATTTCTATAGAATTATTCATTGCTGAGTGGCATTAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGG
               90       100       110       120       130       140       150       160

              170       180       190       200       210       220       230       240
WT_H08 CTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G16_H1 CTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
              170       180       190       200       210       220       230       240

              250       260       270       280       290       300       310       320
WT_H08 CGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCTCTGCTTGGTGTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G16_H1 CGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCTCTGCTTGGTGTTGG
              250       260       270       280       290       300       310       320

              330       340       350       360       370       380       390       400
WT_H08 AGGACCCGCGTTTAGTCGCGGGCCGCCGAAATGCATCGGCTGTTGTATATACAGCTTCCCTGTGTAGTAAATGCTTAGCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G16_H1 AGGACCCGCGTTTAGTCGCGGGCCGCCGAAATGCATCGGCTGTTGTATATACAGCTTCCCTGTGTAGTAAATGCTTAGCT
              330       340       350       360       370       380       390       400

              410       420       430       440       450       460       470       480
WT_H08 TTACACTTTGAAACTTTTATATAACATGCCGAAAAACCCTCAACTTTTGAAAGGTTGACCTCGGATCAGGTAGGAATACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G16_H1 TTACACTTTGAAACTTTTATATAACATGCCGAAAAACCCTCAACTTTTGAAAGGTTGACCTCGGATCAGGTAGGAATACC
              410       420       430       440       450       460       470       480

              490
WT_H08 CGCTGAACTTAAGCATAT
       ::::::::::::::::::
G16_H1 CGCTGAACTTAAGCATATCAATAAGCCGGAGGAA
              490       500       510
```

T. basicola BRIP40192 wild-type (WT_H08) and transformant G17-2 (G17_H12)

```
>>G17_H12                                                             (513 nt)
 initn: 2410 init1: 2410 opt: 2469  Z-score: 1616.6  bits: 308.7 E(10000): 3.1e-88
banded Smith-Waterman score: 2469; 99.8% identity (99.8% similar) in 498 nt overlap (1-498:1-497)

               10        20        30        40        50        60        70        80
WT_H08 TCTTAACCATATGTGAACGTACCTTTTCTAGCTGCTTTGGCAGGTGCCTCTCGGGGCTTCTGCCGGTAGCATTTATAAAC
       :::::::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G17_H1 TCTTAACCATATGTG-ACGTACCTTTTCTAGCTGCTTTGGCAGGTGCCTCTCGGGGCTTCTGCCGGTAGCATTTATAAAC
               10         20        30        40        50        60        70

               90       100       110       120       130       140       150       160
WT_H08 TCTTTATATTTCTATAGAATTATTCATTGCTGAGTGGCATTAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G17_H1 TCTTTATATTTCTATAGAATTATTCATTGCTGAGTGGCATTAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGG
      80        90       100       110       120       130       140       150

              170       180       190       200       210       220       230       240
WT_H08 CTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G17_H1 CTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
     160       170       180       190       200       210       220       230

              250       260       270       280       290       300       310       320
WT_H08 CGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCTCTGCTTGGTGTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G17_H1 CGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCTCTGCTTGGTGTTGG
     240       250       260       270       280       290       300       310

              330       340       350       360       370       380       390       400
WT_H08 AGGACCCGCGTTTAGTCGCGGGCCGCCGAAATGCATCGGCTGTTGTATATACAGCTTCCCTGTGTAGTAAATGCTTAGCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G17_H1 AGGACCCGCGTTTAGTCGCGGGCCGCCGAAATGCATCGGCTGTTGTATATACAGCTTCCCTGTGTAGTAAATGCTTAGCT
     320       330       340       350       360       370       380       390


              410       420       430       440       450       460       470       480
WT_H08 TTACACTTTGAAACTTTTATATAACATGCCGAAAAACCCTCAACTTTTGAAAGGTTGACCTCGGATCAGGTAGGAATACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
G17_H1 TTACACTTTGAAACTTTTATATAACATGCCGAAAAACCCTCAACTTTTGAAAGGTTGACCTCGGATCAGGTAGGAATACC
     400       410       420       430       440       450       460       470

              490
WT_H08 CGCTGAACTTAAGCATAT
       ::::::::::::::::::
G17_H1 CGCTGAACTTAAGCATATCAATAAGCCGGAGGAA
     480       490       500       510
```