I, Essa Suleman, declare that this treatise represents my own unaided work and where necessary due credit has been given. This treatise is in fulfillment of the requirements for the degree, *Magister Scientiae*, in the Faculty of Science at Nelson Mandela Metropolitan University, as per rule 304.5.3.

__________________________ ____________________

E. Suleman DATE

The role of *pacC* in *Aspergillus flavus*

by

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Magister Scientiae

in the Faculty of Science at the

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ABSTRACT

Many microorganisms, and in particular fungi, are able to grow over a wide pH range. Thus, these microorganisms must possess some regulatory mechanism or system that senses the environmental pH signal and ensures that gene expression of certain molecules is tailored to the pH of the environment (Penalva and Arst, 2002). In *Aspergillus* species and several other fungi, pH regulation is mediated by seven genes viz. *palA, palB, palC, palF, palH, palI* and the global pH regulatory gene, *pacC* (MacAbe *et al*, 1996; Negrete-Urtasun, 1999; Denison, 2000). The activated form of the PacC protein activates genes that are required at alkaline pH, e.g. genes coding for alkaline phosphatases, and represses certain genes that are functional at acidic pH, e.g. genes encoding acid phosphatases (Negrete-Urtasun, 1999). PacC (and its homologues) also positively regulates genes involved in penicillin biosynthesis, e.g. the isopenicillin N synthase gene, *ipnA*, in *A. nidulans* (Penalva and Arst, 2002). It has also been hypothesised that *pacC* may negatively regulate aflatoxin biosynthesis, a carcinogenic secondary metabolite in several species of *Aspergillus* (Keller *et al*, 1997).

To elucidate the role of *pacC* a novel method of post-transcriptional gene silencing known as RNA interference was utilized. This method involved the cloning of a partial *pacC* gene fragment first in the forward and then the reverse orientations in a fungal expression cassette to create an RNA interference (RNAi) vector. The unique structure of this vector would allow the cloned fragments to be expressed and the resulting RNA to immediately form a double stranded stem-loop structure or short hairpin RNA (shRNA; McDonald *et al,* 2005). The formation of this shRNA, in turn, would be responsible for activating the endogenous RNA degradation complexes that would lead to mRNA degradation and subsequent gene silencing (Liu *et al*, 2003; Kadotoni *et al, 2003*; McDonald *et al*, 2005).

The results presented here have shown that confirmed *pacC* RNAi mutants produced aflatoxins irrespective of environmental pH (i.e. the mutants produce aflatoxins under acidic and alkaline conditions). Thus, *pacC* is essential for pH regulation of aflatoxin production in *A. flavus.*

There are numerous other biological (e.g. presence of oxylipins, lipooxygenases) and non-biological factors (pH, carbon source etc.) which affect maize colonisation and aflatoxin production by *A. flavus* (Burrow *et al*, 1996; Wilson *et al*, 2001; Calvo,

et al; 2002; Tsitsigiannis *et al*, 2006). However, all the genetic mechanisms involved have as yet not been identified. It has been shown by Caracuel *et al* (2003) that *pacC* acts as a negative virulence regulator in plants and these workers have hypothesised that PacC prevents expression of genes that are important for infection and virulence of the pathogen.

Therefore the physiological effects that *pacC* silencing had on the growth, conidiation and pathogenicity of *A. flavus* mutants were also investigated. The results of this study showed that *pacC* does not play a significant role in primary growth and development but does affect conidial production. SEM results showed that mutants have many "open ended" phialides and poorly developed conidiophores. This would suggest that *pacC* activation of conidial production genes is also required. Furthermore, *pacC* RNAi silencing severely impaired the ability of the *A. flavus* mutants to infect and cause damage on maize. The results obtained here are similar to that of *pacC* null mutants in *A. nidulans, C. albicans* and *F. oxysporum* which also exhibited low pathogenicity (Davis *et al*, 2000; Fonzi, W.A, 2002; Caracuel *et al*, 2003; Bignell *et al*, 2005 and Cornet *et al*, 2005). This study indicates that pathogenicity of *A. flavus* on maize is directly related to the structural integrity of conidia, which in turn is greatly influenced by PacC. This gene is a global transcriptional regulator and may either repress or activate one or many genes in each of the above pathways (Penalva and Arst, 2002). Studies on the genetic mechanisms of *pacC* regulation on these pathways are needed to elucidate the mechanisms of activation or repression of these genes.

Dedicated to my beloved parents

Mohammed Iqbal Suleman
and
Shariffah Suleman

and the rest of my family

Thank you

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The genus *Aspergillus* consists of over 185 species that are ubiquitous in the environment, found principally in soils and decaying vegetation. A large number of species are also closely associated with human foods, particularly cereals, grains, maize and nuts (Pitt and Hocking, 1985; Diener *et al*, 1987; Flaherty *et al*, 1997; Moreno and Kang, 1999). Over 20 species have been reported as causative agents of opportunistic infections in man (Yu *et al*, 1996). A number of species of *Aspergillus* produce mycotoxins of which the major ones are ochratoxin, sterigmatocystin, and aflatoxins (Bhatnagar *et al*, 2003). The most common isolates, *Aspergillus parasiticus, A. flavus* and *A. fumigatus*, are known to produce aflatoxins and *A. nidulans* is known to produce sterigmatocystin. The most common isolate infecting humans is *Aspergillus fumigatus*, the causative agent of aspergillosis.

Many microorganisms and in particular fungi are able to grow over a wide pH range. All of these microorganisms probably have pH homeostatic mechanisms that limit intracellular pH variation. However molecules situated near or beyond the cell's permeability barrier will only be protected from extremes of pH if they are synthesised at the suitable pH (van den Hombergh *et al*, 1996). Expression of genes coding for secreted enzymes and exported metabolites are also influenced by acidic or alkaline conditions and must be expressed at the appropriate environmental pH. Thus these microorganisms must possess some regulatory mechanism or system that senses the environmental pH signal and ensures that gene expression of certain molecules is tailored to the pH of the environment (Penalva and Arst, 2002).

In *Aspergillus* species and several other fungi, pH regulation is mediated by seven genes viz. *palA, palB, palC, palF, palH, palI* and the global pH regulatory gene, *pacC* (MacAbe *et al*, 1996; Negrete-Urtasun, 1999; Denison, 2000). The encoded products of the *pal* genes appear to form the signalling component of the pH signal transduction pathway, which then leads to activation of the pH-regulatory gene *pacC* via proteolytic cleavage. The activated form of the PacC protein activates genes that are required at alkaline pH e.g. genes coding for alkaline phosphatases and represses certain genes that are functional at acidic pH e.g. genes encoding acid phosphatases (Negrete-Urtasun, 1999). PacC (and its homologues) also usually positively regulates genes involved in penicillin biosynthesis in *A. nidulans* (e.g. the isopenicillin N synthase gene, *ipnA*; Penalva and Arst, 2002). It has also been hypothesised that *pacC* may

negatively regulate aflatoxin biosynthesis, a carcinogenic secondary metabolite in several species of *Aspergillus* (Keller *et al*, 1997). Numerous studies have been conducted to determine the mechanism by which *pacC* regulates gene expression in response to pH (MacAbe *et al*, 1996; Negrete-Urtasun, 1999; Denison, 2000, Penalva and Arst, 2002; Arst and Penalva, 2003).

1.2 THE pH REGULATORY GENE *pacC*

1.2.1 Genetics of pH Regulation

Understanding the genetics of pH regulation has mainly been done via genetic mutagenesis. Mutations which affect pH regulation fall into two main categories, viz. alkalinity-mimicking and acidity-mimicking mutations. Alkalinity-mimicking mutations result in similar gene expression to wild type grown under alkaline conditions, irrespective of ambient pH. Acidity-mimicking mutations results in gene expression similar to wild type strains grown under acidic conditions irrespective of ambient pH (Penalva and Arst, 2002). There are also a small number of mutations resulting in a neutrality-mimicking phenotype irrespective of ambient pH whose gene expression is comparable to wild type grown at pH 6.5 or has a heterogeneous mixture of alkalinity-mimicking and acidity mimicking characteristics (Mingot *et al*, 1999; Penalva and Arst, 2002).

Regulation of gene expression by pH is mediated primarily by *pacC* and six *pal* genes (Johannes *et al*, 1996; Denison, 2000; Calvo *et al,* 2002). Mutations in these genes result in alkalinity, acidity or occasionally neutrality mimicry. Acidity, alkalinity and neutrality mimicking mutations have been obtained for *pacC* in various *Aspergillus* species (Caddick *et al*, 1986; MacAbe *et al*; 1996; Tilburn *et al*, 1995; Mingot *et al*, 1999; Diez *et al*, 2002). The genetic and phenotypic variation of *pacC* mutations reflects its direct involvement in ambient pH regulation of gene expression (Penalva and Arst, 2002). There are three *pacC* acronyms used to denote *pacC* mutations viz. *pacC*^{c} referring to constitutively active PacC mutants which mimic wildtype growth at alkaline pH (alkali expressed genes are transcribed and acid expressed genes are repressed); *pacC*$^{+/-}$ which refers to partial loss of function mutants that mimic growth under acidic conditions and show an increase in acid phosphatase levels and penicillin production (*A. nidulans*) and *pacC*$^{-}$ referred to as the loss-of-function class of *pacC* mutants which also mimic growth under acidic conditions irrespective of ambient pH (Caddick *et al*, 1986; MacAbe et al; 1996; Tilburn *et al*, 1995; Mingot *et al*, 1999; Diez *et al*, 2002). *pacC*$^{-}$ mutations relieve repression of acid-expressed genes at alkaline pH (Tilburn *et al*, 1995). A genetic model of pH regulation by *pacC* in *A. nidulans* has been hypothesized (Fig 1.1; Penalva and Arst, 2002).

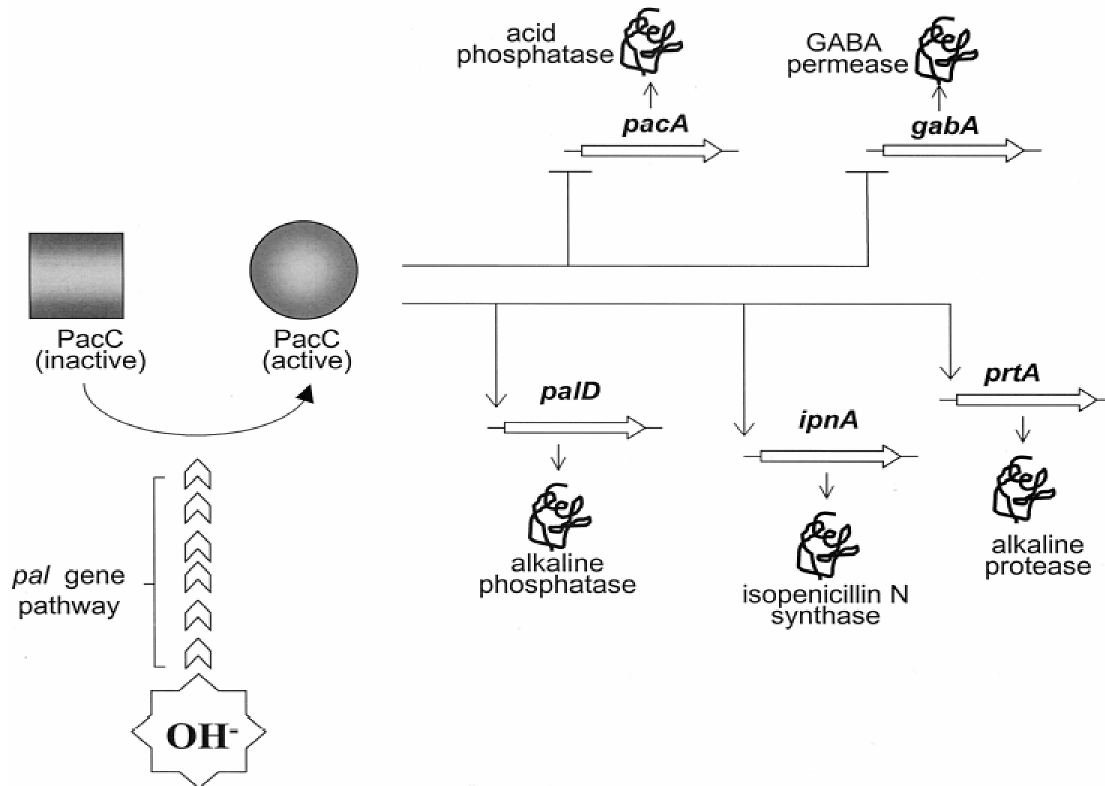

Figure 1.1: Genetic model of pH regulation in *A. nidulans* (Penalva and Arst, 2002).

PacC is synthesized as an inactive protein and is activated via a signal transduced under alkaline pH conditions by the *pal* signalling pathway. The model in Figure 1 shows that, at alkaline pH, the products of the six *pal* genes trigger conversion of the PacC transcription factor to the active form activating the expression of alkali-expressed genes and preventing expression of acid-expressed genes (Penalva and Arst, 2002). The active form of PacC is a transcriptional repressor of acid-expressed genes, such as *pacA* and *gabA*, and a transcriptional activator of alkaline-expressed genes, such as *ipnA*, *prtA*, and *palD*. Mutations inactivating pacC (*pacC*$^{-}$ or *pacC*$^{+/-}$) or the *pal* signalling pathway lead to absence of expression of alkaline-induced genes and derepression of acid-induced genes, which results in acidity mimicry. Gain-of-function *pacC*^{c} mutations bypassing the *pal* signalling pathway (i.e. leading to active PacC at any ambient pH) result in permanent activation of alkaline-induced genes and superrepression of acid-induced genes, which leads to alkalinity mimicry (Penalva and Arst, 2002).

1.2.2 The *pacC* Transcription Factor

The deduced sequence of PacC contains 678 amino acids (Tilburn *et al*, 1995). However mutational analysis has shown that possibly all translation proceeds from methionine at codon 5, resulting in a 674-residue protein (Mingot *et al*, 1999). The most important feature of the

translation product is the presence of three Cys_2His_2 zinc fingers beginning at residue 78 which recognise the core consensus DNA sequence, 5'- GCCARG -3' (Johannes *et al*, 1996; Denison, 2000; Calvo *et al,* 2002*;* Penalva and Arst, 2002; Arst and Penalva, 2003) (Fig 1.2).

The specificity of the PacC zinc finger DNA binding domain has been thoroughly analyzed using site-directed and classical mutagenesis of both target DNA and finger residues involved in critical contacts, quantitative binding experiments, various footprinting techniques, and molecular modelling (Tilburn *et al*, 1995; Arst and Penalva, 2003). Zinc-finger 1 interacts with zinc-finger 2 rather than directly with the target DNA. Zinc finger 2 also interacts with the 5′ moiety of the binding site while finger 3 binds the 3′ moiety (Arst and Penalva, 2003). Partially purified PacC obtained from mycelial extracts and bacterially expressed PacC bind DNA specifically to the target sequence 5′- GCCARG - 3′ with a preference for a thymine (T) at 1bp before the PacC binding site (Tilburn *et al*, 1995; Johannes *et al*, 1996; Denison, 2000; Calvo *et al,* 2002). Nearly every base of the core binding sequence appears to be involved in specific interactions with residues of zinc fingers 2 & 3 (Fig 1.3). A Gln155-to-A4 contact (Fig 1.3) is crucial for binding (Espeso *et al*, 1997; Arst and Penalva, 2003). The almost complete conservation of amino acid sequence in the reading (specificity determining) α-helices of fingers 2 and 3 between PacC and its homologues (e.g. *RIM101p* in *Yarrowia lipolytica, PRR2* in *C. albicans*) strongly suggests that these factors have similar binding specificities (Lambert *et al*, 1997; Arst and Penalva, 2003; Cornet *et al,* 2005).

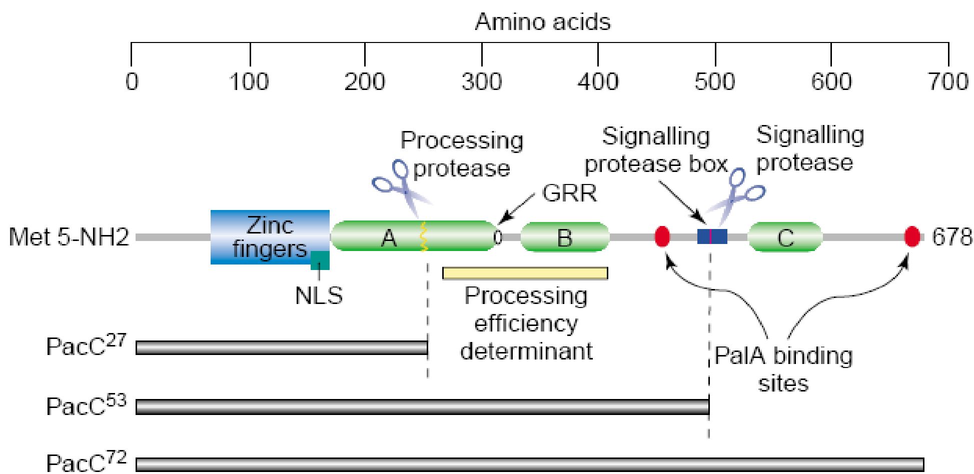

Figure 1.2: Schematic representation of functionally relevant regions of *A. nidulans pacC*. (Arst and Penalva, 2003)

Translation begins at methionine codon 5. A, B and C refer to regions that interact together to maintain the closed conformation. This prevents cleavage by the processing protease. GRR refers to the conserved glycine-rich region in PacC. There is a nuclear localization signal (NLS) in the third zinc finger in the DNA-binding domain. $PacC^{72}$, $PacC^{53}$ and $PacC^{27}$ indicate the 72-kDa translation product, the 53-kDa committed intermediate and the 27-kDa processed PacC forms, respectively**.**

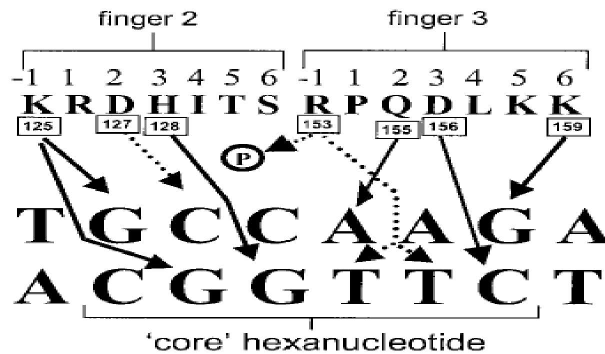

Figure 1.3: DNA binding specificity of PacC (Penalva and Arst, 2002).
Prediction of specific contacts between residues in the reading α-helix of PacC zinc fingers 2 and 3 and the PacC target hexanucleotide. The contacts indicated by the solid lines have been suggested by experimental evidence and modelling. Almost every base in both strands of the target sequence is predicted to establish specific contacts with PacC zinc finger residues. Dotted arrows indicate possible contacts of Asp127 and of Arg153, whose side chain can be in contact with the phosphate backbone or the O-4 atoms of both the T4' and T5' thymines. Zinc finger 1 does not appear to be involved in specific contacts with DNA.

1.2.3 Activation and Repression Roles of PacC

Detailed studies have been conducted to determine the roles of PacC in the promoters of one alkali-expressed gene, *ipnA* (isopenicillin N synthase) and one acid expressed gene, *gabA* (GABA, gaba amino butyric acid) in *A. nidulans* (Espeso and Penalva, 1996; Denison, 2000; Espeso and Arst, 2000). The bidirectional promoter (872bp) between the oppositely transcribed *acvA* (encoding δ-(l-α-aminoadipyl)-L-cysteinyl-D-valine synthethase) and *ipnA* genes contains five GCCARG sites (named ipnA1 to ipnA4B). The site closest to *acvA* (ipnA1) has a negligible affinity for the PacC protein (Espeso and Penalva, 1996). The two PacC binding sites closest to *ipnA* are in opposite orientation (separated by 9bp) forming the double ipnA4AB site at -258bp. The ipnA2 and ipnA3 PacC binding sites are located at -593bp and -502bp relative to the transcription start point, respectively. Although ipnA2 has a five-fold-greater affinity for PacC than ipnA3 or the double ipnA4AB site, deletion of both ipnA3 and ipnA4AB almost completely destroyed the elevation of *ipnA* expression under alkaline growth conditions (Espeso and Penalva, 1996). It was also found that deletion of only ipnA2 reduced expression by nearly 50%, and deletion of ipnA2, ipnA3, and ipnA4AB together reduced expression nearly 20-fold which was almost comparable to expression levels under acidic growth conditions. Deletion of ipnA3 resulted in a five-fold reduction in expression (Espeso and Penalva, 1996), and it has also been suggested by Then-Bergh and Brakhage (1998) that ipnA3 is the most important PacC binding site for *acvA* expression (Penalva and Arst, 2002). Deletion of PacC binding sites eliminates elevated *ipnA* expression which occurs under alkaline growth conditions, thus verifying that

PacC directly activates the expression of an alkali-expressed gene (Fig 1.3) (Penalva and Arst, 2002).

The promoter of *gabA* (which encodes a GABA transporter) has two PacC binding sites, which are adjacent but inversely oriented (Espeso and Arst, 2000). These sites overlap the site for the transcription factor IntA/AmdR, thus PacC and IntA/AmdR compete for binding (Espeso and Arst, 2000; Penalva and Arst, 2002) which implies that PacC directly represses *gabA* expression by blocking induction of IntA/AmdR.

1.2.4 Mutational Analysis of *pacC*

pacC has been genetically analyzed in various *Aspergillus* species, and characterization of *pacC* mutations were vital towards understanding of PacC activation by proteolysis (Espeso *et al*, 1997; Then-Bergh and Brahkage, 1998; Espeso *et al*, 2000; Díez *et al*, 2002; Penalva and Arst, 2002). Deletions which removed between 92 and 412 residues from the C terminus of PacC resulted in an alkalinity-mimicking (*pacC*^{c}) mutant (Tilburn *et al*, 1995; Then-Bergh and Brahkage, 1998; Mingot *et al,* 1999). Thus there is a negative-acting domain in the C-terminal region of PacC which is inactivated by alkaline-ambient-pH signalling. Single-residue changes involving residues 259, 266, 340, 573, and 579 also lead to an alkalinity-mimicking *pacC*^{c} phenotype (Mingot *et al*, 1999; Espeso *et al*, 2000; Penalva and Arst, 2000).

N-terminal deletions result in an acidity-mimicking phenotype (partial loss-of-function *pacC*$^{+/-}$ mutations), and if the truncation extends into the zinc finger domain, a null *pacC*$^{-}$ phenotype results (Tilburn *et al*, 1995; Mingot *et al*, 1999; Denison, 2000; Espeso *et al*, 2000; Penalva and Arst, 2002). Phenotypically *pacC*$^{-}$ mutations result in cryosensitivity at pH = 6.5, low growth rates at ambient temperature, very poor conidiation under alkaline conditions, and overproduction of an unknown brown pigment (Tilburn *et al*, 1995; Mingot *et al*, 1999; Penalva and Arst, 2002; Pinero and Keller, unpublished data). Deleting residues between 299 and 315, at residue 379 and residues 465 through 540 also result in an acidity-mimicking phenotype (Tilburn *et al*, 1995; Mingot *et al*, 1999; Espeso *et al*, 2000). Neutrality-mimicking *pacC*$^{c/-}$ mutations generally occur in the region between alkalinity-mimicking and acidity-mimicking deletion mutations (Mingot *et al,* 1999).

1.2.5 Proteolytic Processing of PacC

There are three different forms of PacC viz. a 72-kDa translation product, a 53-kDa open form and a 27-kDa truncated product that is referred to as the processed form (Orejas *et al*, 1995; Mingot *et al,* 1999; Penalva an Arst, 2002). Under acidic conditions or if the functional *pal* pH

signalling pathway is lacking the 72-kDa translation product predominates, whereas under alkaline growth conditions the processed (27-kDa) form predominates (Orejas *et al*, 1995; Mingot *et al*, 1999). This supports the conclusion that PacC is proteolytically processed in response to alkaline ambient pH in a *pal* pathway dependant manner (Penalva and Arst, 2002). In acidic environments, the 674 residue PacC protein is in a "closed" conformation and is protected from proteolytic activation via intramolecular interactions involving the ≤150 residue C-terminal domain. pH signalling mediated by the *pal* pathway converts PacC to an accessible conformation enabling processing cleavage within residues 252-254 (Fig 1.4) (Díez *et al*, 2002; Arst and Penalva, 2003). It has been determined by Díez *et al* (2002) that activation of PacC requires two proteolytic steps. First, the "closed" translation product is converted to an accessible, committed intermediate by proteolytic cleavage of the C-terminus. This ambient pH-regulated cleavage is required for the final, pH-independent processing reaction (Díez *et al*, 2002, Penalva and Arst, 2002). The signalling protease cleaves PacC between residues 493 and 500, within a conserved 24 residue "signalling protease box" (Díez *et al*, 2002) (Figs 1.2 and 1.4). Most of mutant $pacC^{c}$ mutants occur as a result of C-terminal truncation of PacC leading to an alkalinity-mimicking phenotype. Mutant proteins truncated at or between residues 407 and 578 are proteolytically processed at any pH. This implies that an important function of the C-terminal moiety is to prevent proteolytic cleavage of the "closed" PacC under acidic ambient pH conditions removed by $pacC^{c}$.

The 72kDa *pacC* translation product is relatively inactive as revealed by the strong acidity-mimicking phenotype which is the consequence of non-truncating $pacC^{+/-}$ mutations, thus preventing proteolytic processing and leading to preferential localization of PacC to the cytosol (Penalva and Arst, 2002). In comparison if PacC is truncated after residue 266 it is able to activate alkaline-induced and repress acidic-induced gene expression (Penalva and Arst, 2002).

1.2.6 Regulation of PacC Nuclear Localisation

Localization of PacC to the nucleus is regulated by pH-dependent proteolytic activation (Mingot *et al*, 2001). If pH signalling is absent proteolytic cleavage of PacC does not occur resulting in its localisation within the cytosol. In contrast, pH signalling-mediated conversion of PacC to its processed form results in almost exclusive PacC nuclear localization (Mingot *et al*, 2001; Penalva and Arst, 2002). The molecular mechanism whereby the C-terminal region of PacC prevents its nuclear localization is unknown (Penalva and Arst, 2002). PacC contains a nuclear localization signal between residues 252 and 269 (Penalva and Arst, 2002), which facilitate nuclear localization of the "open" form of PacC. Mingot *et al* (2001) showed that only

the zinc finger region of PacC mediates nuclear localization of the PacC protein and is also involved in DNA binding.

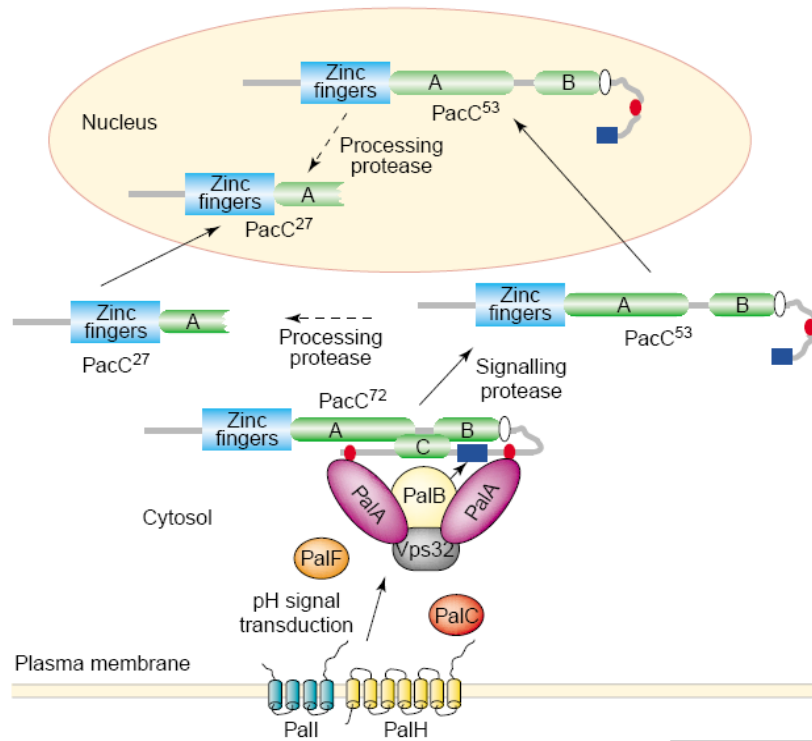

Figure 1.4: Molecular model showing proteolytic processing and nuclear localisation of PacC in response to *pal*-pathway mediated ambient pH signalling (Arst and Penalva, 2003).
One or both of two likely membrane proteins, PalH and PalI, possibly transduce the alkaline pH signal through undefined steps to the signalling protease, which is most probably the PalB protein, which then proteolytically cleaves PacC.

1.3 AFLATOXIN BIOSYNTHESIS

Aflatoxins are of great medical significance because they have been identified as strong hepatocarcinogenic, mutagenic and teratogenic fungal secondary metabolites (Moreno and Kang, 1999; Yu *et al,* 2002; Bhatnagar *et al;* 2003). They are colourless, tasteless and odourless, making their detection in foods difficult (Moreno and Kang, 1999). Aflatoxins belong to a group of polyketide-derived furanocoumarins with at least 16 structurally related variants (Yu *et al,* 2002; Yu *et al*, 2004), and four major derivatives viz. aflatoxins B_1, B_2, G_1 and G_2 (Fig. 1.5).

Aflatoxin B_1 is produced by most *Aspergillus* species and has an extremely high carcinogenic potency. AFB_1 contamination occurs widely in certain food sources e.g. maize and peanuts. The Food and Agriculture Organization (FAO) estimate that 25% of the world's food crops are affected by aflatoxins (Moreno and Kang, 1999). Aflatoxin losses to livestock and

poultry producers include death and more subtle effects, such as immune suppression and reduced growth rates (Moreno and Kang, 1999). Factors such as temperature, nitrogen, carbon source and pH are known to affect aflatoxin production in *A. flavus.*

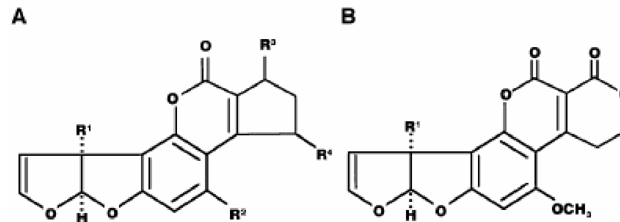

Figure 1.5: Chemical structures of common Aflatoxins (Bhatnagar *et al*, 2003)
(A) The B–type aflatoxins are characterized by a cyclopentane E-ring and fluoresce blue under long wavelength UV light. (B) The G-type aflatoxins have a xanthone ring and fluoresce green.

1.3.1 Aflatoxin Biosynthetic Pathway

Attempts to determine the aflatoxin biosynthetic pathway began with the discovery of the structure of these toxins (Yu et al; 2002). It has been shown that the 25 identified genes involved in aflatoxin biosynthesis are clustered within a 70kb DNA region in the chromosome, corresponding to an average gene length of 2.8 kb (Bhatnagar *et al*, 2003; Yu *et al*, 2004). The entire aflatoxin biosynthetic pathway gene cluster has been sequenced for *A. parasiticus* and *A. flavus*, GenBank nucleotide sequence accession numbers AY371490 and AY510453 respectively (Ehrlich *et al*, 2004; Yu *et al*, 2004).

Generally, aflatoxin biosynthetic pathway genes have been named based on the substrate converted by the gene product, according to their enzymatic functions, or according to their roles in regulation of aflatoxin biosynthesis (Yu *et al*, 2004). A new naming system has been proposed by Yu *et al* (2004) for naming aflatoxin biosynthetic pathway genes in the interests of consistency and uniformity. This new naming system will be used below.

The currently accepted scheme (Fig 1.6) for aflatoxin B_1 biosynthesis is: Acetate building blocks → Anthrone – derivative polyketide → Norsolorinic acid (NOR) → averantin (AVN) → hydroxyaverantin (HAVN) → averufin (AVF) → hydroxyversicolorine (HVN) → versiconal hemiacetal acetate (VHA) → versiconal (VAL) → versicolorin B (VERB) → versicolorin A (VERA) → demethyl-sterigmatocystin (DMST) → sterigmatocystin (ST) → *O*-methylsterigmatocystin (OMST) → AFB_1 (Yu *et al*, 2004).

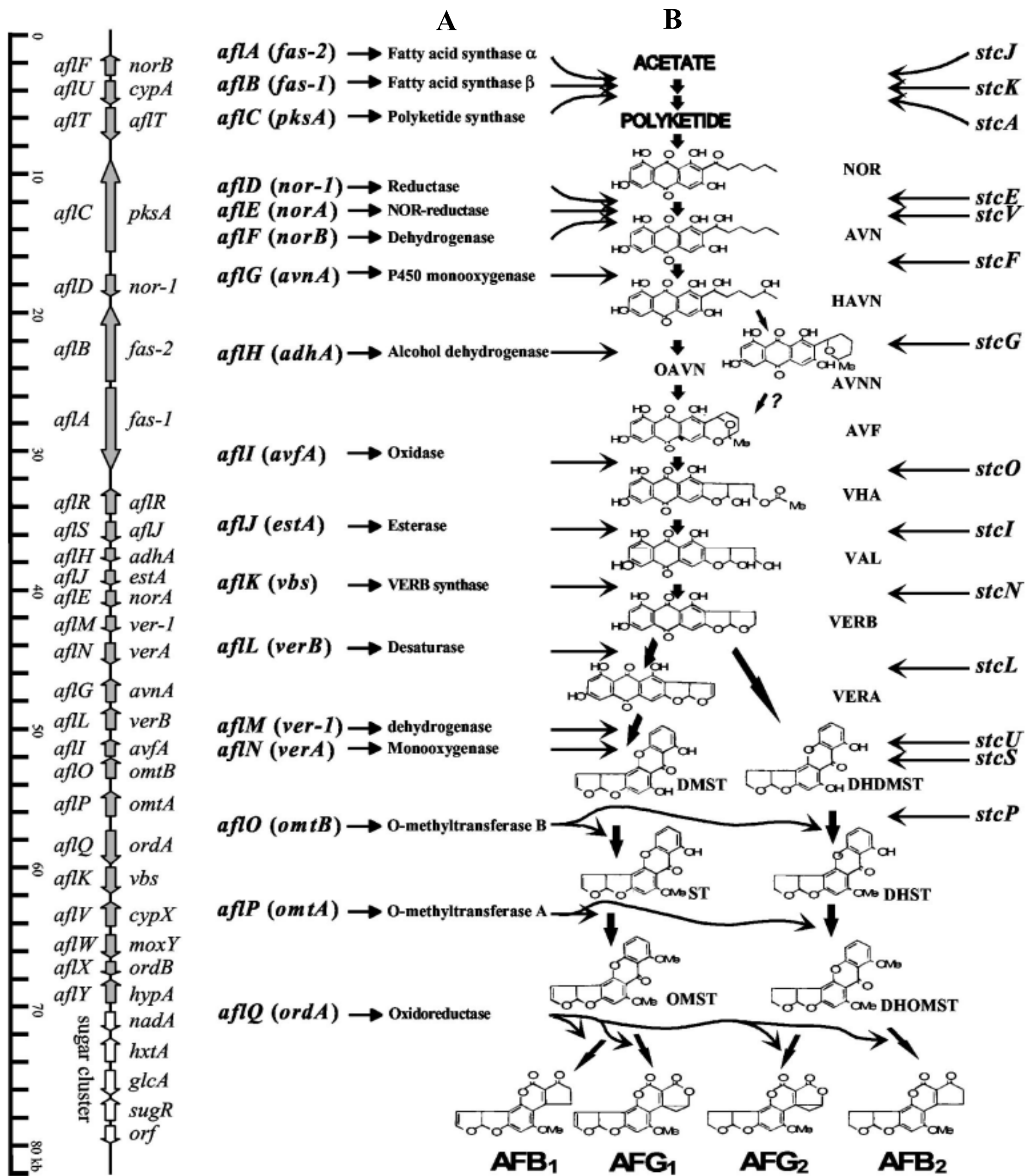

Figure 1.6: Clustered genes and the aflatoxin biosynthetic pathway in *A. flavus*. (Yu *et al*, 2004.
(A) Enzymes involved in aflatoxin biosynthesis. (B) The generally accepted pathway for aflatoxin and sterigmatocystin biosynthesis. Vertical line = 82kb aflatoxin biosynthetic pathway gene cluster and sugar utilisation cluster New gene names and the old gene names are given on the left and right of the vertical line respectively. Arrows along the vertical line indicate direction of transcription and the ruler at the far left indicates the relative sizes of the genes (kb). *A. nidulans* sterigmatocystin biosynthetic pathway genes are shown on the right of panel B.

1.3.2 Regulation of Aflatoxin Biosynthesis

Aflatoxin production by toxigenic *Aspergillus* species is affected by environmental and nutritional factors such as temperature, pH, carbon and nitrogen source, stress factors, lipids, and certain metal salts e.g. Ca^{2+} (Chang *et al*, 1996; Payne *et al,* 1998; Chang *et al*, 2000; Yu *et al*, 2002) . Although numerous studies have been conducted, the molecular mechanisms controlling

these effects are still largely unclear (Yu *et al*, 2002). Some of these factors may affect expression of genes in the aflatoxin pathway, expression of the aflatoxin regulatory gene, *aflR*, or structural genes, possibly due to responses to environmental and nutritional signals by globally acting transcription factors (Yu *et al,* 2002). Aflatoxin accumulation may be affected by some of these nutritional and environmental factors by altering the activity of one or more enzymes involved in aflatoxin biosynthesis (Yu *et al,* 2002). Of the 25 characterised genes involved in aflatoxin (and sterigmatocystin) biosynthesis, only one, *aflR*, appears to encode a transcription factor (Wolushuk *et al*, 1994; Chang *et al,* 1995; Yu *et al,* 1996; Chang *et al,* 1999; Erhlich *et al,* 1999a; Erhlich *et al,* 1999b; Bhatnagar *et al* 2003). Accumulation of structural gene transcripts were prevented by disruption of *aflR* (Yu *et al,* 2002); however introduction of an additional copy of *aflR* in *Aspergillus parasiticus* caused overproduction of aflatoxin biosynthetic intermediates (Chang *et al*, 1995; Yu *et al*, 2002).

Overexpression of *aflR* in *A. flavus* and *A. parasiticus* up-regulates aflatoxin pathway gene transcription and aflatoxin accumulation (Bhatnagar *et al* 2003). Ehrlich *et al* (1999b) showed that aflatoxin biosynthesis and AFLR binding to aflatoxin biosynthetic genes occurred in aflatoxin inducing media (Glucose Mineral Salts) but did not occur in aflatoxin suppressing media (Peptone Mineral Salts) indicating that AFLR binding correlates with aflatoxin biosynthesis. Metabolite feeding studies have shown that a functional *aflR* allele is required for biosynthesis and accumulation of norsolorinic acid (NOR), the first stable intermediate in the aflatoxin biosynthetic pathway, since *aflR* disruption mutants do not produce aflatoxins but otherwise grow normally (Bhatnagar *et al* 2003).

1.3.3 Characterisation and Role of *aflR* in Expression of Aflatoxin Biosynthetic Pathway Genes

The AflR protein, encoded by *aflR,* consists of 444 amino acids with a cysteine-rich motif near its N-terminus, which is homologous to Cys_6-Zn_2 domains of other fungal and yeast GAL4-type transcription factors involved in the regulation of many catabolic pathways (Burger *et al*, 1991; Woloshuk *et al*, 1994; Todd *et al,* 1998; Liu and Chu, 1998; Bhatnagar *et al,* 2003). DNA-binding proteins which have Zn_2Cys_6 domains are important positively acting regulatory factors necessary for expression of multiple genes in a cluster (Ehrlich *et al*, 1999b).

aflR and *aflS* are divergently transcribed from an intergenic region of 758bp that is located between the two genes (Bhatnagar *et al*, 2003). Ehrlich *et al* (1999a) analysed promoter function of the *aflR* gene by using the entire 758-bp intergenic region as well as truncated forms of this region to drive expression of the *E. coli* β-glucuronidase (GUS)-encoding gene, *uidA.* They

found that removal of sequences in the promoter from -758 to -280 had no noticeable effect on promoter activity, but further truncation to -118 enhanced gene expression nearly 5-fold, implying that there may be a negative regulatory element in the region from -280 to -118 (Fig 1.7, Erhlich *et al,* 1999a). Further removal of bases -118 to -100 almost completely eliminates GUS gene expression, deletion from -118 to -107 resulted in two-thirds of decrease in activity (Ehrlich *et al*, 1999a). This implies that the 18-bp region, which overlaps a 10-bp palindrome (-120 to -111) and a purine-rich region, from -100 to -118 appears to be critical for *aflR* promoter activity (Ehrlich *et al*, 1999a). Enzyme mobility shift assays by Espeso and Arst (2000) using nuclear extracts from *A. parasiticus* and oligonucleotide ligands covering the region from -81/-173 revealed the presence of a putative PacC-binding site (5'-GCCARG-3'). Binding to the *aflR* PacC site is consistent with the function PacC in repressing transcription of acid-expressed genes under alkaline conditions, since aflatoxin biosynthesis in *A. flavus* occurs in acidic media, but is inhibited in alkaline media (Bhatnagar *et al,* 2003). It is possible that PacC binding to the -148/ -173 site has a negative effect on *aflR* expression, however as yet this has not been proven.

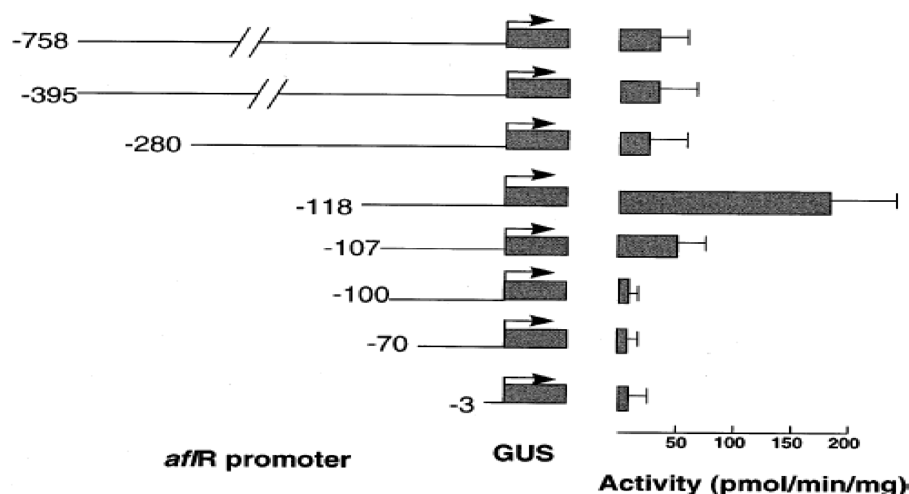

Figure 1.7: Functional activity of the *aflR* promoter truncation mutants based on β-glucoronidase (GUS) activity (Erhlich *et al,* 1999a).

aflS (*aflJ*), encoding AlfS, is divergently transcribed from the *aflR* promoter and has no known sequence homologies with proteins in yeast or fungal databases, but *aflS* may function as transcription enhancer of *aflR* (Bhatnagar *et al*, 2003). Disruption analysis of *aflS* by Meyers *et al* (1998) in *A. flavus* resulted in failure to produce any aflatoxin pathway metabolites even though transcripts for many of the aflatoxin pathway genes are still made, suggesting that AflS does not affect AflR activity. It has also been found by Chang *et al* (2000) that AreA (nitrogen

regulatory protein), binds to GATA sites in the *aflR-aflS* intergenic region, which suggests that regulation of aflatoxin production by nitrogen could be linked to AreA control of *aflR* and *aflS* expression (Bhatnagar *et al,* 2003). It has been demonstrated using a yeast two-hybrid system that AflS binds to the carboxy-terminal region of AflR, thus AflS probably functions as an AflR coactivator/enhancer (Chang *et al*, 1999).

1.3.4 pH Regulation of Aflatoxin Biosynthesis

Many microorganisms experience variations in the pH of their environment. All microorganisms probably have pH regulatory mechanisms to limit the range of intracellular pH variation. Molecules located at or beyond the cell membrane can only be protected from extremes of pH by ensuring that synthesis takes place at the appropriate pH (van den Hombergh *et al*, 1996). Thus it is beneficial to possess a regulatory system that senses environmental pH, allowing for mediation of pH regulated gene expression (van den Hombergh *et al*, 1996). In *Aspergillus nidulans* genes regulated by ambient pH can be classified into three groups viz. those encoding secreted enzymes (e.g. *pacA, xlnB,* encoding acid phosphatase and a xylanase respectively), those encoding permeases (e.g. *gabA* which encodes a GABA transporter that has an acidic pH optimum) and those encoding enzymes involved in the synthesis of exported metabolites e.g. the first two enzymes of penicillin biosynthesis viz. *acvA* and *ipnA* which encodes isopenicillin *N*-synthase (Espeso *et al*, 1993; Espeso and Penalva, 1996; Then-Bergh and Brakhage, 1998; Penalva and Arst, 2002), as well as *stcU* (preferentially acid-expressed) which encodes a ketoreductase that catalyses reduction of versicolorin A, a precursor of sterigmatocystin and aflatoxin biosynthesis (Brown *et al*, 1996; Keller *et al*, 1997; Penalva and Arst, 2002). The *pacC* gene encoding the transcription factor mediating pH regulation is itself preferentially expressed at alkaline pH (Tilburn *et al*, 1995; Penalva and Arst, 2002).

Certain carbon sources and nitrogen have been known to enhance (e.g. glucose) or hinder (e.g. nitrate) aflatoxin biosynthesis in *A. flavus* and *A. parasiticus*. Glucose stimulation is contrary to glucose repression of other secondary metabolites, however recent studies have shown that glucose is not a limiting factor for aflatoxin production in *A. parasiticus* provided that the fungus is grown in acidic environments (pH = 4 to 5). A relationship between aflatoxin production and sclerotial morphogenesis based on changes of both chemical and morphological differentiation in response to pH was determined by Cotty (1988). The effect of pH on aflatoxin biosynthesis is dependant on the composition of the growth media (Calvo *et al*, 2002). As the pH of ammonium based growth medium increased, *A. nidulans* and *A. parasiticus* showed five to ten-fold decreases in mycotoxin production (Keller *et al,* 1997). A mutant *Aspergillus nidulans*

resulting in constitutive activity of the pH regulatory factor resulted in ten-fold lower sterigmatocystin production than wildtype (Keller *et al*, 1997). At pH 4.0 or lower, aflatoxin production is maximal while sclerotial production was reduced by 50% in *A. flavus* (Cotty, 1988; Calvo *et al*, 2002).

Effects of pH have also been observed in studies of nitrogen effects on aflatoxin production. Generally aflatoxin producing *Aspergillus* strains acidify ammonium based media (stimulating aflatoxin production) whereas nitrate based medium (aflatoxin inhibiting) remain neutral (Keller *et al,* 1997). It has also been demonstrated that buffering ammonium media to neutral pH and nitrate media to acidic pH reversed the inducing and repressing effects of nitrogen source on *A. flavus* aflatoxin production on solid medium (Keller *et al,* 1997).

1.3.4.1 *Role of PacC in regulation of aflatoxin biosynthesis*

Fungal development and aflatoxin production in *Aspergillus* species is affected by pH in a complex manner. It has been found that aflatoxin production increases under acidic conditions and is repressed under alkaline conditions. Keller *et al* (1997) hypothesised that control of aflatoxin and sterigmatocystin expression in response to pH could be mediated via the PacC transcriptional factor (Keller, *et al*, 1997; Calvo *et al*, 2002). This hypothesis was supported by the fact that a constitutively activated *A. nidulans* PacC mutant ($pacC^{c202}$, mimicking constant alkali conditions) produced ten-fold less aflatoxin than wild type (Keller *et al*, 1997). At acidic pH, the PacC protein is inactive and cannot bind to target sites, but at alkaline pHs it is cleaved to produce the active form. The activated PacC protein is known to bind to promoters of target genes which have one or more PacC binding sites (5′-GCCARG), activating the expression of alkaline expressed genes, and repressing the expression of acid expressed genes (MacAbe *et al*, 1996; Espeso *et al*, 1997). Since the aflatoxin biosynthesis pathway genes are more highly expressed at acidic pH, it has been suggested that PacC may be a negative regulator of aflatoxin and sterigmatocystin biosynthesis at alkaline pH (Keller *et al*, 1997).

Ehrlich *et al* (1999) have shown that the promoter region of the *A. parasiticus aflR* contains at least one PacC binding site and it has been demonstrated that this site is bound by a protein presumably PacC. Examination of the aflatoxin gene cluster (in *A. flavus*) revealed approximately 122 putative PacC binding sites and in some cases the PacC binding site is situated very near an AflR site (global regulator of aflatoxin/sterigmatocystin biosynthesis). Several promoters of *stc* (sterigmatocystin) genes in *A. nidulans* also contain putative PacC binding sites (Keller *et al*, 1997), thus it is possible that aflatoxin and sterigmatocystin biosynthesis may be regulated by PacC. For example the promoter of the sterigmatocystin

biosynthesis gene *stcU* has a PacC binding site and an AflR binding site that are only two bases apart (Keller *et al*, 1997). The close proximity of these two binding sites implies that PacC may have an effect of aflatoxin/sterigmatocystin production by interacting with AflR or prevention of AflR binding thus repressing expression of aflatoxin biosynthetic pathway genes. There are other genes in the aflatoxin biosynthetic cluster, which have PacC binding sites at important positions in their promoters that may affect their expression e.g. the 1.7 kb intergenic region separating the *aflD* (*nor-1*) and *aflC* (*pksA*) genes has two adjacent PacC sites nearly in the middle that affect expression of *aflC* (Erhlich *et al,* 2002). PacC is also a regulator of genes involved in penicillin biosynthesis (Calvo *et al,* 2002). In *A. nidulans* at high pH, PacC activates expression of the isopenicillin N synthase gene, which is involved in penicillin biosynthesis. In *Penicillium chrysogenum*, PacC activates the expression of the isopenicillin N synthase homologue, *pcbC* (Calvo *et al*, 2002). PacC has also been found to be involved in the regulation of morphogenesis and development, since deletion of the entire PacC coding region results in deficient growth and conidiation (Tilburn *et al,* 1995; Calvo *et al,* 2002). However it remains unknown whether *pacC* directly or indirectly regulates morphogenesis and development.

1.4 RNA INTERFERENCE FOR STUDYING GENE SILENCING

It has become evident over the last 15 years that different organisms are able to target foreign and endogenous nucleic acids for silencing based on the recognition of sequence homology (Cogoni, 2001; Agrawal *et al*, 2003). Gene silencing can be achieved through either suppression of gene expression (transcriptional gene silencing, TGS) or at a posttranscriptional level involving sequence-specific mRNA degradation (Cogoni, 2001; Agrawal *et al*, 2003). The latter is commonly referred to as either posttranscriptional gene silencing (PTGS) or RNA interference (RNAi). RNAi was first discovered by accident in plants (Napoli *et al*, 1990); however RNAi effects have been observed in numerous eukaryotic organisms including human and mouse cell lines (Agrawal *et al*, 2003). Investigations of RNA silencing, referred to as PTGS in plants, quelling in fungi, RNAi in animals and virus-induced gene silencing have led to a universal theory of gene regulation by RNA interference. The are three critical components of RNA interference viz. (i) the presence of a suitable double stranded RNA (dsRNA) inducer which is homologous to the target RNA; (ii) production of siRNAs from the dsRNA inducer and (iii) enzymes which degrade the target RNA in a homology dependant manner (Agrawal *et al*, 2003). In most studies the formation of small-interfering RNA (siRNA) of 21-25nt was found to be critical for initiation of the conserved RNA interference pathway (Elbashir *et al*, 2001; Bernstein *et al*, 2001).

1.4.1 Mechanism of RNA Interference

The RNA interference pathway appears to be widely conserved, ranging from plants, fungi, protozoans, algae, invertebrates and vertebrates (Agrawal *et al*, 2003). There are slight differences with regards to the actual mechanism and proteins involved. However, there is a generally accepted scheme of RNA silencing as shown in Fig 1.8 (Mocellin and Provenzano, 2004).

Generally the introduction of double-stranded RNA (dsRNA) into a cell triggers the RNAi pathway. After entry of the dsRNA into the cell the RNaseIII type enzyme, referred to as Dicer, binds to and digests the dsRNA into similarly-sized (21-26nt) small interfering RNAs (siRNA) (Cogoni, 2001; Agrawal *et al*, 2003; Jana *et al*, 2004; McDonald *et al*, 2005). These RNaseIII type enzymes (Dicer) are evolutionary conserved in flies, fungi, mammals, plants and worms. The siRNAs form a complex with a ~500 kDa multicomponent ribonuclease complex, commonly referred to as the RNA induced silencing complex (RISC) (Cogoni, 2001; Agrawal *et al*, 2003; Jana *et al*, 2004; Mocellin and Provenzano, 2004; McDonald *et al*, 2005). Target mRNA that is homologous to the siRNAs are bound by the RISC complex and cleaved into small fragments resulting in transcriptional silencing of the targeted gene (Cogoni, 2001; Agrawal *et al*, 2003; Jana *et al*, 2004; Mocellin and Provenzano, 2004; McDonald *et al*, 2005). It is thought that the cleaved mRNAs are then degraded by exoribonucleases (Agrawal *et al*, 2003).

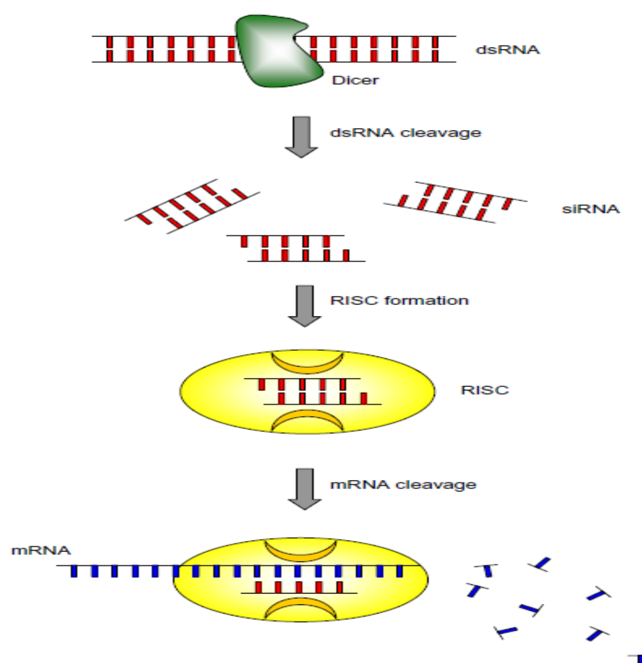

Figure 1.8: Schematic representation of the RNA interference pathway (Mocellin and Provenzano, 2004).

1.4.2 siRNA Synthesis and Delivery Strategies

It should be noted that several strategies for introducing dsRNA within target cells have been developed (Cogoni, 2001; Agrawal *et al*, 2003; Jana *et al*, 2004; Mocellin and Provenzano, 2004; McDonald *et al*, 2005). Each of these synthesis and delivery methods have specific advantages and disadvantages (Table 1.1). Chemical synthesis of siRNAs by industrial processes is becoming increasingly popular since the method is very rapid and high purity RNA is produced. However, the sequence of the target gene must be thoroughly analysed to find sequences having the highest probability of forming siRNAs that are of high specificity and acitivity (Mocellin and Provenzano, 2004). In vitro siRNA synthesis which relies upon the T7-phage polymerase is an alternative, which produces separate sense and antisense siRNA strands. Alternatively, long dsRNAs can be cleaved by recombinant RNase-III to produce multiple siRNAs. Although the last method is easy to implement it carries the potential drawback of generating non-specific siRNAs (Agrawal *et al*, 2003; Mocellin and Provenzano, 2004). The major drawback with these methods is that the siRNAs must be delivered directly into the target cell. Delivery can be difficult although newer methods employing specially developed delivery methods and transfection reagents have greatly increased the probability of these siRNAs entering the intended cell and thus cause silencing of the target gene. Furthermore, the effects of gene silencing using in-vitro synthesised siRNAs are usually transient (Agrawal *et al*, 2003; Mocellin and Provenzano, 2004). Gene expression usually recovers after 96 to 120 hours or 3 to 5 cell divisions after transfection thereby hindering long term studies of the effects of silencing on the target gene (Mocellin and Provenzano, 2004).

siRNAs can also be produced by polymerase-III promoter-based DNA plasmids or expression cassettes (Agrawal *et al*, 2003; Mocellin and Provenzano, 2004; McDonald *et al*, 2005). These constructs contain a portion of the target DNA cloned into an expression vector at two sites which are separated by a spacer region. The target DNA fragment is cloned into the first site in a 5' → 3' orientation and into the second site in an inverse orientation (3' → 5'). These inversely orientated fragments separated by a spacer region are referred to as Inverted Repeat Transgenes (IRTs) (McDonald *et al*, 2005). Transcription of these IRTs begins at a specific initiation sequence, as determined by the promoter to produce short hairpin RNAs (shRNAs) also referred to just as hairpin RNAs (hpRNAs), which are then processed into siRNAs by Dicer (Mocellin and Provenzano, 2004; McDonald *et al*, 2005). The effects of vector-mediated production and delivery of siRNAs can be sustained as long as two months after transfection in mammalian cells and much longer in organisms such as fungi and yeasts. There is growing interest in the use of viral vector-mediated RNAi. Adenoviral and retroviral vectors have been

reported to produce siRNAs in vivo and stable RNAi is obtained using this method although there are ethical and potential biohazardous issues associated with viral mediated RNAi.

Table 1.1: Comparison of siRNA delivery methods (Mocellin and Provenzano, 2004).

Method	Advantages	Disadvantages
Chemical or enzymatic synthesis	Rapid No need to test individual siRNA Chemical: high purity	Transient RNAi Needs transfection Variable purity & specificity Chemical: expensive
DNA plasmid vector or casette	Less expensive Stable RNAi	Labor intensive Needs transfection
Viral vector	Stable RNAi May be effective in cells resistant to transfection with dsRNA/plasmids	Labor intensive Potential biohazard

In fungi, effects of gene silencing by RNA interference have usually been investigated using plasmid vector mediated production of IRT's to silence specific genes. Promoters such as the glyceraldehye phosphate dehydrogenase (*gpdA*) promoter and strong fungal terminators e.g. the tryptophan terminator (*trpC*) are very often used in the construction of fungal RNAi vectors (McDonald *et al*, 2005; Hammond and Keller, 2004). Traditionally the complex life cycles and general difficulty of performing targeted gene replacements in many fungi have hampered genetic studies (Liu *et al*, 2001). However, with the advent of RNA interference, genetic studies in many organisms that were previously not amenable to classical mutagenesis techniques can now occur. Several studies using RNA interference have been conducted on fungi such as *C. neoformans; F. graminearum; A. flavus* and also the human pathogenic fungus *A. fumigatus* to determine the role of various genes which regulate virulence, pathogenicity and secondary metabolite production (Liu *et al*, 2001; Mouyna *et al*, 2004; McDonald *et al*, 2005; Hammond and Keller, 2004).

This study focused on the construction of an RNA interference vector and the effect that *pacC* silencing had on aflatoxin production, vegetative growth, conidiation and pathogenicity of *A. flavus* in an effort to further characterize the role of the wide domain pH-regulatory gene, *pacC*.

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CHAPTER TWO

RNA INTERFERENCE OF *pacC* AND ITS EFFECTS ON AFLATOXIN PRODUCTION

2.1 INTRODUCTION

pacC is a conserved wide domain pH regulatory gene, which controls the secretion of enzymes such as alkaline and acid phosphatases or permeases in response to environmental pH (MacAbe *et al*, 1996; Denison, S.H, 2000). This gene is required for *Aspergillus* survival and growth in environments with varying pH. In *Aspergillus parasiticus* the *pacC* gene encodes a 678 amino-acid protein that is activated under alkaline conditions by proteolysis of approximately 60% of the carboxyl terminus (Orejas *et al,* 1995; Mingot *et al,* 1999; Espeso *et al*, 2000; Penalva and Arst, 2002). *pacC* is also autoregulated by the protein binding to PacC sites (5' - GCCARG - 3') in its own promoter thus amplifying the alkaline pH signal.

A great deal about *pacC* has been uncovered by investigating *pacC* regulation of penicillin production in *A. nidulans* (Orejas *et al,* 1995; Tilburn *et al*, 1995). It is also thought that *pacC* may regulate other secondary metabolite genes such as those for aflatoxin and sterigmatocystin biosynthesis in other *Aspergillus sp*. Although aflatoxin production increases under acidic conditions and decreases under alkaline conditions (Keller *et al*, 1997), the role of *pacC* in this process has not been determined for *A. flavus*. Thus this study will aim to understand the role that *pacC* plays in aflatoxin biosynthesis in *A. flavus*.

In order to characterize the role of *pacC* a novel method of post-transcriptional gene silencing known as RNA interference was utilized. This method involved the cloning of a partial *pacC* gene fragment in first the forward and then the reverse orientations in a fungal expression cassette to create an RNA interference (RNAi) vector. The unique structure of this vector would allow the cloned fragments to be expressed and the resulting RNA to immediately form a double stranded stem-loop structure or short hairpin RNA (shRNA), (McDonald *et al,* 2005). The formation of this shRNA, in turn, would be responsible for activating the endogenous RNA degradation complexes that would lead to mRNA degradation and subsequent gene silencing (Liu *et al*, 2001; Kadotani *et al, 2003*; McDonald *et al*, 2005).

In this chapter it has been shown that confirmed *pacC* RNAi mutants produced aflatoxins irrespective of environmental pH (i.e. the mutants produce aflatoxins under acidic and alkaline conditions). Thus, *pacC* is essential for pH regulation of aflatoxin production in *A. flavus.* It was

also observed that silencing *pacC* affects the growth, conidiation and germination of *A. flavus*. These observations will be investigated further in Chapter 3.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

Chemical reagents for media, buffers and supplements were purchased from Sigma-Aldrich (Germany), restriction enzymes (*Asc*I, *Eco*RI, *Hin*dIII, *Nco*I, and *Not*I) and the 1kb DNA ladder from New England Biolabs (Massachussetts, USA), Qiagen gel extraction and plasmid isolation kits from Whitehead Scientific, TripleMaster *Taq* DNA polymerase from Eppendorf (Germany) and PCR primers from Integrated DNA Technologies (IDT, USA). All reagents used were of the highest molecular biology grade.

2.2.2 Fungal Strains and Growth Conditions

Strains of *Aspergillus spp.* used for this study were *A. flavus* 8610 (*argB*$^-$; *pyrG*$^-$), obtained from N.P. Keller (UWI), *A. flavus* MRC2527WT (obtained from the Medical Research Council of South Africa) and *pyrG*$^-$ mutants of *A. flavus* MRC2527 (Suleman, 2003). Routine culturing was performed by growing fungal strains on either glucose minimal medium (GMM) supplemented with L-arginine [50ml/l 20x salts with $NaNO_3$ (7mM); 1ml/l trace elements; 55.5mM glucose (dextrose); 1.0g/l L-arginine; adjusted to pH 6.5 with NaOH]. For solid media, agar (15g/l) was added to the liquid broth before autoclaving.

2.2.3 Isolation of Fungal Genomic DNA

Genomic DNA was isolated according to the Keller lab manual (NP Keller, UWI). Several loopfuls of *Aspergillus* conidia were inoculated into 20ml of GMM supplemented with 5.0 g/l yeast extract and incubated at 29°C overnight (16-24 hours). Thereafter, the mycelial mat was collected and pressed between paper towels to remove as much of the liquid broth as possible. Each mycelial mat was then aliquoted into eppendorf tubes (approx. 250mg/tube), snap frozen in liquid nitrogen and lyophilised for 24 hours. Lyophilized mycelia was crushed to a fine powder using sterile toothpicks, resuspended in 700µl of LETS buffer [0.1M LiCl; 20mM EDTA (pH 8.0); 10mM Tris-HCl (pH 8.0); 0.5% SDS] and thoroughly mixed using the toothpick. The mixture was then incubated at room temperature for 5 minutes. The suspension was extracted by mixing with 700µl of phenol:chloroform:isoamyl alcohol (25:24:1) and then left at room temperature for 5 minutes. The suspension was centrifuged for 10 minutes at 16,500x*g* in a microfuge at 4°C. The supernatant was then transferred into a new eppendorf tube, extracted

with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged for 10 minutes at 16,500x*g* at 4°C. DNA was then precipitated by transferring the supernatant to a new eppendorf tube, adding 1ml of 95% ethanol, mixing and centrifuging at 16,500xg at 4°C to pellet the DNA. The supernatant was then removed, the pellet washed with 70% ethanol and centrifuged at 16,500x*g* for 2 minutes to pellet the DNA. The supernatant was then discarded and the pellet air-dried for 5 minutes at room temperature. The pellet was then re-suspended in 30µl TE buffer (pH 8.0) and finally treated with 2µl RNAse (10mg/ml stock) by incubating at 50°C for 30 minutes to inactivate DNases and to digest RNA. Thereafter the DNA was stored at -20°C until needed. DNA concentration was determined via agarose gel electrophoresis by comparing band intensities with bands of a 1Kb DNA marker (NEB) having known concentrations of DNA.

2.2.4 Isolation, Cloning and Phylogenetics of a Partial *pacC* Fragment

The *A. oryzae pacC* sequence (GenBank accession number AB035899) was used to design forward (*pacC* NcoHind F1.1: 5' - AGT CGC **CCA TGG AAG CTT** CTG CTA CCA CGA CAT CCT CGG - 3') and reverse (*pacC* AscNot R1.1: 5' - TAG TA**G GCG CGC CGC GGC CGC** GGG TTC GCT GGT TGA GGT GG - 3') PCR primers to amplify an approximately 750bp region from *A. flavus* 8610 and MRC2527WT strains. *Nco*I (5' - CCA TGG - 3') and *Hin*dIII (5' - AAG CTT - 3') restriction sites were added to the 5'- end of *pacC* NcoHind F1.1 and *Asc*I (5' - GGC GCG CC - 3') and *Not*I (5' - GCG GCC GC - 3') restriction sites were added to the 5' - end of the *pacC* AscNot R1.1 to facilitate directional cloning of the two PCR fragments in the forward and reverse directions.

PCR was performed on genomic DNA isolated from *A. flavus* MRC2527 and *A. flavus* 8610. PCR conditions were 5 minutes at 95°C for initial denaturation followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension of 7 minutes at 72°C. Typical reaction mixtures contained 1U TripleMaster *Taq* DNA polymerase, 2.5µl 10x High Fidelity buffer with 2.5mM $MgCl_2$, 250µM dNTP's, 20pM of each primer and 40ng of template DNA brought up to a final volume of 25µl with sterile ddH_20. Amplified PCR products and a 1kb DNA marker were electrophoresed on a 1% (w/v) TAE (pH 8.0) agarose gel containing 5µg/ml ethidium bromide at 10V/cm for 45 minutes and visualised on a UV transilluminator. Photographs of agarose gels were taken using the AlphaImager 3400 (Alpha Innotech) imaging system. The appropriate band was excised using sterile surgical blades from the agarose gel and purified using the QiaGen gel extraction kit, and the DNA stored at 4°C until required.

Aliquots of purified *pacC* fragments from *A. flavus* 8610 and MRC2527 strains were sent to Rhodes University (Grahamstown) for sequencing. BLAST searches were performed on the sequencing data to confirm the identity of the amplified sequences. Sequences were aligned using the Vector NTI (Invitrogen) to determine the degree of homology between sequences. Phylogenetic analysis was also performed on the sequencing data and *pacC* sequences from other fungal species including related *Aspergillus sp.* using the Vector NTI software.

2.2.5 Construction of the RNA Interference Vector pESA*pacCi*2.2

The base RNAi plasmid, pTMH44.1 (courtesy of N.P. Keller, University of Wisconsin, USA) was the shuttle vector containing the expression cassette used for construction of the *pacC* RNA interference vector. pTMH44.1 has an approximately 280bp spacer fragment (containing unique *Nco*I and *Asc*I sites at the 5' end of the spacer and unique *Bam*HI, *Not*I and *Hin*dIII sites at its 3' end) placed between the *A. nidulans gpdA* promoter and *trpC* terminator. pTMH44.1 also has the ampicillin resistance gene (*ampR*), facilitating selection of bacterial transformants by resistance to ampicillin.

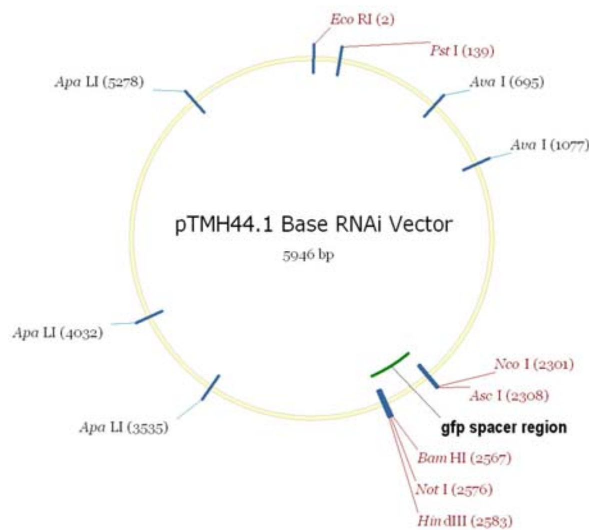

Figure 2.1: Restriction map of pTMH44.1, the base RNAi plasmid (N.P. Keller, unpublished data).

2.2.5.1 *Cloning of the pacC Inverted Repeat Transgene 1 into pTMH44.1 to Generate pESApacCi1.1*

pESApacCi2.2 (RNAi vector) was constructed in a similar manner to that described by McDonald *et al* (2005). The purified *pacC* fragment and pTMH44.1 were sequentially digested with *Not*I and *Hin*dIII overnight at 37°C. Typical restriction digests contained 10 units of each

enzyme, appropriate buffer/s, BSA (if necessary), 0.5µg DNA and water in a total reaction volume of 25µl. Digested products were electrophoresed on a 1% (w/v) agarose gel at 10V/cm for 45 minutes; the bands were excised and purified from the gel using the QiaGen gel extraction kit. The purified *Not*I/*Hin*dIII digested *pacC* fragment (referred to as *pacC* Inverted Repeat Transgene 1, *pacC*IRT1) was ligated into the *Nco*I/*Hin*dIII linearised pTMH44.1 plasmid using the RapidLigation system (Promega), transformed into *E. coli* JM109 strains, selected for by plating on Luria-Bertani agar containing 100µg/ml ampicillin (LB-Amp) and incubating overnight at 37°C. Random colonies appearing on the plates after 16-24 hours were inoculated into 5ml LB+Amp broth and incubated at 37°C overnight with shaking at 180rpm. Plasmids were isolated from broth cultures using the QiaGen plasmid isolation kit (QiaGen). Plasmid DNA from transformants was then digested with *Not*I & *Hin*dIII for 4 hours at 37°C, and electrophoresed as described before. Clones having two bands (the 5.9kb pTMH44.1 and 750 *pacC* fragments) were designated as pESApacCi1.1 and selected for further genetic manipulations.

2.2.5.2 *Cloning of the pacC Inverted Repeat Transgene 2 into pESApacCi1.1 to Generate pESApacCi2.1*

pESApacCi1.1 and the gel purified *A. flavus pacC* PCR fragments were then digested overnight at 37°C with *Asc*I and *Nco*I (referred to as *pacC* Inverted Repeat Transgene 2 - *pacC*IRT2). Digested fragments were electrophoresed and purified as before. The purified *Nco*I/*Asc*I digested *pacC*IRT2 fragment was cloned into the *Nco*I/*Asc*I linearised pESA *pacC* 1.1 plasmid as described in section 2.2.5.1. Plasmid DNA from transformants was digested with *Asc*I and *Nco*I for 4 hours at 37°C and clones having two clear bands (the 6.6kb pESApacCi1.1 plasmid and 750 *pacC*IRT2 fragments) were selected to confirm the presence of the complete *pacC* Inverted Repeat Transgene. Plasmid DNA from these clones were digested with *Hin*dIII and those yielding two fragments (the 5.6Kb pTMH44.1 and 1.8kb inverted repeat construct) selected for further genetic manipulation and designated as pESApacCi2.1.

2.2.5.3 *Cloning of the pyrG marker gene into pESApacCi2.1 to generate the RNA Interference Vector pESApacCi2.2*

The *Aspergillus pyrG* gene (encoding orotidine-5'-phosphate decarboxylase) was used to as the selectable marker (Woloshuk *et al*, 1989; d'Enfert, 1996) in order to select for fungal transformants by cloning into pESApacCi2.1. The *pyrG* cassette was obtained from plasmid

p74.1 (N.P. Keller, UWI) by digestion with *Eco*RI and pESApacCi2.1 was linearised by digestion with *Eco*RI. Fragments were gel isolated and ligated as previously described. Plasmid DNA from bacterial transformants was then separately digested with *Eco*RI and *Hin*dIII for 4 hours at 37°C and electrophoresed as described in section 2.2.4 to verify insertion of the *pyrG* gene. Bacterial clones selected for plasmid DNA isolation and fungal transformations were then referred to as pESApacCi2.2.

2.2.6 Fungal Transformation

2.2.6.1 *Generation of Protoplasts*

Confluent cultures of *A. flavus* 8610 (*pyrG*$^-$; *argB*$^-$) and *A. flavus* MRC2527 (*pyrG*$^-$) were grown on stabilised minimal media plates [50ml/l 20x salts with $NaNO_3$ (7mM); 1ml/l trace elements; 55.5mM glucose (dextrose); 1.0g/l yeast extract; 16g/l agar adjusted to pH 6.5 with NaOH] containing the appropriate supplements (1.0g/l L-arginine; 0.56g/l uracil; 1.26g/l uridine) and 1.2M sorbitol for 2-3 days at 28°C. Spores were collected by scraping into 10 ml sterile ddH_2O containing 0.1% Tween-80. Spores were counted in a haemocytometer and $1x10^9$ spores were inoculated into 500ml of sterile defined minimal medium [25ml/l 20x salts with $NaNO_3$ (7mM); 0.5ml/l trace elements; 55.5mM glucose (dextrose); 0.5g/l yeast extract adjusted to pH 6.5 with NaOH, containing the appropriate supplements (1.0g/l L-arginine; 0.56g/l uracil; 1.26g/l uridine)] and incubated at 28°C on a rotary shaker (300 rpm) for approximately 8 hours or until the cultures had an abundance of young germlings in small aggregates.

The cultures were harvested by filtration through sterile Miracloth (Sigma-Aldrich) and the mycelia washed with 50 ml wash solution (0.6 M $MgSO_4$). Thereafter 1.0g of mycelia was transferred to a 125ml Erlenmeyer flask, resuspended in 5 ml osmotic medium (1.2 M $MgSO_4$; 10 mM sodium phosphate buffer (Appendix A), adjusted to pH 5.8 with 1M Na_2HPO_4) and vortexed gently. 100mg of Fluka lysing enzyme (Sigma-Aldrich) and 1.2 mg BSA per ml of osmotic medium was added to the Erlenmeyer flask, mixed and incubated on ice for 5 minutes. Thereafter, the flasks were shaken for 4 hours at 80rpm at 28°C. Protoplast formation was monitored microscopically every 30 minutes. Once sufficient protoplasts had begun to form, the cell suspension was transferred directly into a sterile 30ml Corex tube and very gently overlayed with 10 ml trapping buffer (0.6M sorbitol; 0.1M Tris-HCl at pH 7.0) and centrifuged at 2500x*g* for 15 minutes at 4°C. The protoplasts were removed from the interface and transferred into a new 15ml Falcon tube. The remaining trapping buffer was then removed and the pellet resuspended in the remaining osmotic medium. The cells were overlayed once more with 10 ml trapping buffer and centrifuged as above. Protoplasts were again removed from the interface and

pooled with those from the first centrifugation step. The protoplast suspension was diluted with at least 1 volume of STC buffer (1.2M sorbitol; 10 mM Tris.HCl at pH 7.5; 10 mM $CaCl_2$) and centrifuged at 3500x*g* for 8 minutes. The supernatant was discarded and the pellet resuspended in 0.2ml STC buffer and stored on ice. Samples of the protoplasts were diluted 500 times and counted using a haemocytometer to determine protoplast concentration.

2.2.6.2 *Protoplast Transformation*

Approximately 10µg of pESApacCi2.2 was digested with *Eco*RI at 37°C for 4 hours to linearise the vector and release the *pyrG* gene. Plasmid DNA was then mixed with STC buffer to a total volume of 100µl. Thereafter, 100µl of protoplast solution ($1x10^7$ protoplasts) was mixed with the plasmid DNA and incubated for 50 minutes on ice. Thereafter 1.25 ml of PEG solution (60% PEG4000; 10 mM $CaCl_2$; 10mM Tris.HCl at pH 7.5) was added to the DNA-protoplast mixture and mixed gently by turning the tube on its side and rotating it. The tube was then incubated at room temperature for 20 minutes. Thereafter 5 ml of STC buffer was added to the tube and mixed gently. Aliquots (1ml) were taken and mixed with liquid top-agar (GMM), plated onto selective media [GMM adjusted to pH 4.0 using 19.3mM/l Na_2HPO_4 and 15.35mM/l citric acid (Pinero and Keller, unpublished data), supplemented with sorbitol and L-arginine but lacking uridine and uracil] and incubated overnight at 28°C. Transformations were separately performed on protoplasts generated from *A. flavus* 8610 and *A. flavus* MRC2527.

Putative transformants were transferred to GMM (supplemented with 1g L-arginine per litre) plates buffered to pH 4.0 (using 19.3mM/l Na_2HPO_4; 15.35mM/l citric acid) (Pinero and Keller, unpublished data) to allow for recovery of mutants. Once transformants had sufficiently recovered, they were transferred to fresh GMM plus arginine agar plates buffered to pH 4.0 and incubated at 28°C.

2.2.7 PCR Based Screening of Transformants

Several loopfuls of conidia from the transformants were inoculated into liquid GMM supplemented with 5g yeast extract per litre and incubated at 28°C overnight (16-24 hours) for genomic DNA isolation (see 2.2.3).

2.2.7.1 *PCR Screening via Amplification of IRTs 1 and 2*

PCR on transformants was carried out to facilitate genetic screening for the presence of the pESApacCi2.2 RNA interference vector using the forward pESA 2.1 IR F1 (5'- TTG AGA CAT CAC CAT GGA AGC TTC TGC - 3') and reverse pESA 2.1 IR R1 (5'- CAG TAA CGT TAA

GTG GAT CTC AAG CTT CTG C -3') primers. These primers contained partial vector/insert sequences and were therefore designed to precisely anneal at the junctions between vector and insert. PCR reaction conditions were similar to that described in section 2.2.4 except that extension times were increased to 2 minutes.

2.2.7.2 *PCR Screening via Partial Amplification of ampR*

Fungal transformants generated in this study would harbour the *ampR* (ampicillin resistance marker for bacterial transformation) gene and this gene was used to screen fungal clones for the presence of pESApacCi2.2 (RNA interference vector). PCR primers were designed to amplify an approximately 700bp region of the *ampR* gene using the forward (AmpR F1: 5' - CAT CCA TAG TTG CCT GAC TCC CC - 3') and reverse (AmpR R1: 5' - TCA ACA TTT CCG TGT CGC CC - 3') primers. PCR was performed with reaction conditions as described in section 2.2.4.

2.2.8 Southern Hybridization

Approximately 10µg genomic DNA isolated from each fungal transformant was digested with *Eco*RI overnight at 37°C. Thereafter the restriction digests were electrophoresed on a 1% agarose gel at 1V/cm for 6-8 hours (or until the DNA loading dye had migrated at least ¾ the length of gel) (Sambrook *et al*, 1989). After electrophoresis the agarose gel was blotted onto positively charged nylon membrane (HyBond N^+, AEC Amersham). The DNA was depurinated by soaking the gel in depurination solution (0.25 M HCl) for 10 minutes at room temperature with gentle agitation. The depurination solution was then removed and the gel rinsed several times with sterile deionised water. Thereafter, the DNA was denatured by soaking the gel in denaturation solution (1.5 M NaCl; 0.4M NaOH) for 15 minutes at room temperature with gentle agitation. A gel transfer apparatus was then set as described by Sambrook *et al* (1989) and continuing capillary transfer for 18 hours.

Nylon membranes were pre-hybridised overnight using a phosphate-SDS [0.5M phosphate buffer[1] (pH 7.2); 1mM EDTA (pH 8.0); 7% (w/v) SDS] at 65°C prior to addition of the labelled probe. Probes were synthesized by first digesting pESApacCi2.2 with *Hin*dIII to release the cloned 1.8kb fragment that corresponds to the RNA interference region of the plasmid. This 1.8kb fragment was gel isolated and purified and approximately 25ng of the DNA was radioactively labelled using the MegaPrime DNA labelling kit (AEC Amersham) and 5µl

[1] 0.5M phosphate buffer contains 80g Na_2HPO_4, 4ml of 85% H_3PO_4 and ddH_2O to 1liter.

Redivue α-dCTP^{32} (150μCi/μl; AEC Amersham) according to the manufacturer's instructions for 1 hour at 37°C. The labelling reaction was stopped by the addition of 5μl of 0.2M EDTA (pH 8.0). Labelled probe was boiled for 5 minutes and then cooled on ice for 5 minutes to maintain the probe as single-stranded DNA. The denaturation process was repeated and after cooling on ice, the probe was centrifuged briefly to collect the contents at the bottom of the tube. The probe was then added to the blot already soaking in prehybridization buffer. Hybridization was allowed to proceed for 18 hours (overnight) at 65°C. Thereafter the blot was removed and washed in 2x SSC, 0.2% SDS buffer at 65°C for 15-20 minutes, and then washed in 1x SCC, 0.1% SDS buffer at 65°C for 15-minutes. The blot was then wrapped in cling-wrap, placed in a steel X-ray cassette, together with X-ray film (Hyperfilm, AEC Amersham) and an intensifying screen. The blot was left to expose at -80°C for at least 16 hours, and thereafter the autoradiograph was developed. Transformants exhibiting positive signals were selected and the hybridization procedure repeated to confirm the presence of the RNA interference cassette in these clones.

2.2.9 Northern Hybridization

2.2.9.1 *RNA Isolation and Quantification*

Approximately 1x10^6 spores from untransformed 8610 and three confirmed fungal mutants (TpacCi87, TpacCi88 and TpacCi96) were separately inoculated into 10ml 20% YES (20% yeast extract; 2% sucrose) buffered to pH 4 and pH 8 in Erlenmeyer flasks and incubated at 28°C. Mycelia were harvested after incubation for 38 hours. The mycelial mat was collected and blotted dry using paper towels. This was then aliquoted into eppendorf tubes, snap frozen in liquid nitrogen and lyophilised for 24 hours. Thereafter dried mycelia were crushed to a fine powder using sterile toothpicks with the final amount being no more than a 100μl equivalent. 1ml of Trizol reagent (Invitrogen) was added to the ground mycelium and gently mixed with the toothpick to ensure complete homogenisation. The mycelium/trizol mixture was allowed to stand at room temperature for 5 minutes. Thereafter 200μl chloroform was added and the mixture shaken (by hand) for 15 seconds and left at room temperature for 3 minutes. The mixture was then centrifuged at 11,000x*g* for 15 minutes at 4°C.

The aqueous phase was transferred to a new eppendorf tube and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.6) for 5 minutes at room temperature and then centrifuged for 15 minutes at 11,000x*g* at 4°C. The aqueous layer was obtained and 500μl isopropanol added to the mixture which was then vortexed and incubated at room temperature for a further 10 minutes, and thereafter centrifuged at 11,000x*g* for 10 minutes at 4°C. The supernatant was discarded, the RNA pellet briefly air-dried, re-suspended in 40μl

DEPC (diethyl pyrocarbonate, Sigma Aldrich) treated H_2O and heated at 65°C for 10 minutes. 1µl (undiluted or 1:5 dilution) aliquots were loaded into an RNA Nano chip (Agilent Technologies) and quantified on the Expert 2100 BioAnalyser (Agilent Technologies). RNA was stored at -80°C until required.

2.2.9.2 *Electrophoresis and Blotting*

An equal amount of RNA (15µg) of each sample was mixed with 15µl RNA loading buffer/sample [1µl DEPC-H_2O; 3µl 10x MOPS; 4µl 37% formaldehyde; 9µl formamide] and heated at 68° for 10 minutes. Thereafter samples were briefly centrifuged in a microfuge to concentrate samples to the bottom of the eppendorf tubes, the entire sample loaded onto a 1.2% agarose-1.5% formaldehyde gel and electrophoresed at 30V for 6 hours in 1x MOPS running buffer [100ml 10x MOPS buffer (Sigma-Aldrich); 20ml 37% (12.3M) formaldehyde (Sigma-Aldrich); 880ml DEPC-H_2O]. After electrophoresis, the gel was photographed using the AlphaImager (AlphaInnotek) imaging system. The gel was rinsed in DEPC treated distilled water with gentle shaking for 15 minutes and blotted as described in section 2.1.9.

2.2.9.3 *Hybridization*

PCR was performed on genomic DNA from *A. flavus* 8610 using the *pacC* NcoHind F1.1 and *pacC* AscNot R1.1 primers as described in section 2.1.4 and the 750bp band gel purified as described previously. This PCR fragment was labeled and hybridizations performed as described in section 2.2.8 with the exception that the hybridization temperature was reduced to 45°C. Thereafter the blot was removed and washed in wash buffer (2x SSC; 0.2% SDS) at 45°C for 15-20 minutes. The wash was repeated with diluted wash buffer (1x SSC; 0.1% SDS) at 45°C for 15 minutes. The blot was then wrapped in cling-wrap, placed in a steel X-ray cassette together with X-ray film (Hyperfilm, AEC Amersham) and an intensifying screen and exposed at -80°C for 120 hours, before the autoradiograph was developed.

Northern hybridizations were repeated with the 28S rDNA probe to verify the presence of equal amounts of RNA in the RNA blots (positive control). The *pacC* probe (above) was stripped from each membrane by rinsing in DEPC-treated boiling water for 5 minutes. This was repeated thrice to ensure that all bound probe was removed. PCR was performed on genomic DNA from *A. flavus* 8610 to amplify the 28S rDNA fragment using primers rDNA F1 (5' - AAA CCA ACC GGG ATT GCC - 3') and rDNA R1 (5' - GTC GTT TAC GAC CAT TAT GCC - 3'). Reaction conditions were as described in section 2.2.4 except that the annealing temperature was 50°C and

extension time was 1 minute. The PCR product was gel purified as described before and the probe labeled and hybridized as above.

2.2.10 Aflatoxin Production

2.2.10.1 *Aflatoxin Induction*

Untransformed *A. flavus* MRC2527, *A. flavus* 8610 and each mutant (TpacCi87, TpacCi88, TpacCi96) were inoculated into 10ml YES (2% yeast extract; 20% sucrose) broth buffered to pH 4.0 (aflatoxin inducing) with 1 x 10^6 spores to induce aflatoxin production (Fente *et al*, 2001). These inoculations were repeated with 10ml YES broth buffered to pH 8.0 (aflatoxin suppressing). Cultures were grown as stationary cultures at 30°C for 4 days in the dark (Trail *et al*, 1995). After 4 days the cultures were analysed for aflatoxin production.

2.2.10.2 *Aflatoxin Extraction*

Aflatoxins were extracted by a modification of the method described by Criseo *et al* (2001). Mycelia were harvested and ground in a mortar and pestle. The ground mycelia and liquid broth were transferred to a sterile 50ml culture tube; 20ml chloroform was added to the culture and vortexed for 5 minutes. The homogenised mixture was centrifuged at 10,000x*g* for 15 minutes. After centrifugation, the aqueous layer was discarded, the chloroform layer filtered through Whatman No. 1 filter paper and the filtrate collected in a separating funnel. The chloroform layer was again filtered through anhydrous sodium sulphate (Na_2SO_4) and the filtrate collected. The filtration steps were repeated twice and the extracts pooled. The pooled extracts were evaporated to dryness under N_2 gas. The dried extracts were re-suspended in 200µl chloroform, transferred to sterile eppendorf tubes and stored in the dark at -20°C.

2.2.10.3 *Thin Layer Chromatography (TLC) for Aflatoxin Detection*

Aflatoxins from purified extracts were detected by TLC on silica gel plates. This was done by spotting 10µl aliquots of toxin from each isolate onto a pre-marked TLC plate. Chloroform:acetone:water (91:9:1) was used as the developing solvent and development allowed to occur for 45 to 60 minutes (Suleman, 2003). The plates were dried and viewed under long wavelength UV (365nm) light, which causes B group aflatoxins to fluoresce a bright blue colour. Photographs showing aflatoxins fluorescing blue under UV light were taken using the AlphaImager (AlphaInotech) imaging system.

2.3 RESULTS AND DISCUSSION

2.3.1 Construction of RNA Interference Vector pESApacCi2.2

A portion of the *pacC* gene was successfully amplified via PCR using primers *pacC* NcoHind F1.1 and *pacC* AscNot R1.1 as shown in Figure 2.2A.

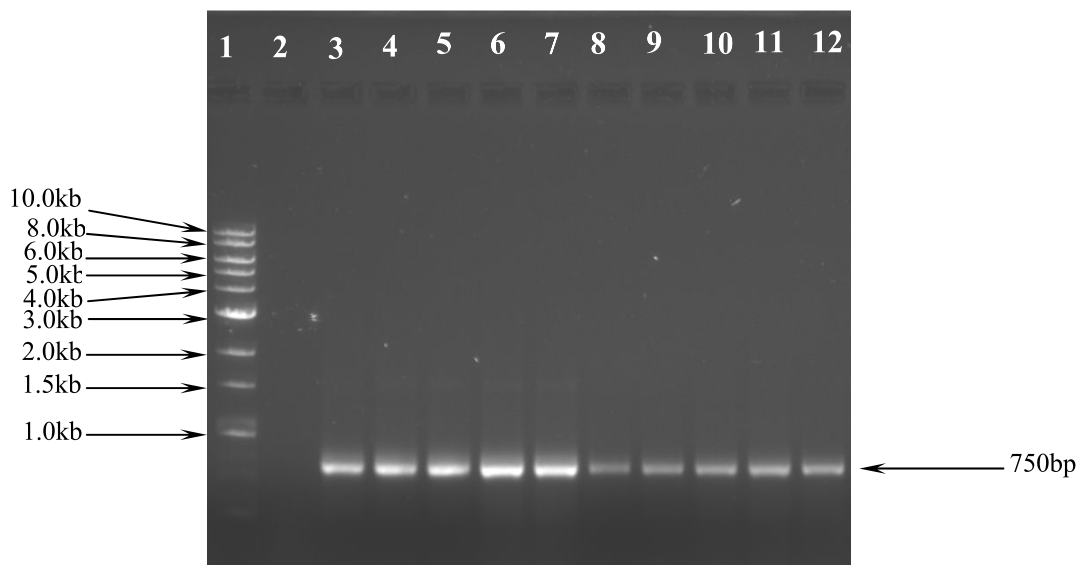

Figure 2.2A: Agarose gel electrophoresis (1%) showing amplification of the partial *pacC* fragment from *A. flavus* MRC2527WT.
Lane 1: 1kb DNA ladder (NEB - the 3kb band has a concentration of 125ng/µl and was used for determining concentration of other bands); Lane 2: Blank (negative control); Lanes 3-12: 750 *pacC* fragment.

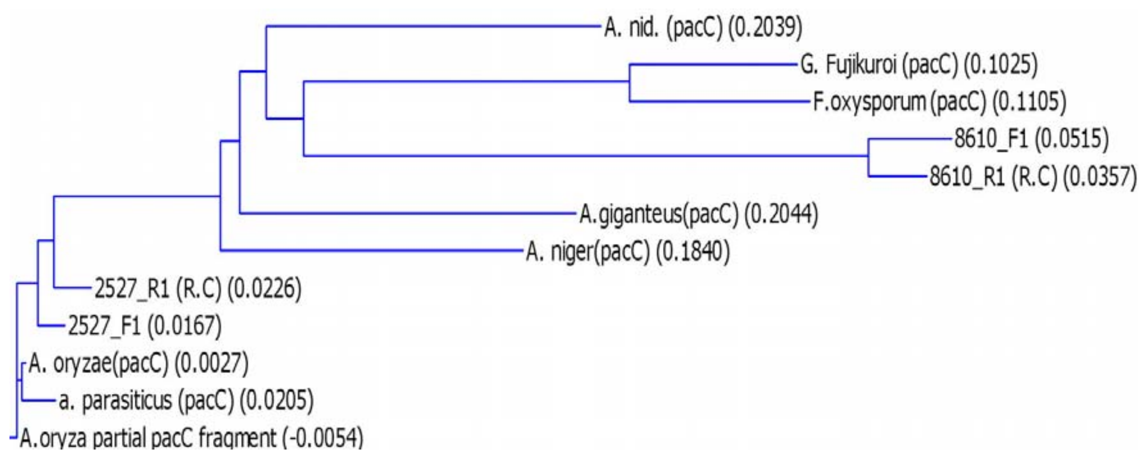

Figure 2.2B: Phylogenetic tree showing relationship between amplified *pacC* sequences of *Aspergillus flavus* 8610 and *Aspergillus flavus* 2527 with sequences from *A. oryzae, A. nidulans, A. parasiticus, A. niger*, *A. giganteus*, *G. fujikuroi* and *F. oxysporum.*
The phylogenetic tree was constructed using VectorNTI software.

PCR generated an expected 750bp fragment based on the *A. oryzae pacC* gene sequence obtained from GenBank. Since the *A. oryzae* gene sequence was used to design primers for *A.*

flavus, this indicates a high degree of homology between the *pacC* genes of *A. oryzae* and *A. flavus*. This is not surprising since it is thought that *A. oryzae* was derived from *A. flavus* (Kurtzman *et al*, 1986). At the commencement of this study the *A. flavus pacC* gene sequence was not available, although sequence data was available for closely related organism's *viz. A. nidulans, A.oryzae, A. niger* and *A. parasiticus*. Tominaga *et al* (2005) have shown that several aflatoxin biosynthetic cluster genes in *A. oryzae* have between 97% to 99% homology to *A. flavus. A. oryzae* belongs to the *Aspergillus* section *Flavi*, which includes *A. flavus* and *A. parasiticus.* It is thought that *A.oryzae* is highly related taxonomically to *A. flavus* and share 90% sequence homology (Kurtzman *et al*, 1986; Tominaga *et al*, 2005).

When the sequence of the amplified *A. flavus* partial *pacC* fragment was compared to those in the GenBank database using the BLAST (Altschul *et al*, 1997) tool, the amplified *pacC* sequence was found to be 98% homologous to the sequence of the *A. oryzae* (E-value = 0) and *A. parasiticus pacC* (E-value = 0) genes (see Appendix B). Phylogenetic analysis using the partial *pacC* fragment indicated that the amplified fragment clustered together with the *pacC* genes of *A. oryzae, A. nidulans, A. parasiticus, A. niger*, *A. giganteus*, *G. fujikuroi* and *F. oxysporum* (Fig 2.2B). These results together indicated that the fragment amplified in this study was from the *pacC* gene.

This gel purified Inverted Repeat Transgene 1 (IRT1) fragment was successfully ligated into the pTMH44.1 base RNAi plasmid as evidenced by the 750bp fragment isolated from pESApacCi1.1. Similarly the Inverted Repeat Transgene 2 (IRT2) fragment was successfully ligated into pESApacCi1.1 as evidenced by 1.8kb fragment (2x 750bp PCR products plus 300bp spacer; Fig 2.3).

The cloning method employed here ensured that the expressed mRNA would form a hairpin loop structure (See Appendix C). The success of vector-mediated RNA interference is dependent upon the presence of inverted repeat sequences present in the vector to efficiently activate the RNA silencing mechanism in many fungal species. This has been observed in several fungi including *Neurospora crassa, Magnaporthe oryzae*, *Cryptococcus neoformans*, *Aspergillus fumigatus* and *A. nidulans* (Catalanotto *et al*, 2004; Hammond and Keller 2004; Kadotani *et al*, 2003; Liu *et al*, 2001; Mouyna *et al*, 2004). It has been hypothesized that IRT's produce hairpin RNA (hpRNA) that is digested into small interfering RNA (siRNA) species by an RNAse III enzyme such as Dicer thus activating RNA silencing of endogenous genes that are homologous to the sequences contained in the hpRNA (McDonald *et al*, 2005).

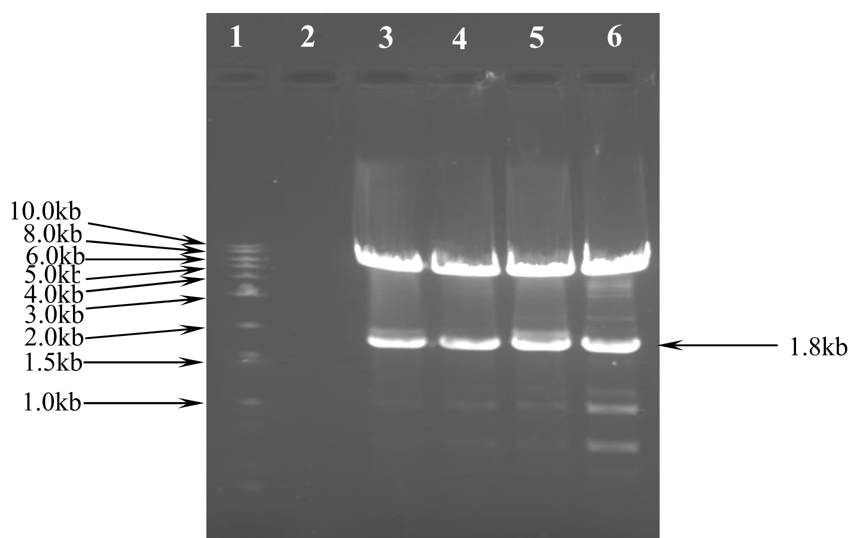

Figure 2.3: Agarose gel electrophoresis of pESApacCi2.1 after digestion with *Hin*dIII.
Lane 1: 1kb DNA ladder; Lane 2: Blank; Lane 3: pESApacCi2.1 DNA from bacterial clone 4; Lane 4: pESApacCi2.1 DNA from bacterial clone 8; Lane 5: pESApacCi2.1 DNA from clone 9 and Lane 6: pESApacCi2.1 DNA from clone 10.

In the final step a 3kb *pyrG* gene was subcloned into the *Eco*RI site of pESApacCi2.1 to form the complete RNA interference vector pESApacCi2.2. This was essential since the *A. flavus* strains used for transformation in this study lacked the ability to synthesize uracil and uridine (the pyrimidine biosynthesis gene, *pyrG*, was mutated). Complementation of uridine/uracil auxotrophic mutants via the orotidine-5'-phosphate decarboxylase-encoding gene (*pyrG*) results in reversion to uracil/uridine prototrophy, enabling selection of positive transformants (d'Enfert, 1996; Rose *et al,* 2000). The *pyrG* gene was used as a marker to select for transformants by restoring uracil/uridine prototrophy.

From virtual digests using Vector NTI software it was predicted that pESApacCi2.2 (complete RNAi vector) would give band sizes of 3.0kb and 7.5kb when digested with *Eco*RI and 1.8kb, 2.7kb and 6.0kb when digested with *Hin*dIII. However, only one clone had the complete IRT motif and the *pyrG* gene. This clone exhibited two bands of 3.0kb and 7.5kb when digested with *Eco*RI (lane 4, Fig. 2.4) and three bands of 1.8kb, 3.8kb and 5.0kb (Lane 13, Fig. 2.4). Although the *Eco*RI digest gave the predicted band sizes, the *Hin*dIII digest gave anomalous results on agarose gels.

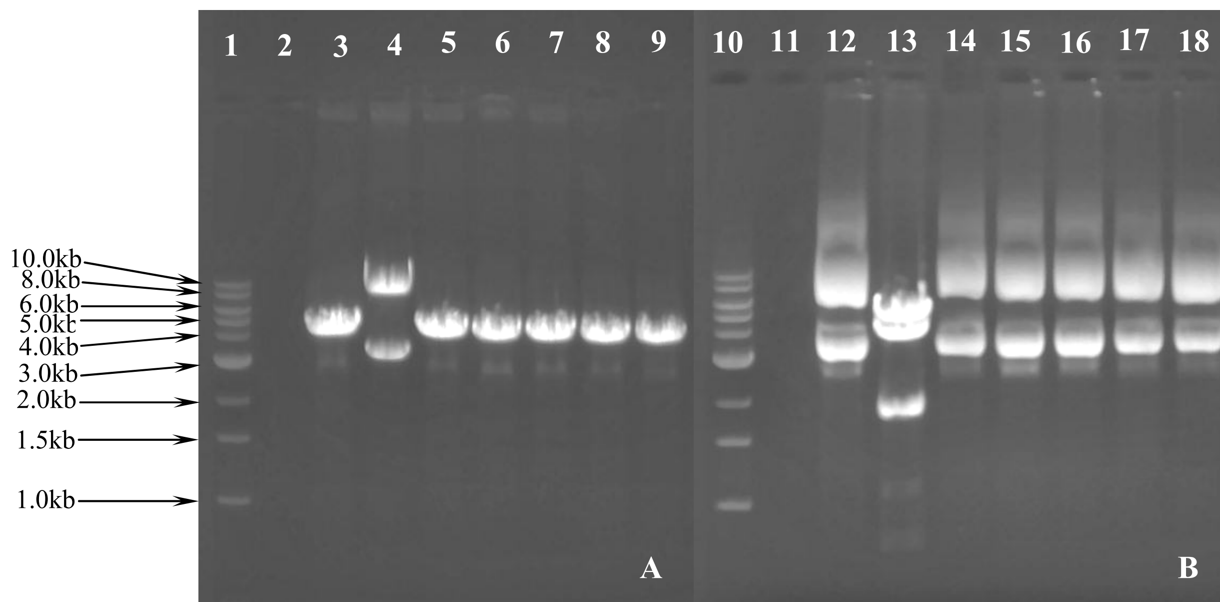

Figure 2.4: Agarose gel electrophoresis of pESApacCi2.2 bacterial clones digested with (A) *Eco*RI and (B) *Hin*dIII.
Lane's 1 and 10: 1kb DNA ladder; Lanes 2 and 11: Blank; Lanes 3-9 and lanes 12-18: bacterial clones harbouring pESApacCi2.2 vector DNA.

After examination of the anomalous result from the *Hin*dIII digest, it was hypothesized that the *pyrG* gene in the bacterial clone was inserted into the reverse orientation and therefore anomalous band sizes were obtained on agarose gels. To test this hypothesis the *pyrG* sequence was reversed in Vector NTI and again analyzed by performing a virtual digest. The predicted sizes were then found to be 1.8kb, 3.74kb and 4.985kb which is almost exactly that obtained on agarose gels. Thus the *pyrG* gene was cloned in the reverse orientation into the pESApacCi2.2 plasmid.

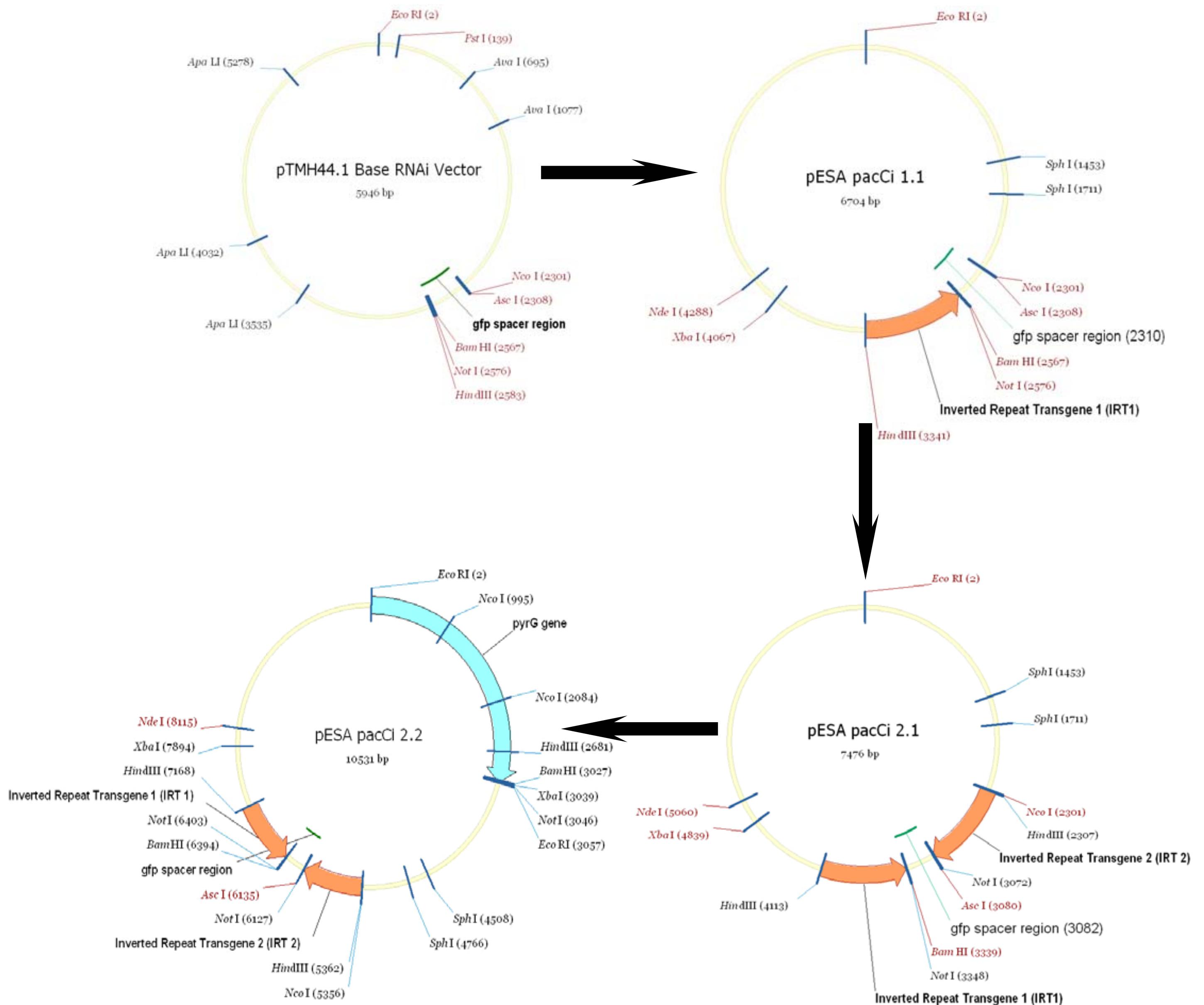

Figure 2.5: Schematic plasmid maps showing the various intermediate stages during construction of the pESApacCi2.2 RNA interference vector.
Arrows indicate direction of construction i.e. pTMH44.1 → pESApacCi1.1 → pESApacCi2.1 → pESApacCi2.2.

2.3.2 Transformation of *A. flavus* 8610 (*pyrG*$^-$; *argB*$^-$) and *A. flavus* MRC2527WT (*pyrG*) strains with pESApacCi2.2

The generation of fungal protoplasts was found to be critical in order for successful transformation of the recipient fungal strains (*A. flavus* 8610 and *A. flavus* MRC2527) with the

pESApacCi2.2 RNAi plasmid. Protoplasts (Fig. 2.6) were successfully generated by lysing the fungal cells walls of young germlings. Minimum protoplast concentrations of at least 5 x 10^7 protoplasts/ml were consistently obtained for *A. flavus* 8610 and *A. flavus* MRC2527. Several attempts to transform *A. flavus* 8610 and *A. flavus* MRC2527 with circular pESApacCi2.2 proved to be unsuccessful. Although the reasons for this are unknown, it is possible that the circular plasmids could not integrate into the genome or that the plasmid concentration was too low. However, when the plasmid was digested with *Eco*RI prior to transformation, *A. flavus* 8610 only was successfully transformed. It has been reported that digestion of the vector improves the transformation efficiency and single-copy integration due to restriction enzyme-mediated integration (REMI) (Schiestl and Petes, 1991; Shi *et al,* 1995; d'Enfert, 1996). Digestion of pESApacCi2.2 released the *pyrG* gene allowing it to replace the non-functional *pyrG* in the recipient strains by either homologous recombination or ectopic integration, while the rest of the RNAi plasmid could integrate ectopically into the genome. It appeared that the South African isolate *A. flavus* MRC2527 *pyrG*$^-$ was not suitable for fungal transformations. It is hypothesised that this strain regenerated its cell wall faster than *A. flavus* 8610 and therefore would not be easily transformed (unconfirmed data).

Successful transformation systems rely on cloning vectors with appropriate genetic markers for selection of positive transformants (Woloshuk *et al*, 1989). There are two classes of selection systems used with filamentous fungi. The first class employs resistance to metabolic inhibitors (e.g. hygromycin and benomyl) for selection, while the second class utilizes the conversion of auxotrophic mutations such as *trpC*, *argB* and *pyrG* to prototrophy (Woloshuk *et al*, 1989). Markers which employ resistance to metabolic inhibitors are the most advantageous since any fungal strain which is sensitive to the inhibitors can be used as a potential recipient in transformation. However, markers requiring recipient strains to have an auxotrophic mutation are in many cases difficult to acquire. Unfortunately, genes which exhibit resistance to metabolic inhibitors cannot be used as genetic markers in *A. flavus,* which is insensitive to most available metabolic inhibitors. The benomyl resistance genes from *N. crassa* and *A. nidulans* are poorly expressed in *A. flavus* (Woloshuk *et al*, 1989). Thus, a transformation system was used which resulted in conversion of auxotrophic mutants to prototrophy.

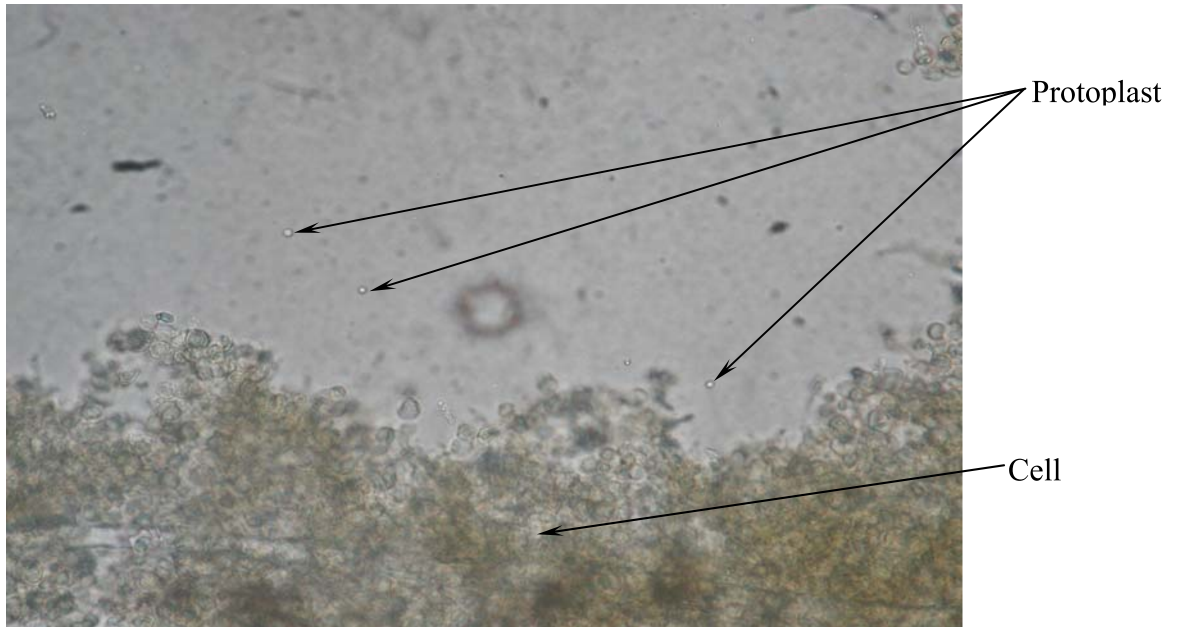

Figure 2.6: Formation of *Aspergillus flavus* 8610 protoplasts (arrows) after treatment with lysing enzymes from *Trichoderma harzianum*.

2.3.3 Screening Fungal Transformants via PCR

Transformants were screened via PCR for the presence of the pESApacCi2.2 RNA interference vector. Primers designed to anneal at the plasmid/insert junction failed to yield the expected band size of 1.8kb from any of the transformants. All transformants and untransformed *A. flavus* 8610 exhibited a 3kb band and several bands less than 1.5kb in size. Surprisingly, the positive control showed a 1.5kb band (Fig. 2.7). Increasing the annealing temperature also did not result in the correct band size appearing for any of the transformants. The plasmid was constructed to deliberately form a hairpin-loop structure to induce RNA silencing. It was thus concluded that the plasmid vector formed stable secondary structures which hindered PCR (see Appendix C). Therefore, anomalous bands obtained are due to non-specific binding of the primers.

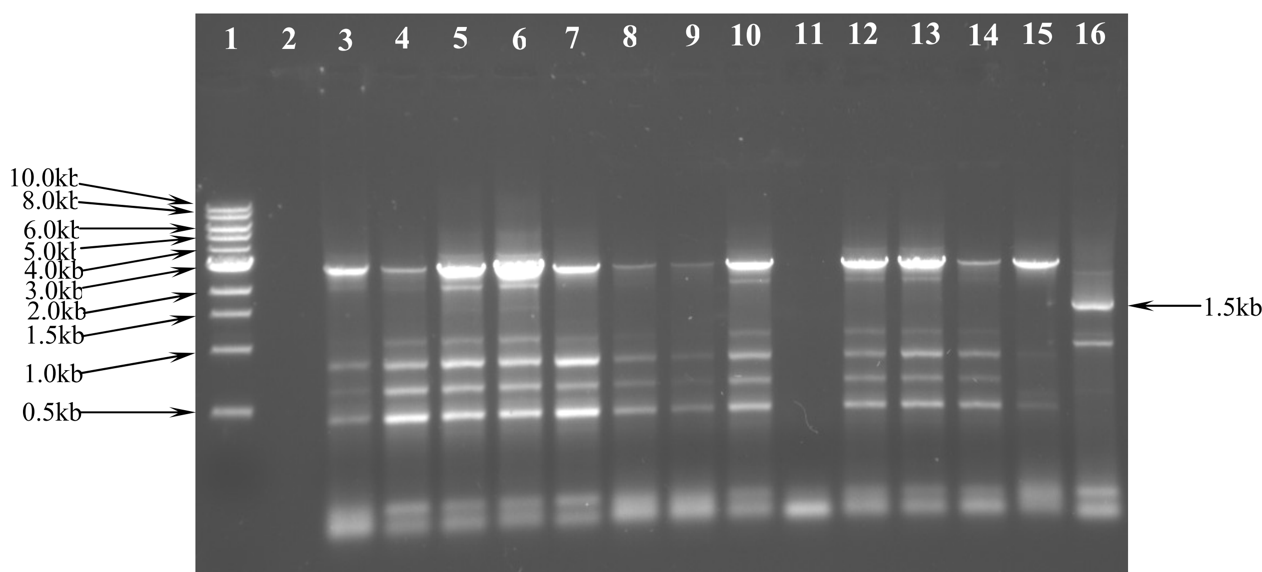

Figure 2.7: PCR screening of transformed and untransformed fungal strains.
Lane 1: 1kb DNA ladder; Lane 2: Blank; Lane 3: TpacCi1; Lane 4: TpacCi14; Lane 5: TpacCi18; Lane 6: TpacCi21; Lane 7: TpacCi25; Lane 8: TpacCi28; Lane 9: TpacCi47; Lane 10: TpacCi52; Lane 11: TpacCi81; Lane 12: TpacCi86; Lane 13: TpacCi90; Lane 14: TpacCi94; Lane 15: untransformed *Aspergillus flavus* 8610 (negative control); Lane 16: pESApacCi2.2 (positive control).

PCR screening of the transformants using the AmpR primers was successful (Fig. 2.8). Three transformants (TpacCi87, TpacCi88 and TpacCi96) showed a bright band of 750 similar to that of the two positive controls (pESApacCi2.2 and pUC18 plasmids) which are known to have the *ampR* gene (Fig 2.8). Untransformed *A. flavus* 8610 and *A. flavus* MRC2527WT genomic DNA did not show the expected 750bp band since the fungus does not normally possess ampicillin resistance and therefore should not harbour the *ampR* gene.

Owing to the difficulty experienced in attempting to directly amplify the 1.8kb IRT fragment, PCR amplification of the *ampR* gene proved an efficient and alternate means of screening for putative fungal transformants. It was expected that the 1.8kb IRT region would be attached to the *ampR* gene since both were introduced into fungal protoplasts as a single fragment. However, this is only indirect evidence for the presence of the IRT. It is possible that a portion of the IRT region was truncated from pESApacCi2.2 during transformation and that the *ampR* gene remained intact. It was therefore necessary to confirm the presence of the 1.8kb IRT within the selected transformants via Southern hybridization.

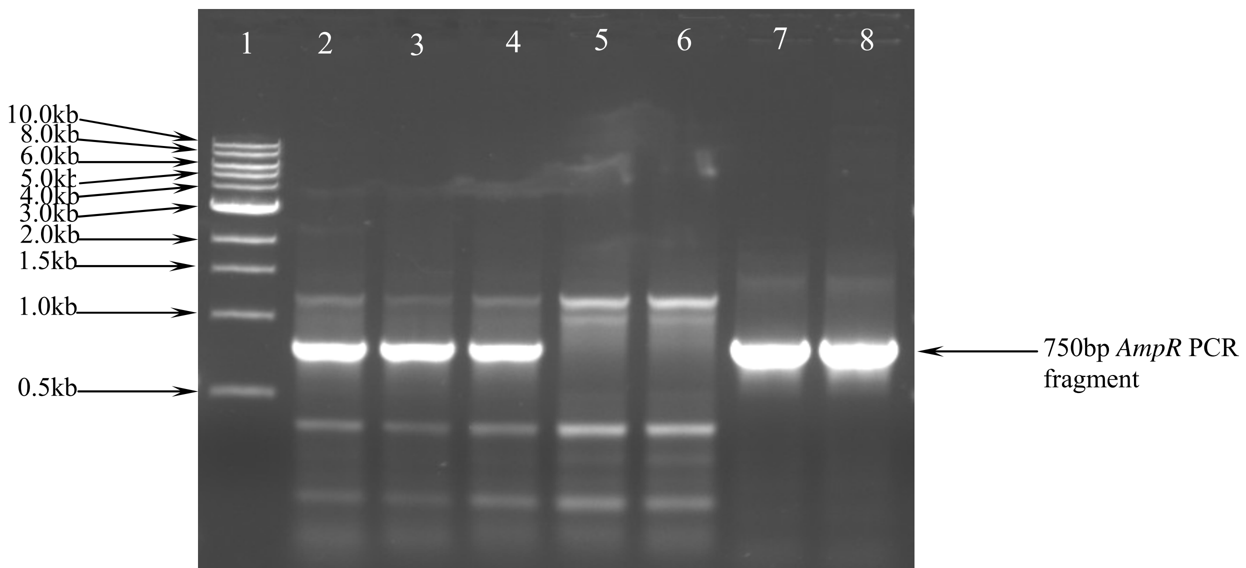

Figure 2.8: PCR screening of fungal transformants and untransformed fungal strains using the ampR primers.
Lane 1: 1kb DNA ladder; Lane 2: TpacCi87; Lane 3: TpacCi88; Lane 4: TpacCi96; Lane 5: *A. flavus* 8610 (negative control); Lane 6: *A. flavus* MRC 2527WT (negative control); Lane 7: pESApacCi2.2 (positive control); Lane 8: pUC18 (positive control).

2.3.4 Southern Hybridization to Confirm the Presence of the RNAi Construct in Fungal Transformants

The radioactively-labelled 1.8kb IRT fragment of pESApacCi2.2 successfully hybridized to the 1.8kb fragment in the control lane which contained the IRT (Fig 2.9). Similarly, transformants TpacCi87 (lane 2), TpacCi88 (lane 3) and TpacCi96 (lane 4) exhibited similar sized bands corresponding to that of the positive control indicating the presence of three positive mutants harbouring the required RNAi construct. The other putative transformants that were selected were negative for the presence of the 1.8kb IRT and thus did not contain the required RNAi construct. These transformants only received the *pyrG* gene during transformation of protoplasts and this restored their ability to synthesise uracil facilitating growth on media lacking uracil and uridine.

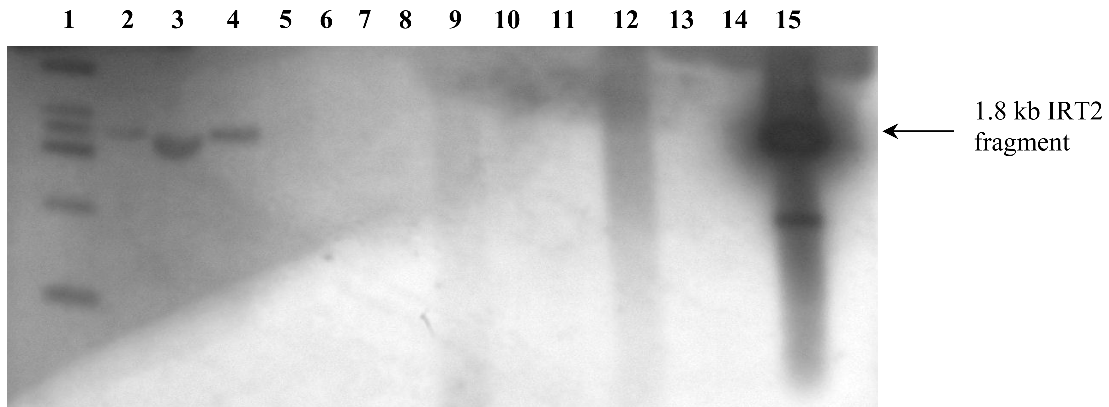

Figure 2.9: Autoradiograph of fungal transformants after *Hin*dIII digests of genomic DNA confirming the presence of the RNAi construct (lanes 2-4).
Lane 1: 1kb DNA ladder; Lane 2: TpacCi87; Lane 3: TpacCi88; Lane 4: TpacCi96; Lane 5: TpacCi58; Lane 6: TpacCi66; Lane 7: TpacCi68; Lane 8: TpacCi76; Lane 9: TpacCi97; Lane 10: TpacCi99; Lane 11: TpacCi100; Lane 12: *A. flavus* 8610 (negative control); Lane 13: Blank; Lane 14: Blank; Lane15: pESApacCi2.2 plasmid (positive control).

Genomic DNA from untransformed 8610 did not show any bands, thus indicating that it did not possess the pESApacCi2.2 RNAi vector and was negative as expected. To ensure that the bands obtained by Southern hybridizations were real and not the result of random non-specific binding of the *pacC* IRT probe, hybridizations were performed using the purified 1.8kb *pacC* IRT fragment at 65°C overnight and wash steps were performed using 1x SCC and again with 0.1x SSC. The probe was highly specific to detect the presence of the complete 1.8kb IRT while hybridization at 65°C ensured that the probe would bind to sequences that are highly homologous and prevented random-non specific binding of the probe. The blots were subjected to high stringency washes (section 2.2.8) to ensure that any non-specific, weakly hybridising probe would be removed during the wash steps. The combination of a highly specific probe, high hybridization temperature, high stringency wash steps and banding profile similar to that of the positive control are sufficient to conclude that the bands obtained for the Southern blot are correct and that TpacCi87, TpacCi88 and TpacCi96 are indeed positive transformants (i.e. mutants) harbouring the pESApacCi2.2 RNA interference vector.

2.3.5 Northern Analysis to Confirm *pacC* Silencing

RNA was successfully isolated from the selected strains as evidenced by the bright 18S and 28S rRNA bands (Fig. 2.10A). Probing the blots with a ^{32}P-labelled *pacC* PCR fragment indicated that *pacC* was expressed only under alkaline conditions (pH 8.0) by untransformed *A.*

flavus 8610. Fungal mutants harbouring the RNAi construct did not express *pacC* at pH 8.0. Hybridization with the 28S probe indicated that there was sufficient RNA present in all lanes for radioactive detection to occur (positive controls).

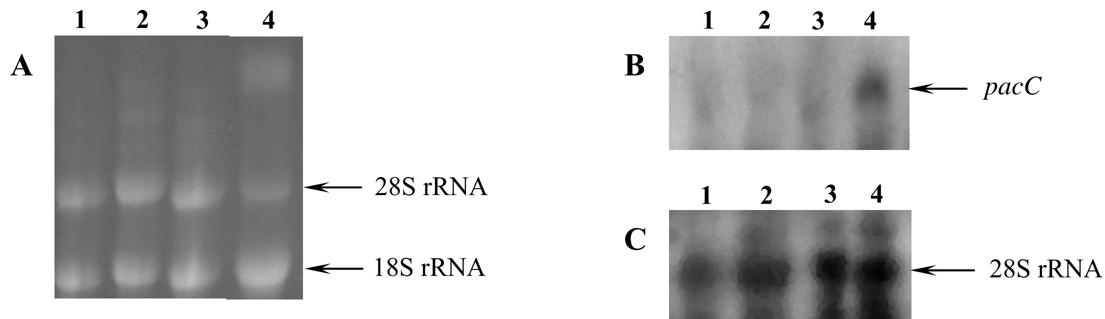

Figure 2.10: (A) Formaldehyde-agarose gel electrophoresis of total RNA (15µg) isolated from cultures grown at pH 8.0. (B) RNA probed with *pacC* gene fragment. (C) RNA probed with 28S rDNA fragment.
Lane 1: TpacCi87; Lane 2: TpacCi88; Lane 3: TpacCi96; Lane 4: Untransformed *A. flavus* 8610.

Northern hybridizations suggested that the 1.8kb IRT silenced expression of the *pacC* gene through the well characterised pathway of RNA interference. It has been shown that RNA silencing of the aflatoxin regulatory gene, *aflR*, with an *aflR* IRT correlated with the presence of *aflR*-specific 25nt siRNAs (Hammond and Keller, 2005; McDonald *et al*, 2005) which are characteristic of the RNA interference pathway. Suppression of expression observed by McDonald *et al* (2005) was thought to occur as a result of degradation of transcripts by RISC-associated siRNAs derived from the *aflR* IRT. Also IRT based silencing of genes in other fungi have been verified (Liu *et al*, 2001; Kadotoni *et al*, 2003; Catalanotto *et al*, 2004). McDonald *et al* (2005) have also reported the presence of Dicer and RNA silencing enzymes in *Fusarium* and *Aspergillus* species. Thus the absence of bands in the Northern blots probed with *pacC* can be attributed to silencing of the *pacC* gene via the RNA interference pathway.

2.3.6 Effect of *pacC* silencing on Aflatoxin Production

It was hypothesised that since the *aflR* promoter has a putative *pacC* binding site, suppression of *pacC* expression via RNA interference would alter pH-dependant induction of aflatoxin such that *pacCi* mutants would produce aflatoxins in aflatoxin inducing medium (20% YES) irrespective of environmental pH. This study found that all isolates produced aflatoxins when grown under acidic conditions (20% YES buffered to pH 4.0) although the concentration of aflatoxins produced by the TpacCi mutants appeared to be lower than that of the untransformed *A. flavus* 8610 strain (Fig. 2.11A). This difference could be due to differences in

growth between untransformed 8610 and the mutants, since the mutants were found to produce less mycelia (wet weight) than untransformed *A. flavus* 8610 (data not shown). As a consequence secondary metabolism and therefore aflatoxin biosynthesis would be delayed in the mutants resulting in the production of less aflatoxin. When grown under alkaline conditions (20% YES, pH 8.0), only the TpacCi mutants produced aflatoxins whereas untransformed *A. flavus* 8610 did not produce any aflatoxins (Fig. 2.11B). This result confirms that *pacC* is a negative regulator of aflatoxin biosynthesis at alkaline pH in *A. flavus*.

It is widely known that aflatoxin biosynthesis is dependant on environmental pH (Cotty, 1988; Keller *et al*, 1997). Normally, aflatoxin production is induced under acidic pH (pH 4.0 - 4.5) and suppressed under alkaline conditions (pH 8.0) in an aflatoxin-inducing (e.g. 20%YES or sucrose low salts) medium (Cotty, 1988; Keller *et al*, 1997). It has been hypothesized that suppression of aflatoxin production occurs via the PacC protein binding to specific sites (5'-GCCARG-3') in the *aflR* promoter (Keller *et al*, 1997). This binding would suppress *aflR* expression thereby suppressing aflatoxin biosynthesis. This study therefore confirms the hypothesis that PacC is a negative regulator of aflatoxin production. It does not indicate which gene/s were affected. Although Keller *et al* (1997) suggests that suppression is due to PacC binding to the *aflR* promoter, it is also possible that PacC may bind to any one of the other 121 putative PacC binding sites in the aflatoxin gene cluster thereby suppressing aflatoxin production.

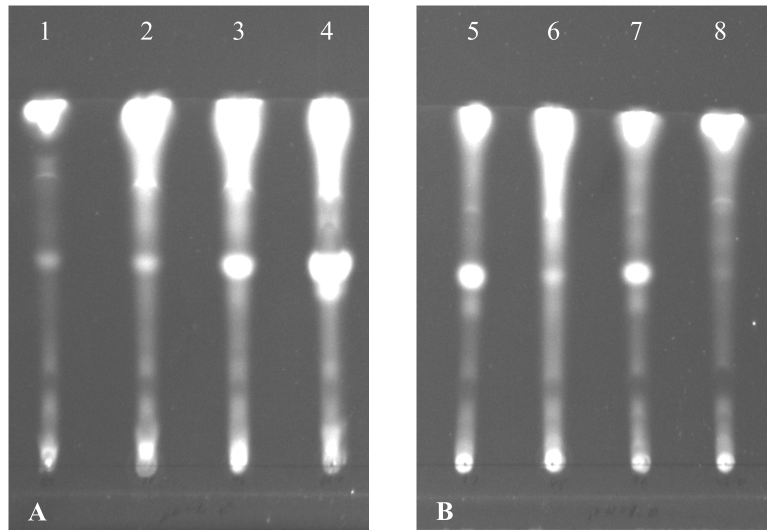

Figure 2.11: Thin layer chromatography (TLC) of aflatoxins isolated from TpacCi mutants grown in 20% YES (in triplicate) buffered to pH 4.0 (A) and pH 8.0 (B) in the dark at 28°C for four days.
Lanes 1 & 5: TpacCi87; Lanes 2 & 6: TpacCi88; Lanes 3 & 7: TpacCi 96; lanes 4 & 9: Untransformed *A. flavus* 8610.

Software based analysis of the 70kb aflatoxin biosynthetic cluster of *A. flavus* has shown that there are 122 PacC sites located within this cluster. Studies have shown that several important genes within the aflatoxin biosynthetic cluster (e.g. *aflC*, *aflD*), including the global regulator of aflatoxin biosynthesis, *aflR*, have both *pacC* and *aflR* binding sites within their promoters (Erhlich *et al*, 1999 and 2002). When *pacC* is expressed under alkaline conditions, the PacC protein binds to these recognition sites preventing the activators (such as the AFLR protein) of these genes from binding (competitive inhibition). This prevents their expression and further suppresses aflatoxin biosynthesis. Since *pacC* was not expressed under alkaline conditions in the TpacCi mutants, expression of genes in the aflatoxin biosynthetic cluster was not suppressed thus resulting in aflatoxin biosynthesis. Thus *pacC* plays a role in suppression of aflatoxin biosynthesis under alkaline conditions and silencing of *pacC* relieves this inhibition under alkaline conditions.

2.4 GENERAL DISCUSSION AND CONCLUSION

Screening for transformants via PCR proved to be more difficult and time consuming in this study possibly due to the target sequences forming very stable hairpin-loop secondary structure characteristic of Inverted Repeat Transgenes (IRT's). However, when primers were designed to amplify vector sequences that are not normally part of the fungal genome such as the *ampR* gene, PCR was found to be an extremely quick, efficient and accurate means of screening for the desired genetic trait in the transformants. As discussed above this is only indirect evidence of the presence of the desired genetic construct since any genetic recombination occurring could cause the construct to be non-functional. Therefore further evidence in the form of Southern and Northern hybridizations had to be performed.

Southern hybridization to detect the 1.8kb IRT fragment was more successful than PCR. This is probably due to the fact that digested DNA was denatured by and transferred with NaOH which prevented the formation of the secondary structures associated with IRT's. Thus Southern hybridization provided confirmation that the putative transformants (TpacCi87, TpacCi88 and TpacCi96) harboured the desired genetic construct. This however did not prove that the pESApacCi2.2 RNAi construct was functional. However, Northern analysis, with a probe designed to detect the expression of *pacC* showed that the *pacC* RNAi mutants did not produce any detectable transcripts at pH 8.0. This implied that the pESApacCi2.2 RNAi construct was functional and silenced the *pacC* gene in all three mutants (TpacCi87, TpacCi88 and TpacCi96). Thus the generation of an RNA interference vector for silencing the *pacC* gene in *A. flavus* was successful.

It has been shown that vectors having IRT's are efficient activators of RNA silencing (McDonald *et al*, 2005). The vector mediated RNA silencing mechanism is mediated by the formation of hairpin-RNAs (hpRNA), which are then cleaved by an RNAseIII type enzyme (e.g. Dicer) into 21nt-26nt short interfering RNAs (siRNA). These siRNA are then integrated into an RNA-induced silencing complex (RISC; Bernstein, *et al*, 2001; Hammond *et al*, 2004;) which bind to the complementary mRNAs leading to its degradation (Hammond *et al*, 2000; Elbashir, *et al*, 2001; Hammond *et al*, 2004; McDonald *et al*, 2005).

Previous studies have shown that transformation of *A. flavus* and *Fusarium graminearum* with vectors having IRT sequences complementary to the *aflR* gene (for *A. flavus*) and *tri*6 (for *F. graminearum*) (transcriptional activators of mycotoxin biosynthesis) resulted in the silencing of these genes and concomitant reduction in mycotoxin production (McDonald *et al*, 2005). From the above experiments and results it can be concluded that aflatoxin production is controlled by environmental pH through the activity of *pacC*. In this study aflatoxin biosynthesis

was found to occur in RNAi mutants irrespective of the environmental pH of the culture medium. Since the pH-regulatory gene, *pacC*, was effectively silenced (Fig 2.10), the suppression of aflatoxin biosynthesis by PacC was alleviated at an alkaline pH resulting in aflatoxin production. This confirms other findings that *pacC* is a negative regulator of mycotoxin biosynthesis (Flaherty *et al*, 2003).

Thus, RNA silencing is a useful tool for the study of the molecular mechanisms of various pathways in fungi, including mycotoxin production. It may also prove to be a valuable tool for the prevention of mycotoxin contamination of agricultural products (McDonald *et al*, 2005).

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CHAPTER THREE

PHYSIOLOGICAL EFFECTS OF *pacC* SILENCING ON *A. flavus* MUTANTS

3.1 INTRODUCTION

A. flavus is a one of several *Aspergilli* that infects maize, peanuts and cereals, causing food spoilage and producing aflatoxins that are potent carcinogens. Tilburn *et al* (1995) have previously suggested that *pacC* mutations in *Aspergillus nidulans* resulting in loss of function, i.e. *pacC*$^-$ mutants, mimic growth at acidic pH and additionally grow and conidiate poorly. However, detailed investigation of the physiological growth of the mutants such as conidiation, toxin production, growth rates, germination rates and pathogenicity were not determined.

Numerous studies have shown that pathogenicity of maize by *A. flavus* and subsequent aflatoxin production is enhanced by abiotic factors such as drought, high temperature, low rainfall and nitrogen stress (Moreno and Kang, 1999). There are numerous other biological (e.g. presence of oxylipins, lipooxygenases) and non-biological (pH, carbon source, etc.) which affect maize colonisation and aflatoxin production by *A. flavus* (Burrow *et al*, 1996; Wilson *et al*, 2001; Calvo, *et al*; 2002; Tsitsigiannis *et al*, 2006). However, all the genetic mechanisms involved have as yet not been identified. It has been shown by Caracuel *et al* (2003) that *pacC* acts as a negative virulence regulator in plants and these workers have hypothesised that PacC prevents expression of genes that are important for infection and virulence of the pathogen.

This study investigated the physiological effects that *pacC* silencing had on the growth, conidiation and pathogenicity of *A. flavus* mutants.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Chemical reagents for media, buffers and supplements were purchased from Sigma-Aldrich (Germany). All reagents were of the highest molecular biology grade.

3.2.2 Growth and Conidiation

To determine the effect that *pacC* RNA interference had on growth and conidiation, fungal clones were cultured by a modification of the methods of Cotty (1988) and Flaherty *et al* (2003). Mutants TpacCi87, TpacCi88, TpacCi96 and untransformed *A. flavus* 8610 were grown on

glucose minimal media (GMM) + arginine [50ml/l 20x salts[2] with $NaNO_3$ (7mM); 1ml/l trace elements[3]; 55.5mM dextrose; 15g/l agar; 1.0g/l L-arginine] plates for 6 days and spores harvested by flooding the plates with 5ml 0.1% Tween 20 and scraping the surface. $1x10^6$ spores (in 0.1% Tween 20) of each culture were then inoculated onto the centre of GMM + Arg agar plates buffered to pH 4.0 (using 19.3mM/l Na_2HPO_4 and 15.35mM/l citric acid) as well as on GMM + Arg agar plates buffered to pH 8.0 (using 47.3mM/l Na_2HPO_4 and 2.7mM/l NaH_2PO_4) (Pinero and Keller, unpublished data) and incubated at 28°C for 7 days. All inoculations were done in triplicate. Colony diameters were measured daily and an average determined. Growth curves were plotted (using Microsoft Excel) and the generated data statistically analysed using analysis of variance (ANOVA). After colony diameters were determined (on the seventh day) the plates were flooded with 5ml 0.1% Tween 20 and the conidia harvested. Dilutions of the conidial suspensions were made and the conidia quantified using a haemocytometer.

3.2.3 Germination Rate

Sterile microscope slides were separately coated with 2.5ml of GMM + Arg agar (see section 3.2.2) buffered to pH 4.0 or pH 8.0 as required. The microscope slides were placed on filter paper (moistened with 0.1% $CuSO_4$ to prevent bacterial contamination) on the bottom of sterile Petri plates. Each slide was inoculated with 1000 spores of the appropriate fungal mutant or untransformed *A. flavus* 8610 in triplicate. The plates were incubated at 28°C for 16 hours. Conidia were microscopically examined for any signs of germination every two hours. Young spore germlings were counted, an average calculated and the percentage spore germination determined. The data was statistically analysed using analysis of variance (ANOVA).

3.2.4 Pathogenicity Assays

Maize seeds (early pearl variety, Agricol, Port Elizabeth, South Africa) were surface sterilised for 1 minute in 10% Jik bleach solution (NaOHCl) and rinsed twice for 1 minute in sterile distilled water (McDonald *et al*, 2005). The embryo of each seed was wounded and each inoculated with 1000 spores from either the fungal mutants or *A. flavus* 8610. Each fungal culture was grown on GMM + Arg media buffered to either pH 4.0 or pH 8.0. Ten seeds were inoculated per fungal strain before transfer to a humidity chamber composed of moist filter paper on the bottom of a sterile Petri plate. 100% humidity was maintained by placing a water-filled

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[2] 20x salts with $NaNO_3$ (1 litre): $NaNO_3$ (141mM); KCl (139.5mM); $MgSO_4.7H_20$ (65.24mM); KH_2PO_4 (223.39mM); ddH_20 (1000ml).

[3] Trace elements (100ml): $ZnSO_4.7H_20$ (2.2g); H_3BO_3 (1.1g); $MnCl_2.4H_20$ (0.5g); $FeSO_4.7H_20$ (0.5g); $CoCl_2.7H_20$ (0.16g); $CuSO_4.5H_20$ (0.16g); $(NH_4)_6.Mo_7O_{24}.4H_20$ (0.11g), Na_4EDTA (5g); ddH_20 (100ml).

cap inside each Petri dish. This was done in triplicate. The plates were covered and sealed with parafilm and incubated in the dark at 28°C for 5 days (McDonald *et al*, 2005). After 5 days, 3 seeds from each replicate visually exhibiting the most conidial growth were selected and resuspended in 500µl sterile 0.1% Tween 20 and vortexed for 2 minutes to release any conidia from the seed. Dilutions of the spore suspensions were made and counted with a haemocytometer. The generated data was statistically analysed by analysis of variance (ANOVA).

3.2.5 Electron Microscopy

Each fungal mutant and untransformed *A. flavus* 8610 was grown on agar plates (as described in section 3.2.2) buffered to either pH 4.0 or pH 8.0. The plates with fungal colonies were then sent to the Electron Microscopy Unit (Rhodes University, Grahamstown) and subjected to Scanning Electron Microscopy (SEM) to determine the changes that *pacC* silencing had induced on the external ultra-structure of the mycelium and conidia of each fungal mutant.

3.3 RESULTS AND DISCUSSION

3.3.1 Radial Vegetative Growth

The growth of each mutant and that of untransformed *A. flavus* 8610 on solid GMM + Arg media buffered to pH 4.0 or pH 8.0 were compared. After 7 days, untransformed *A. flavus* 8610 (Fig. 3.1) appeared to have similar radial growth and conidiation characteristics on GMM + Arg at both pH 4.0 and pH 8.0 i.e. the vegetative growth of 8610 at an alkaline pH was visually similar to growth at acidic pH.

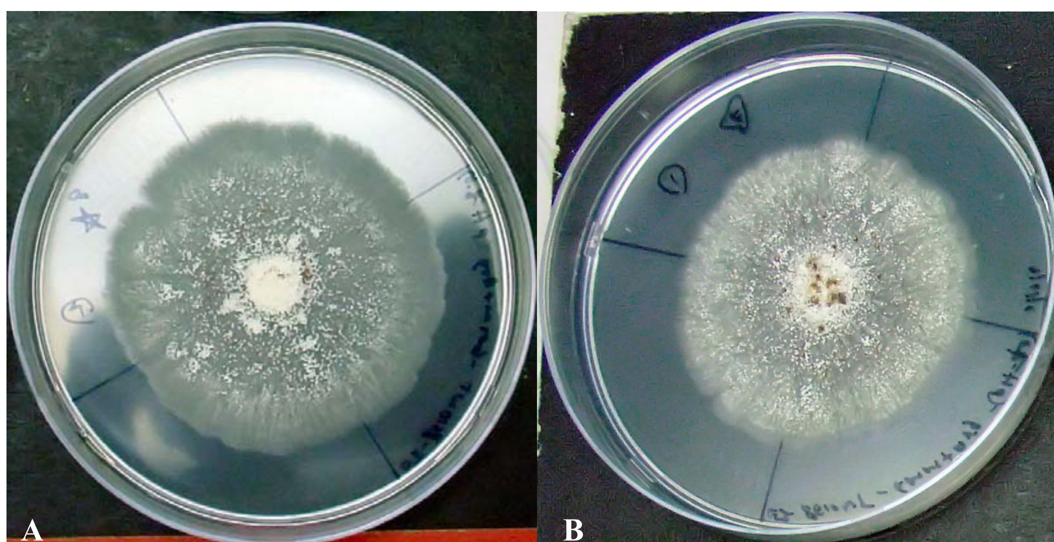

Figure 3.1: Untransformed *A. flavus* 8610 growing on GMM + Arg pH 4.0 (A) and on GMM + Arg pH 8.0 (B).

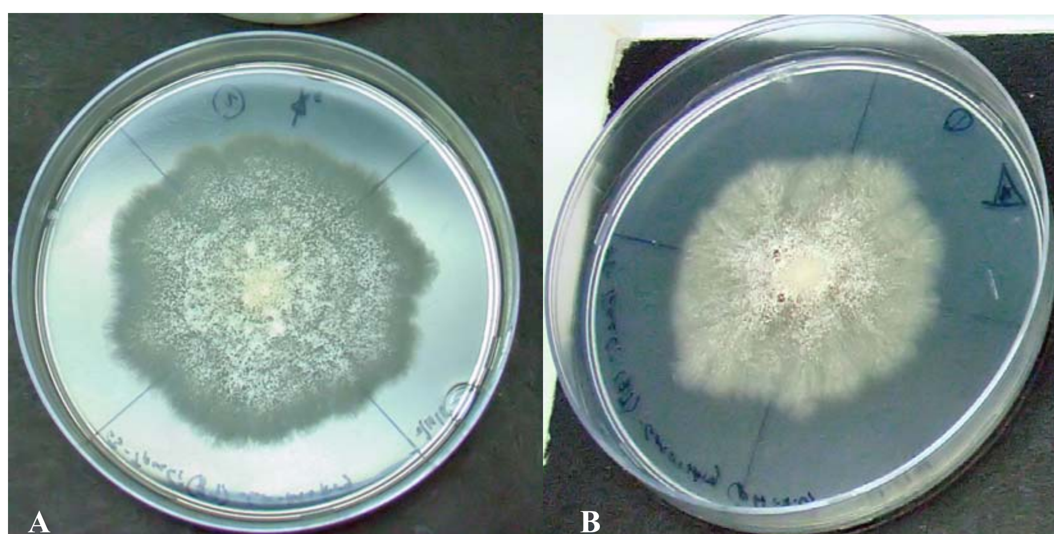

Figure 3.2: TpacCi87 growing on GMM + Arg pH 4.0 (A) and pH 8.0 (B).

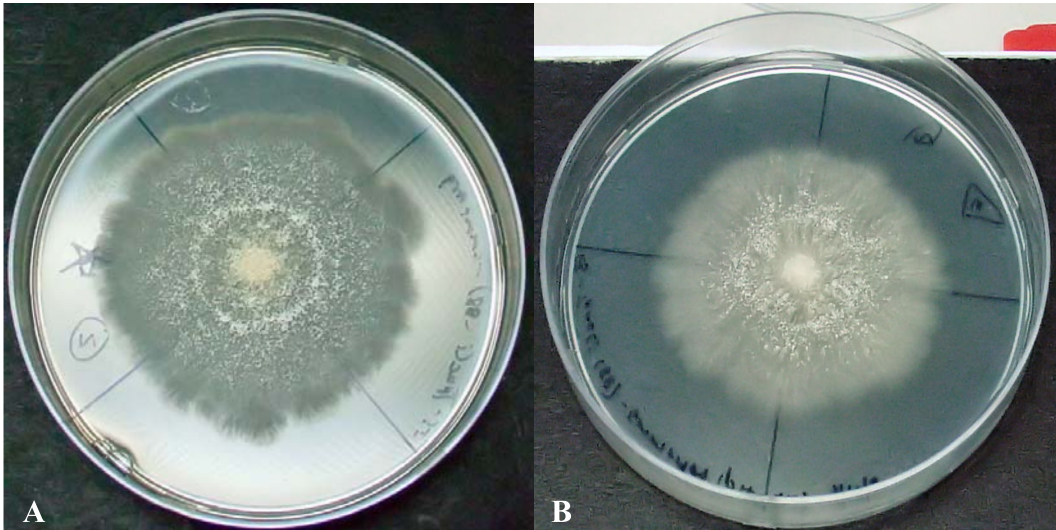

Figure 3.3: Growth of TpacCi88 on GMM + Arg at pH 4.0 (A) and pH 8.0 (B).

In contrast each mutant exhibited differences in vegetative growth at the different pHs. Visual inspection of TpacCi87 indicated that this mutant produced more conidia and grew faster at pH 4.0 than at pH 8.0 (Fig. 3.2). Growth on alkaline media (pH 8.0) resulted in less "fluffy" mycelial production and proceeded slowly across the surface of the agar. Sparse aerial mycelia and conidia were produced. Growth on acidic media was the opposite i.e. rapid growth across the surface of the agar with more aerial mycelia and better conidiation being exhibited.

TpacCi88 also produced more conidia and grew faster on GMM + Arg buffered to pH 4.0 than on GMM + Arg buffered to pH 8.0 (Fig 3.3). Growth on alkaline media (pH 8.0) was slow with sparse aerial mycelia and less conidia compared to acidic media. When compared to the other transformants TpacCi88 produced the least amount of conidia and grew the slowest.

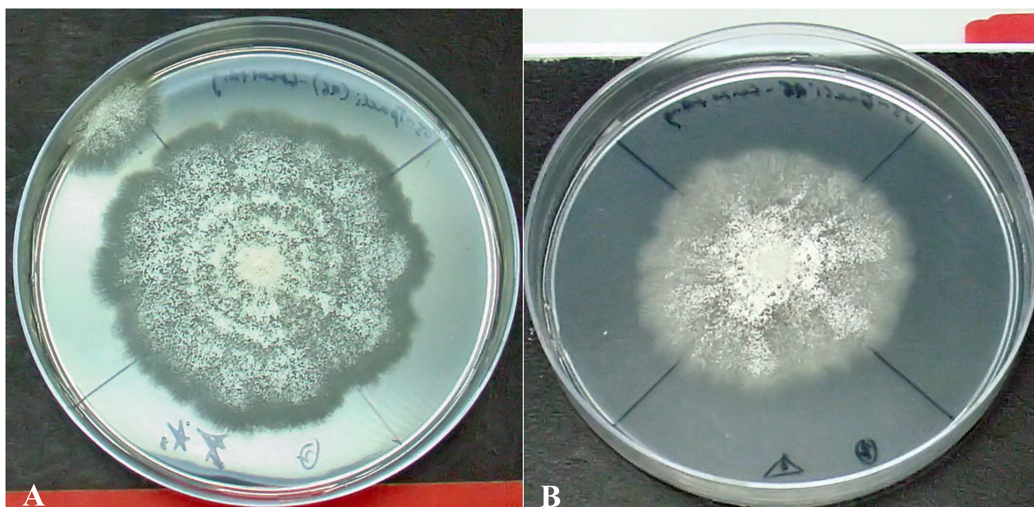

Figure 3.4: Growth of TpacCi96 on GMM + Arg pH 4.0 (A) and pH 8.0 (B).

TpacCi96 produced more conidia and grew faster at pH 4.0 than at pH 8.0 (Fig 3.4). In contrast to the other mutants growth of TpacCi96 on alkaline media (pH 8.0) was slow but the production of aerial mycelia and conidia at pH 8.0 was greater. Of all the mutants, growth of TpacCi96 on acidic media was rapid with more aerial mycelia and profound conidiation and had the closest resemblance to that of untransformed *A. flavus* 8610.

Tilburn *et al* (1995) indicated that *pacC* null mutations (*pacC*$^{-}$) exhibit poor growth, conidiation and occasionally brown pigmentation at alkaline pH (pH 8.0) and similar effects of *pacC* disruption mutants were seen in *Fusarium verticilliodes* (Flaherty *et al*, 2003). Visual examination of colony growth for the *pacC* RNA interference mutants (above) do seem to resemble the results of Tilburn *et al* (1995), and not that of Flaherty *et al* (2003). The latter indicated that at an alkaline pH, radial growth was completely inhibited. It is possible that there are different mechanisms of *pacC* functioning in different organisms or that other related genes may be affected differently in different organisms.

3.3.2 Growth Rates

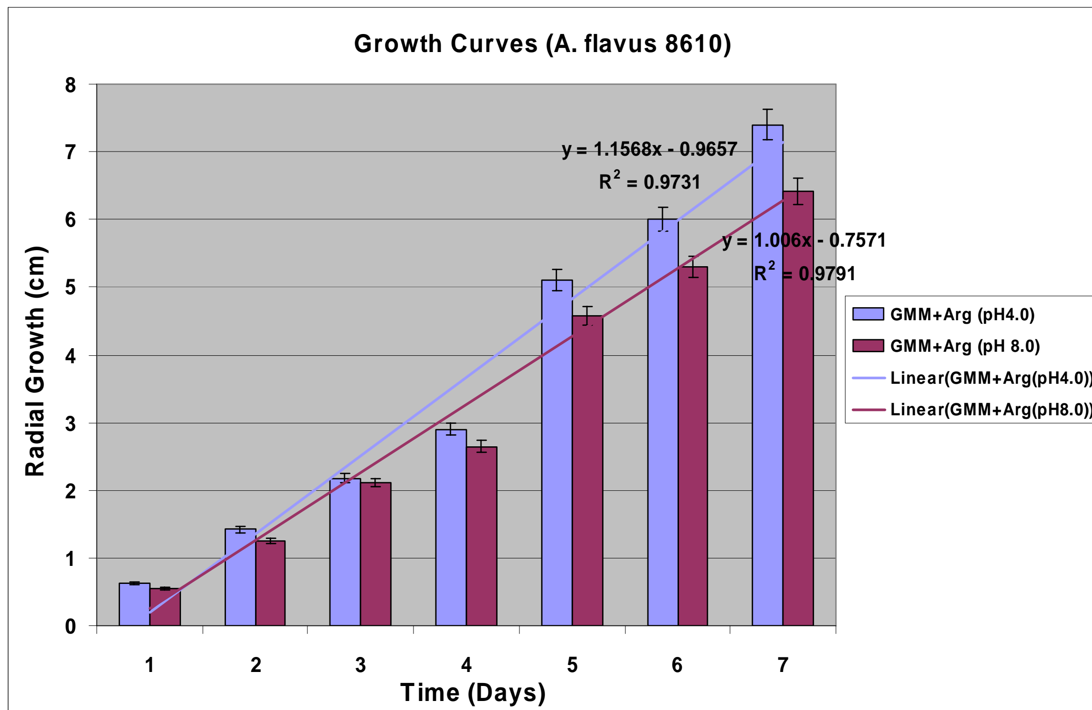

Figure 3.5: Growth curve of *A. flavus* 8610 on GMM + Arg buffered to pH 4.0 and pH 8.0. Error bars indicate standard deviation.

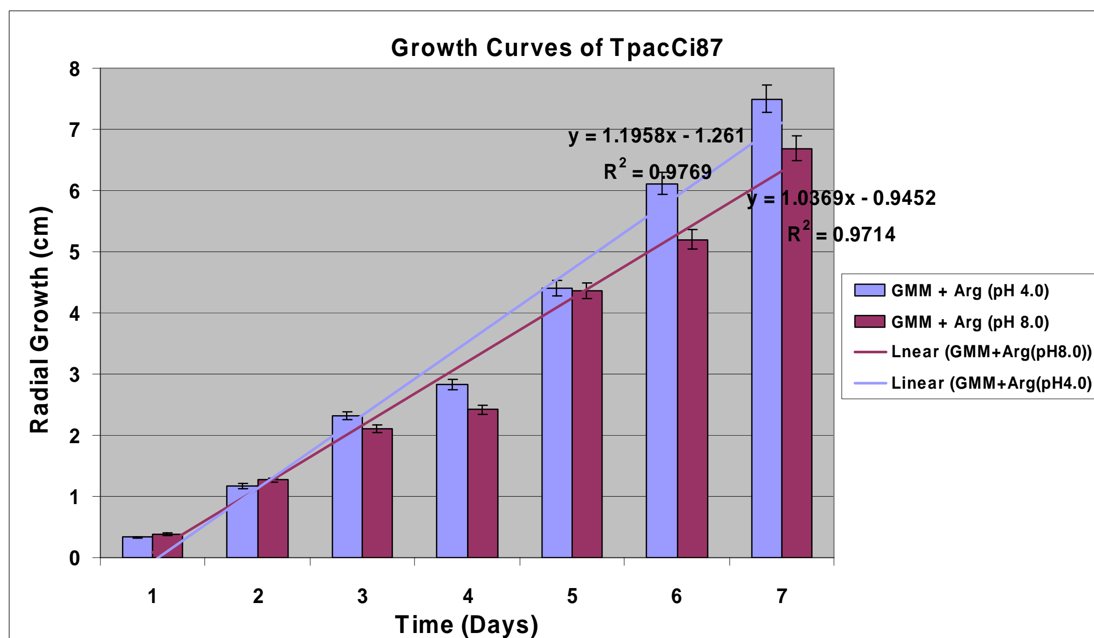

Figure 3.6: Growth curve of TpacCi87 on GMM + Arg buffered to pH 4.0 and pH 8.0. Error bars indicate standard deviation.

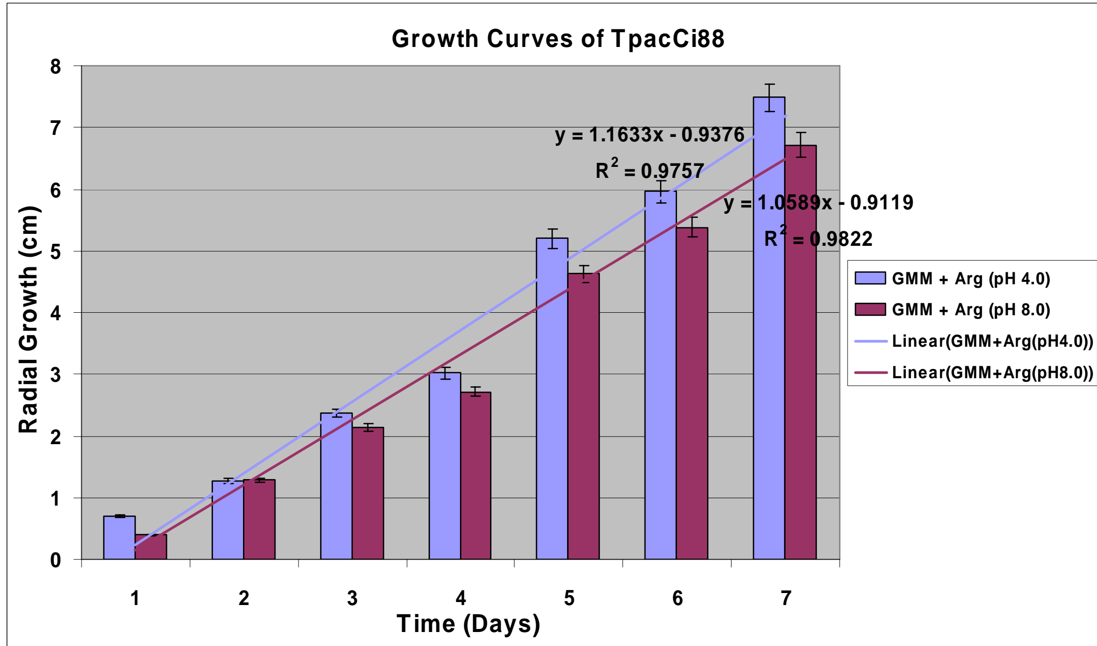

Figure 3.7: Growth curve of TpacCi88 on GMM + Arg buffered to pH 4.0 and pH 8.0. Error bars indicate standard deviation.

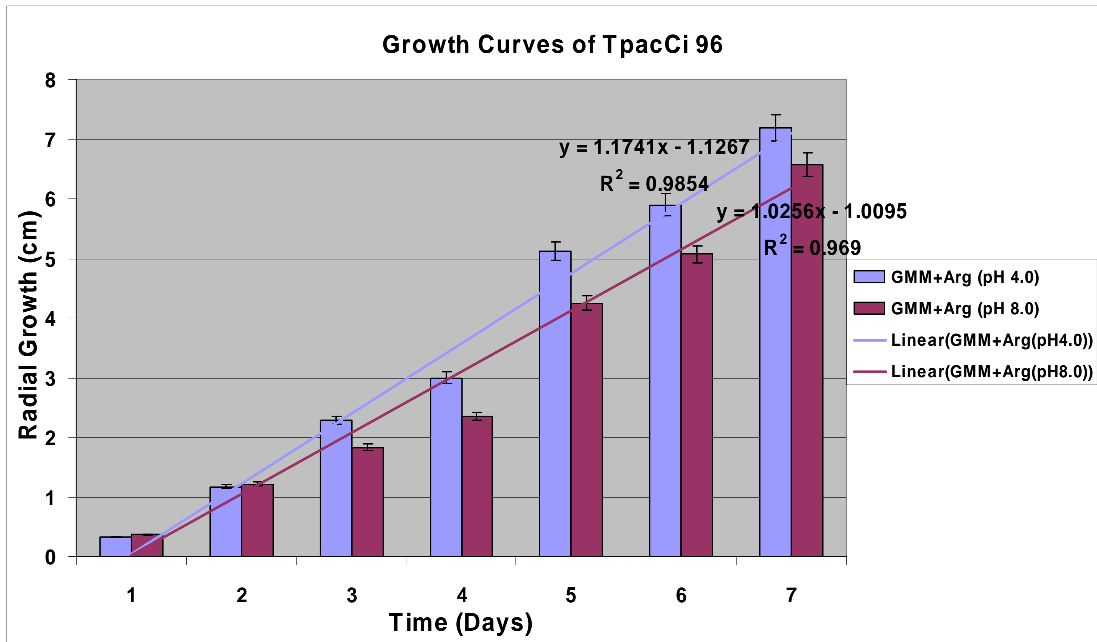

Figure 3.8: Growth curve of TpacCi96 on GMM + Arg buffered to pH 4.0 and pH 8.0. Error bars indicate standard deviation.

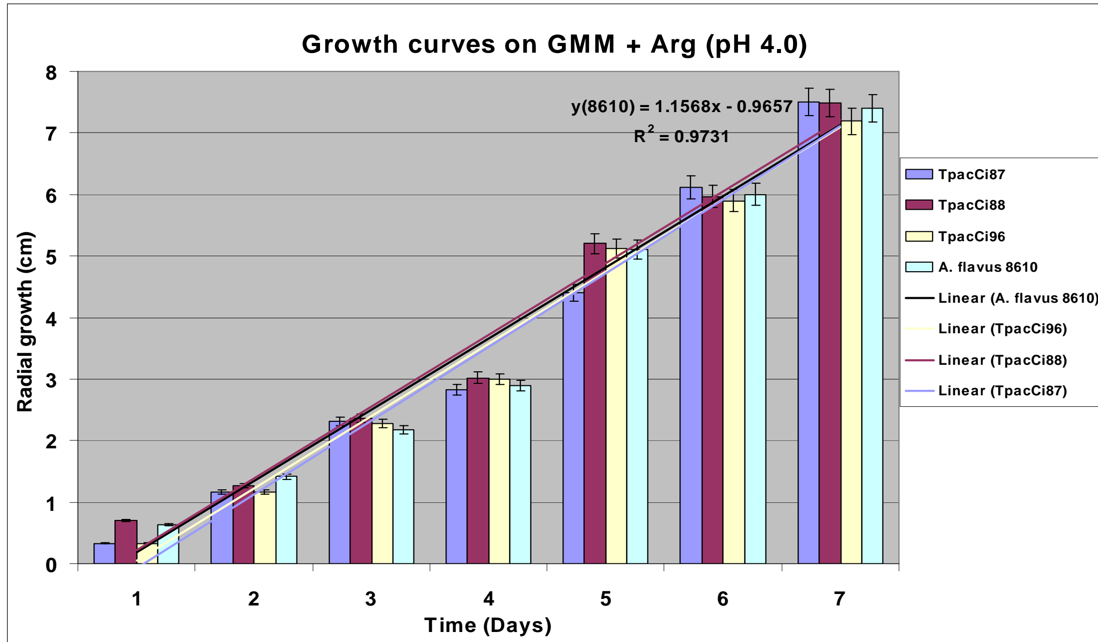

Figure 3.9: Growth curves *pacC* mutants and untransformed *A. flavus* 8610 on GMM + Arg buffered to pH 4.0.
Error bars indicate standard deviation and the trend line equation is shown for *Aspergillus flavus* 8610 only.

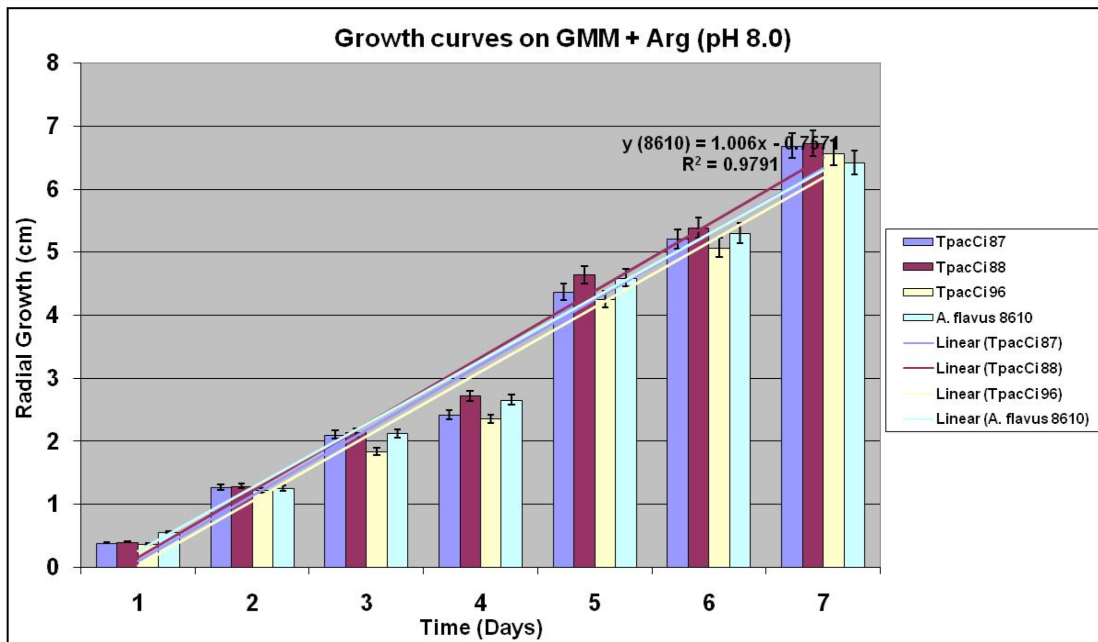

Figure 3.10: Growth curves *pacC* mutants and untransformed *A. flavus* 8610 on GMM + Arg buffered to pH 8.0.
Error bars indicate standard deviation and the trend line equation is shown for *Aspergillus flavus* 8610 only.

All fungal strains (clones and untransformed 8610) were found to grow slower at an alkaline pH than at an acidic pH (Figs. 3.5 - 3.8) generally after three days post-inoculation. At a given pH (either pH 4.0 or pH 8.0), radial growth rates were found to be almost identical ($P > 0.99$) for all fungal strains (Figs. 3.9 and 3.10). Therefore there appeared to be no discernable differences in the rate of fungal growth among the fungal clones and untransformed 8610 at either pH 4.0 or pH 8.0.

The results indicate that silencing of the *pacC* gene does not appear to affect radial growth of fungal clones at a given environmental pH when compared to untransformed *A. flavus* 8610 since the growth rates were found to be almost identical. However, although the growth rates of the mutants were similar to wildtype, visual inspection of cultures showed that *pacC* RNA interference mutants produced sparse aerial mycelia and crept along the surface of the agar whereas untransformed *A. flavus* 8610 produced abundant aerial mycelia and conidia when grown at alkaline pH.

It appears that the effect of *pacC* null mutations varies between different organisms. Tilburn *et al* (1995) suggested that *A. nidulans pacC* null mutants were slow growing and conidiated poorly irrespective of pH when grown on minimal medium. However, no data was presented to support their observations. Disruption of the *F. verticilliodes PAC1* gene resulted in no growth being detected in null mutants at alkaline pH (Flaherty *et al*, 2003). A null mutation of *YlRIM101* (*pacC* homolog) in *Yarrowia lipolytica* did not affect growth at acid or alkaline pH but blocked mating and sporulation (Lambert *et al*, 1997). Similar effects were obtained for null mutants of the *pacC* homologue, *PRR2* in *C. albicans* except that medium-dependent defects in filamentation were observed (Ramon *et al,* 1999). Our results most closely resembles that of Lambert *et al*, (1997) in that *A. flavus pacC* null mutations affected the production of aerial mycelia, conidia and aflatoxin (Chapter 2) at alkaline pH but did not affect the growth rates of the organism at either acidic or alkaline pH.

3.3.3 Conidial Production

Substantial differences in conidiation were observed between the mutants and untransformed *A. flavus* 8610 after 7 days of growth (Fig. 3.11). Conidial production of TpacCi87, TpacCi88 and TpacCi96 grown at pH 8.0 was significantly less than conidial production when grown on acidic media ($P < 0.00003$). TpacCi87 ($P < 0.0001$) and TpacCi88 ($P < 0.000005$) mutants produced significantly less conidia (1.5×10^8 and 7.21×10^7 conidia/ml respectively) on GMM + Arg (pH 4.0) when compared to that of untransformed *A. flavus* 8610 (2.33×10^8). This equates

to a 35.54% and 69.11% decrease in conidial production for TpacCi87 and TpacCi88 respectively compared to untransformed *A. flavus* 8610.

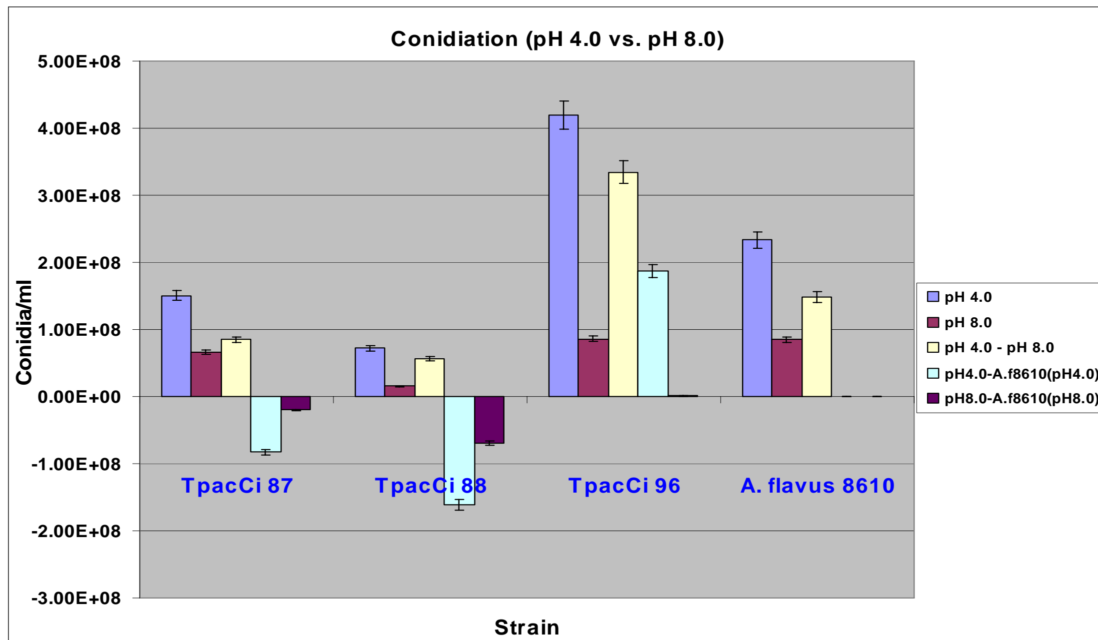

Figure 3.11: Conidial production of *pacC* RNA interference mutants (TpacCi87, TpacCi88 and TpacCi96) and untransformed *A. flavus* 8610 on GMM + Arg buffered to pH 4.0 and pH 8.0. Error bars indicate standard deviation.

Similarly, on GMM + Arg (pH 8.0) TpacCi87 ($P < 0.002$) and TpacCi88 ($P < 0.00001$) produced significantly fewer conidia (6.54×10^7 and 1.54×10^7 conidia/ml, respectively) than untransformed *A. flavus* 8610 (8.5×10^7). This equates to a 23.04% and 81.86% reduction in conidial production when compared to untransformed *A. flavus* 8610.

TpacCi96 produced significantly ($P < 0.000005$) more conidia (4.2×10^8 conidia/ml) than untransformed *A. flavus* 8610 (2.33×10^8 conidia/ml) on GMM + Arg (pH4.0). This equates to an 80% increase in conidial production over *A. flavus* 8610. Again, conidial production on GMM + Arg (pH 8.0) of TpacCi96 was nearly identical ($P > 0.82$) (8.58×10^7 conidia/ml) to untransformed *A. flavus* 8610 (8.5×10^7 conidia/ml) equating to a 0.98% increase in conidial production.

This large decrease in conidial production by *pacC* null mutants at both pH 4.0 and pH 8.0 has also been observed by Flaherty *et al* (2003), who reported that *PAC1* null mutants produced 77% and 95% less conidia at pH 4.0 and pH 8.0, respectively, than the wildtype *F. verticilliodes* strain. Although conidial production of two fungal mutants (TpacCi87 and TpacCi88) was also severely hampered when grown on both acidic and alkaline media, one mutant (TpacCi96) was

not adversely affected by silencing of the *pacC* gene. This was evidenced by an increase in conidial production at both acidic and alkaline pH. TpacCi96 is a confirmed RNAi mutant because Southern hybridization indicated the presence of an intact RNAi construct, Northern analysis confirmed the lack of *pacC* transcripts and aflatoxin biosynthesis was observed at an alkaline pH (aflatoxin inhibiting). Thus TpacCi96 is a very puzzling mutant since it behaves as predicted with respect to aflatoxin production but does not exhibit poor growth and conidiation as determined for *pacC* null mutations (Tilburn *et al*, 1995; Flaherty *et al*, 2003).

It has been hypothesized that in *A. nidulans* occasionally IRT's are "lost" during sexual crosses or during normal growth (McDonald *et al*, 2005; Hammond and Keller, unpublished data). Although it is possible that a similar event may have occurred here, it seems highly improbable since aflatoxin production is elevated in TpacCi96, as it is for the other mutants. The simplest explanation would be that during transformation, the plasmid vector integrated at or near a gene essential for regulating conidial production. It is possible that this integration led to the activation of conidiation genes such as the pathway specific regulator of conidiation *brlA* (Clutterbuck, 1969; Calvo *et al*, 2002). Alternatively, integration could have occurred at a regulatory gene that negatively regulates conidiation such as *pkaA* (Shimizu and Keller, 2001; Calvo *et al*, 2002), thus eliminating a conidiation block. Therefore, further work to clarify the reason/s for an increase in TpacC96 conidial production will have to be done.

3.3.4 Germination Rate

Spores of *A. flavus* 8610 exhibited no differences ($P > 0.92$) in percentage germination between pH 4.0 and pH 8.0 at each sampling time between 16 and 24 hours post inoculation (Fig 3.12). 100% spore germination was achieved after 22 hours (pH 4.0) and 24 hours (pH 8.0). Percentage germination of the mutants TpacCi87 (Fig 3.13), TpacCi88 (Fig 3.14) and TpacCi96 (Fig 3.15) at pH 4.0 was found to be at least 10 percent higher than that at pH 8.0. Each mutant initially exhibited a lag in germination between 16 and 20 hours followed by a sharp increase in germination after 20 hours of incubation at both pH 4.0 and pH 8.0.

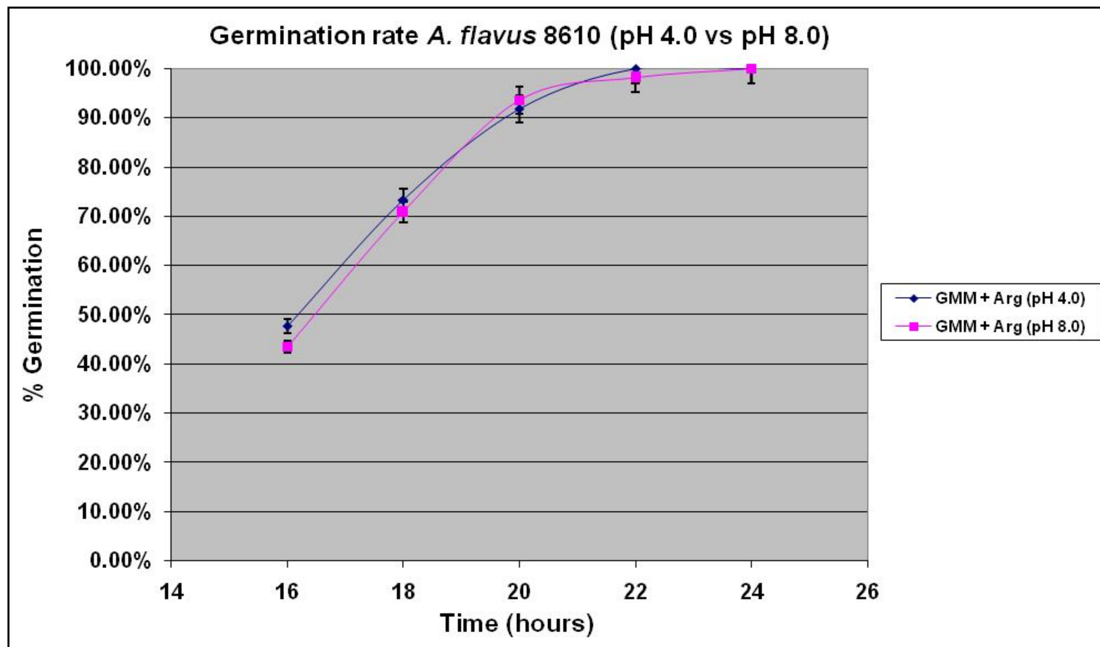

Figure 3.12: Germination rates of untransformed *A. flavus* 8610 on GMM + Arg buffered to pH 4.0 and pH 8.0.
Error bars indicate standard deviation.

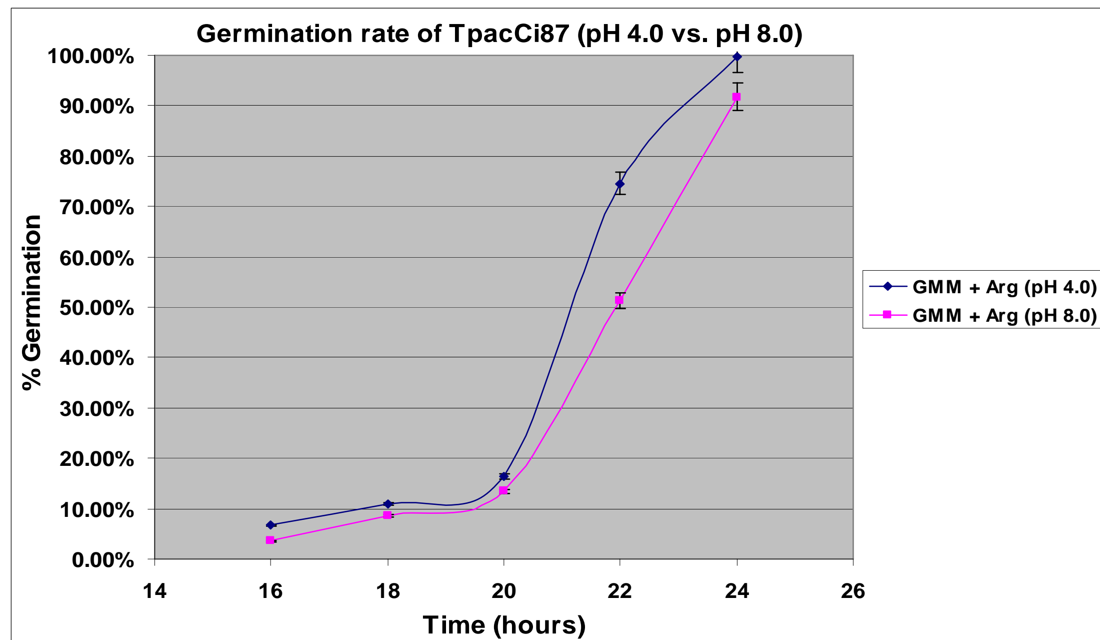

Figure 3.13: Germination rates of *pacC* RNA interference mutant TpacCi87 on GMM + Arg buffered to pH 4.0 and pH 8.0.
Error bars indicate standard deviation.

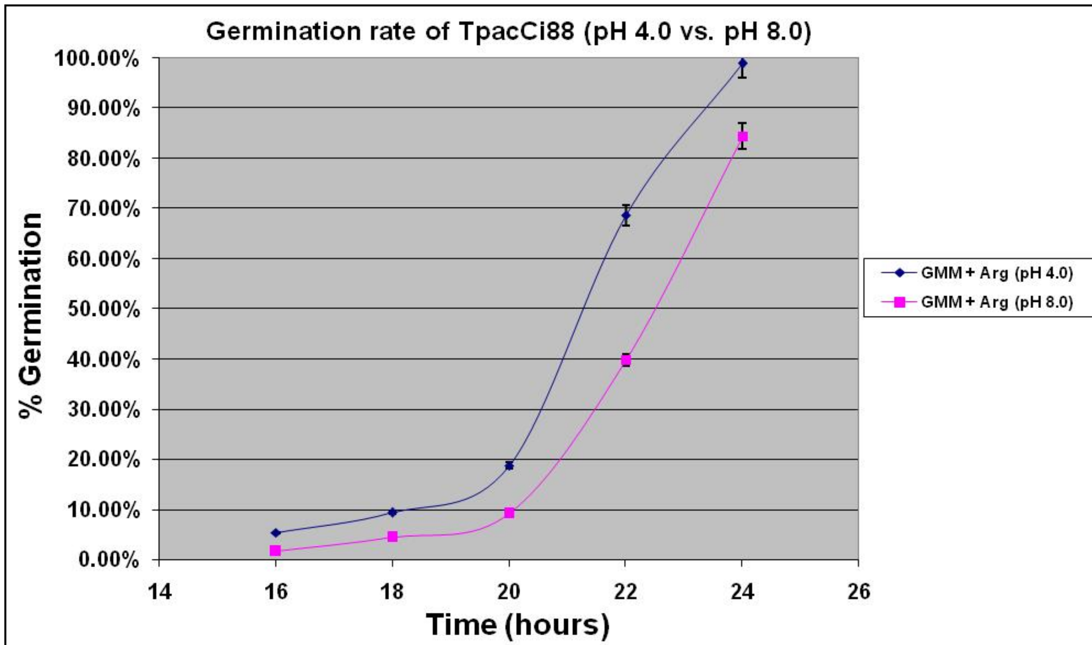

Figure 3.14: Germination rates of *pacC* RNA interference mutant TpacCi88 on GMM + Arg buffered to pH 4.0 and pH 8.0.
Error bars indicate standard deviation.

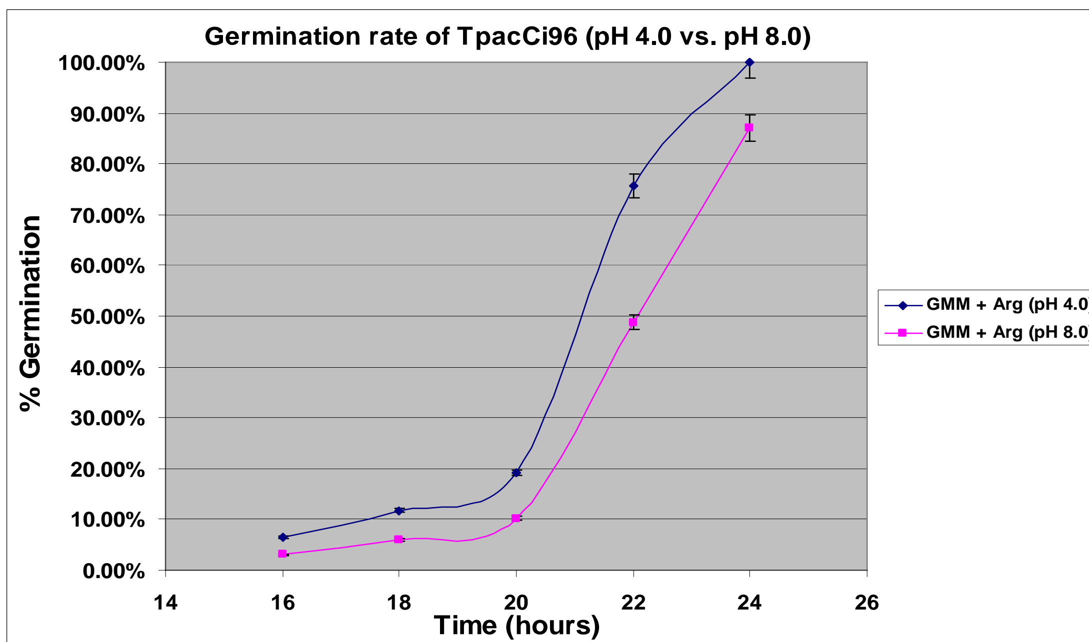

Figure 3.15: Germination rates of *pacC* RNA interference mutant TpacCi96 on GMM + Arg buffered to pH 4.0 and pH 8.0.
Error bars indicate standard deviation.

Prior to 16 hours the percentage of germinated spores was extremely low. It was therefore decided to incubate the spores for 16 hours before commencing counts. Comparison of the germination profiles of untransformed and mutant strains indicated that untransformed *A. flavus*

8610 germinated more rapidly than all mutant strains at both pH 4.0 and pH 8.0 (Figs 3.16 and 3.17). Therefore 8610 reached the 100% germination level more rapidly than any of the mutants. Germination profiles of the mutant strains were nearly identical at both pH 4.0 and pH 8.0 and, as mentioned previously, all mutants exhibited a lag phase followed by a rapid germination phase during the period of observation. After 24 hours the percentage spore germination of fungal mutants on acidic media was almost 100% (Fig 3.16) when compared to between 80 and 90% (Fig 3.17) germination when grown on alkaline media.

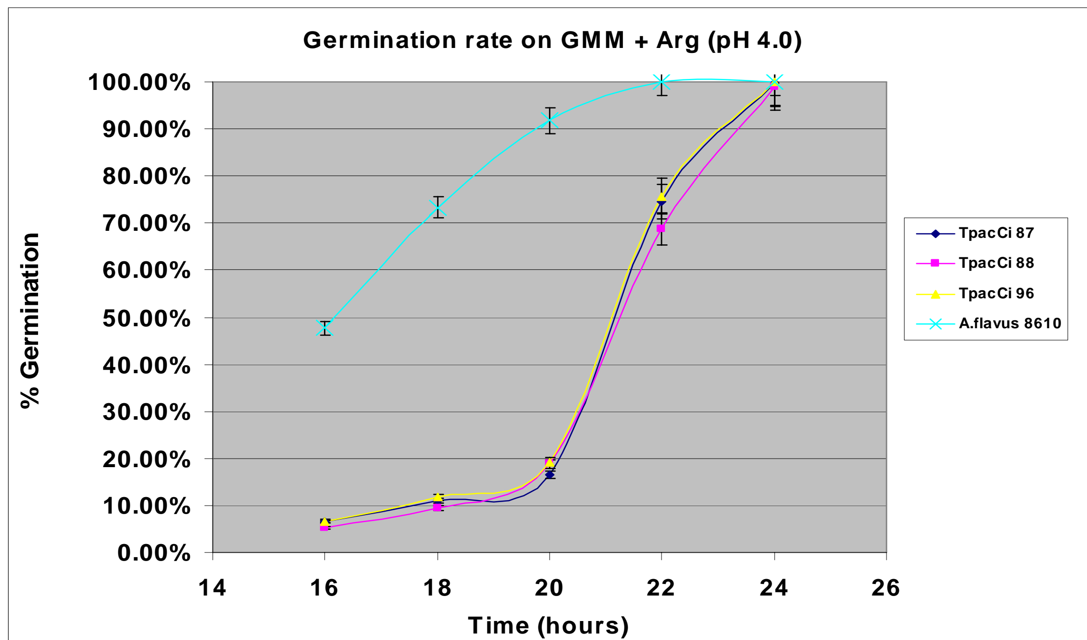

Figure 3.16: Germination rates of *pacC* RNA interference mutants (TpacCi87, TpacCi88 and TpacCi96) and untransformed *A. flavus* 8610 on GMM + Arg buffered to pH 4.0.
Error bars indicate standard deviation.

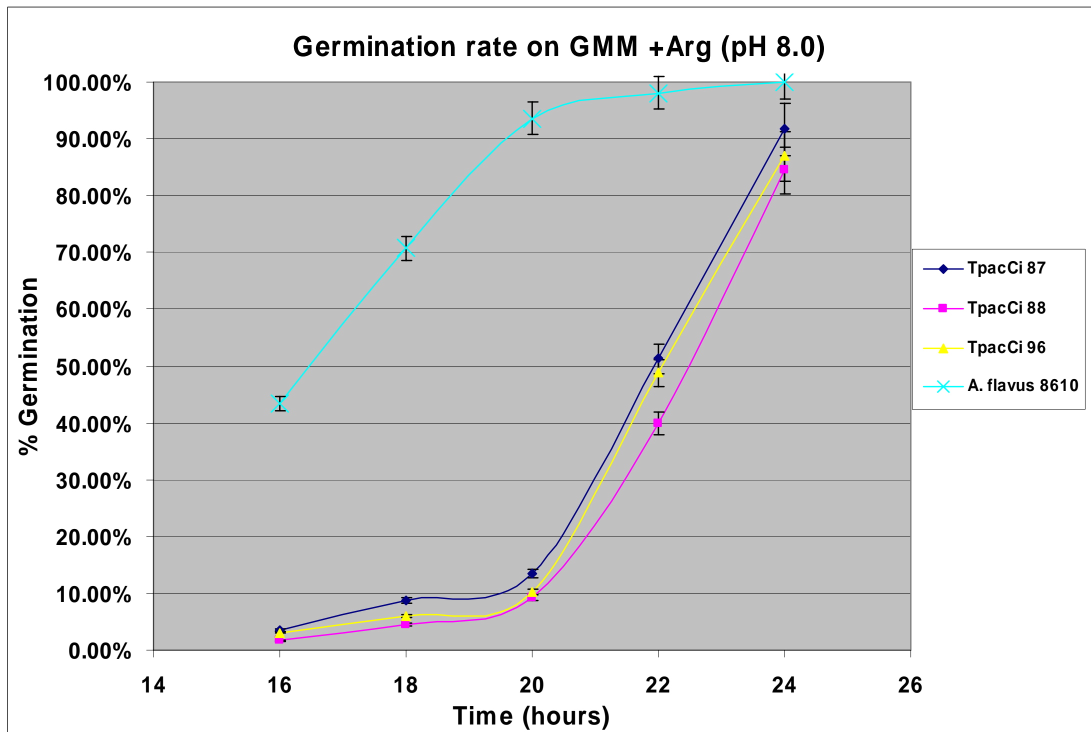

Figure 3.17: Germination rates of *pacC* RNA interference mutants (TpacCi87, TpacCi88 and TpacCi96) and untransformed *A. flavus* 8610 on GMM + Arg buffered to pH 8.0.
Error bars indicate standard deviation.

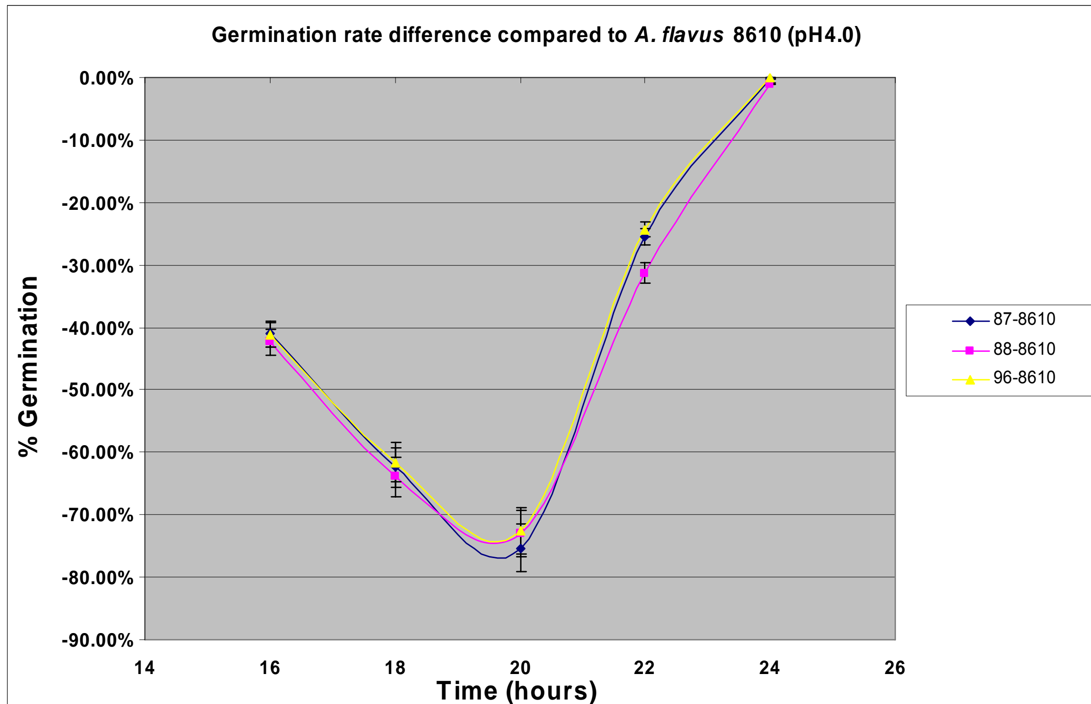

Figure 3.18: Difference in germination rates between fungal transformants (TpacCi87, TpacCi88 and TpacCi96) and untransformed *A. flavus* 8610 after growth on acidic media (GMM + Arg, pH 4.0).
Error bars indicate standard deviation.

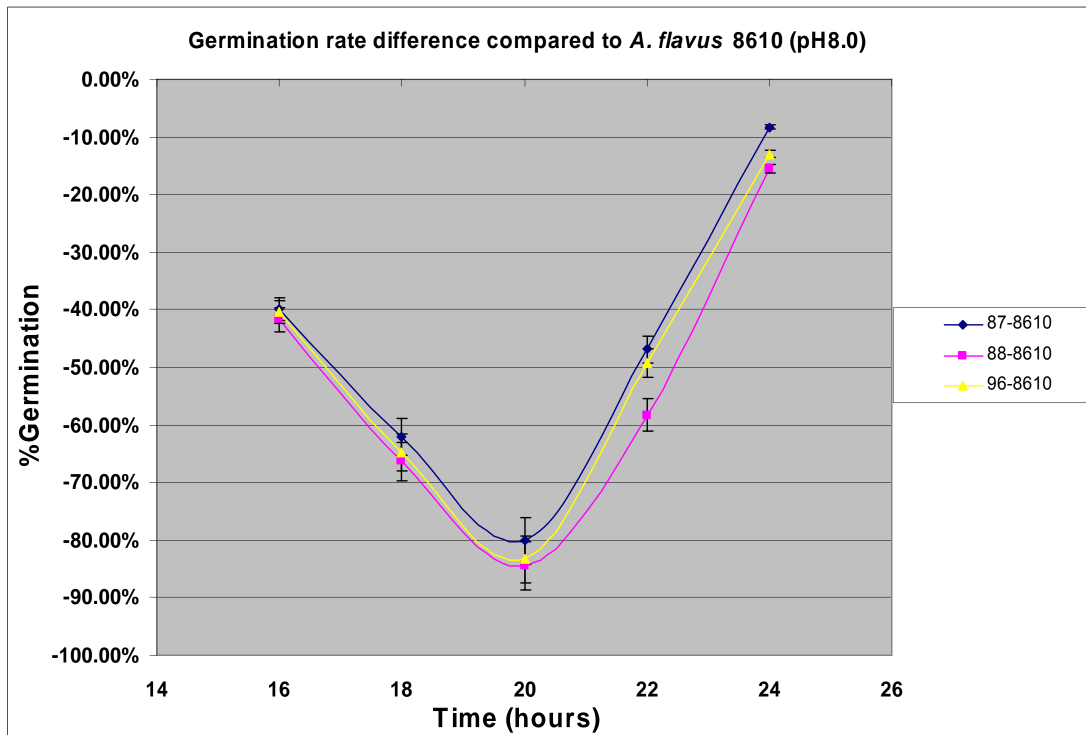

Figure 3.19: Difference in germination rates between fungal transformants (TpacCi87, TpacCi88 and TpacCi96) and untransformed *A. flavus* 8610 after growth on acidic media (GMM + Arg, pH 8.0).
Error bars indicate std. deviation.

A comparison of the difference in germination rates between untransformed 8610 and mutants indicates that there were significant differences in the germination rates on both acidic and alkaline media. Between 16-20 hours, the germination rate of the mutants was lower than that of untransformed *A. flavus* 8610 on both acidic and alkaline media (Figs 3.18 and 3.19). However, between 20-24 hours the germination rate of the fungal mutants exceeded that of untransformed *A. flavus* 8610 until the difference was very nearly zero.

It has been shown that germination rates of pathogenic *Aspergillus* species e.g. *A. flavus, A. fumigatus* and *A. nidulans* is not severely affected by pH (Araujo and Rodrigues, 2004). The perceived negative germination rate between 16 and 20 hours (Figs 3.18 and 3.19) of the fungal mutants (when compared to 8610) could be due to the mutant strains having longer "Lag Phases" which is attributable to silencing of the *pacC* gene. Therefore, it may be deduced that *pacC* silencing reduced the induction of the appropriate genes involved in germination but did not completely inhibit these genes from being transcribed. This data suggests that conidial germination is not solely dependent on *pacC* expression. It has also been hypothesised that spores of filamentous fungi need to be in close proximity to one another before they begin germinating in a manner similar to quorum sensing in other organisms (Hogan, 2006; Semighini

et al, 2006). Thus silencing of the *pacC* gene may have also affected such a "quorum sensing mechanism" leading to a delay in germination.

3.3.5 Pathogenicity Assays

Pathogenicity was assessed visually by examination of fungal colonization of maize seeds (Figs 3.20 – 3.22) and by spore counts (Fig 3.23). Spores of all mutants grown at pH 4.0 were pathogenic on maize kernels and exhibited abundant colonization on all inoculated seeds producing 9.56 x 10^7 conidia/ml (TpacCi87), 6.79 x 10^7 conidia/ml (TpacCi88) and 2.14 x 10^8 conidia/ml (TpacCi96). This was similar to that obtained for untransformed 8610 which produced 5.28 x 10^7 conidia/ml. Again, TpacCi96 produced more conidia than untransformed 8610. This is identical to the results obtained for conidial production on chemically defined media (Fig 3.11, above).

In contrast spores of mutants grown at pH 8.0 were less pathogenic on maize kernels and did not produce easily detectable signs of seed colonization. Mycelial growth and conidiation of TpacCi87 and TpacCi88 was very sparse. TpacCi96 was the only mutant that produced some visual signs of seed colonization but at a reduced level when compared to untransformed 8610. TpacCi87, TpacCi88 and TpacCi96 mutants produced less spores (1.15 x 10^5 conidia/ml, 4.72 x 10^4 conidia/ml and 2.59 x 10^5 conidia/ml respectively) than untransformed 8610 (2.78 x 10^6 conidia/ml).

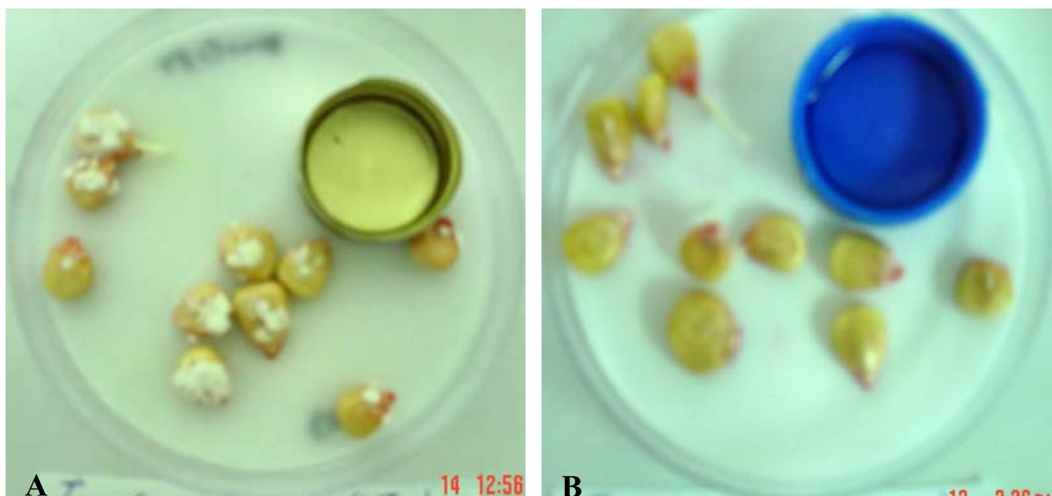

Figure 3.20: Maize kernels infected with TpacCi87 spores.
(A) Kernels inoculated with spores harvested from glucose minimal medium supplemented with arginine at pH4.0. (B) Kernels inoculated with spores harvested from glucose minimal medium supplemented with arginine at pH 8.0.

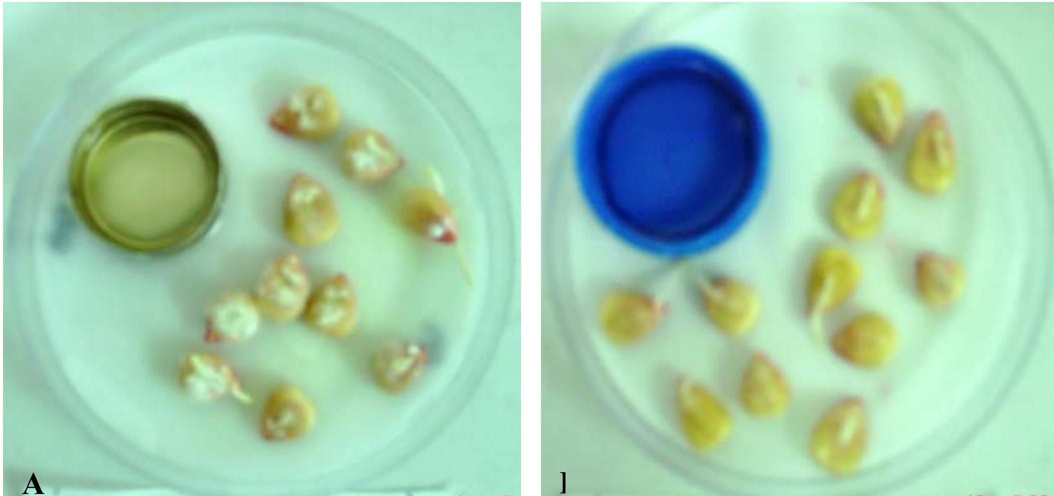

Figure 3.21: Maize kernels infected with TpacCi88 spores.
(A) Kernels inoculated with spores harvested from glucose minimal medium supplemented with arginine at pH4.0. (B) Kernels inoculated with spores harvested from glucose minimal medium supplemented with arginine at pH 8.0.

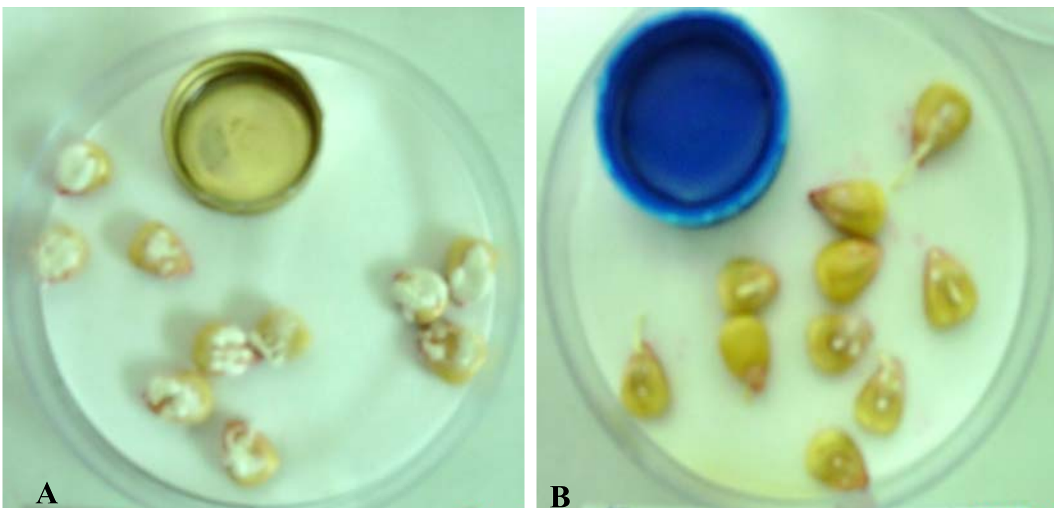

Figure 3.22: Maize kernels infected with TpacCi96 spores.
(A) Kernels inoculated with spores harvested from glucose minimal medium supplemented with arginine at pH4.0. (B) Kernels inoculated with spores harvested from glucose minimal medium supplemented with arginine at pH 8.0.

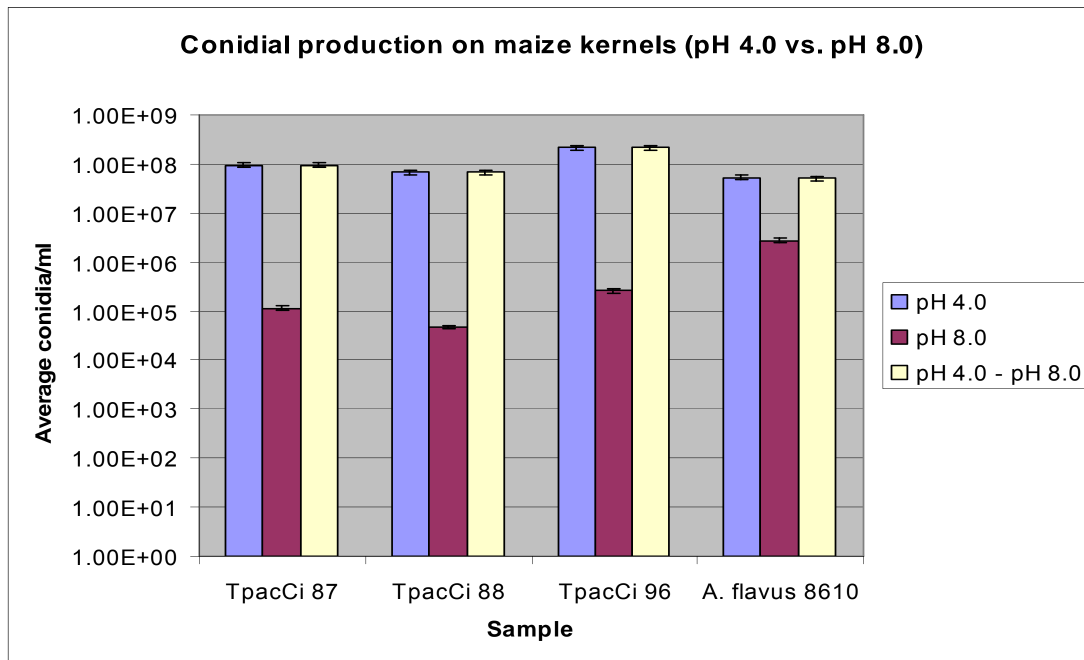

Figure 3.23: Conidial production of *pacC* RNA interference mutants (TpacCi87, TpacCi88 and TpacCi96) on maize kernels.
Spores were harvested after 5 days from infected maize kernels in triplicate and the average conidia/ml determined. NB: Scale on Y-axis is a Logarithmic scale, to facilitate comparison of conidial production (conidia/ml) from maize kernels infected with spores harvested from GMM + Arg (pH4.0 and pH 8.0).

Infection of maize kernels with spores harvested from GMM + Arg (pH 8.0) plates resulted in extremely attenuated virulence and 99.88% (TpacCi87), 99.93% (TpacCi88) and 99.87% (TpacCi96) reduction in conidial production compared to maize kernels infected with spores harvested from acidic media (GMM + Arg, pH 4.0). Statistical analysis on the data obtained for conidial production on maize kernels verified this ($P > 0.00003$; $P > 0.007$ and $P > 0.02$ for TpacCi87, TpacCi88 and TpacCi96 respectively). This data correlates to conidial production on synthetic media buffered to pH 4.0 and pH 8.0 (section 3.3.3). Similar results have been obtained for *PAC1* (*pacC* homologue) in *F. verticilliodes.*

From the results obtained in this study, it can be concluded that silencing of the *pacC* gene via RNA interference in the fungal mutants (TpacCi87, TpacCi88 and TpacCi96) severely affected their capacity to infect maize kernels and cause disease. Disruption of the *pacC* gene (or its homologues) in *A. nidulans*, *C. albicans* and *F. oxysporum* also resulted in extremely attenuated virulence of these organisms (Davis *et al*, 2000; Fonzi, 2002; Caracuel *et al*, 2003; Bignell *et al*, 2005 and Cornet *et al*, 2005). Thus the *pacC* gene is critical for pathogenicity of *A. flavus.* It should be noted that while the *pacC* null mutants characterised in this study are able to

produce aflatoxins (Chapter 2) at both acidic and alkaline pH, the virulence of these strains on maize is severely attenuated (> 99%) when grown on alkaline media.

3.3.6 Electron Microscopy of Mutants

SEM results of untransformed 8610 were virtually identical irrespective of the pH of the growth media (Fig 3.24). Conidia were formed in long chains which are characteristic of *A. flavus*. Phialides and conidiophores were well developed and appeared identical whether grown at pH 4.0 or pH 8.0. Mutants grown at pH 4.0 (Figs 3.25A, 3.26A; 3.27A) indicated no differences in morphology to that of untransformed 8610. The conidiophores and conidia of all mutants at acidic pH were well developed and echinulate which is characteristic of *A. flavus*.

At pH 8.0 conidia of TpacCi87 (Fig 3.25B) and TpacCi88 (3.26B) showed distinct morphological differences from that of pH 4.0. TpacCi87 and TpacCi88 exhibited many collapsed conidiophores with immature phialides and a reduction in the number of conidia. A large number of phialides were "open-ended" and did not appear to produce conidia. In contrast growth of TpacCi96 on alkaline media did not affect the morphology of conidia (Fig 3.27B). These conidia resembled that of untransformed 8610.

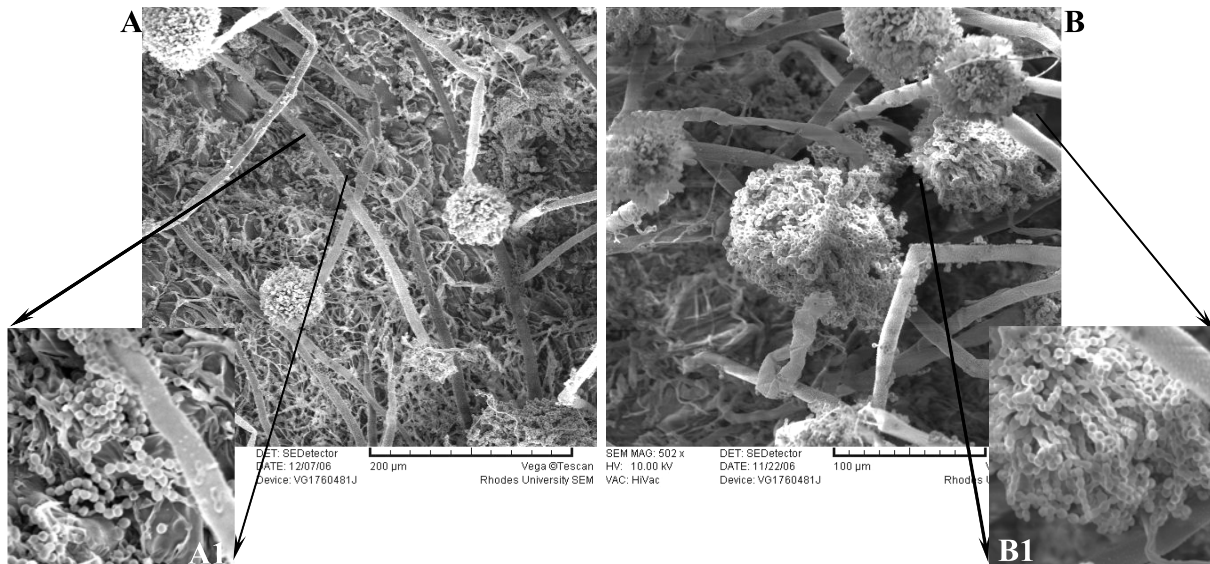

Figure 3.24: Scanning electron microscopy (SEM) of untransformed *A. flavus* 8610 grown on GMM + Arg buffered to pH 4.0 at 335X (A) and 2000X (A1) magnification and pH 8.0 at 502X (B) and 2000X (B1) magnification.

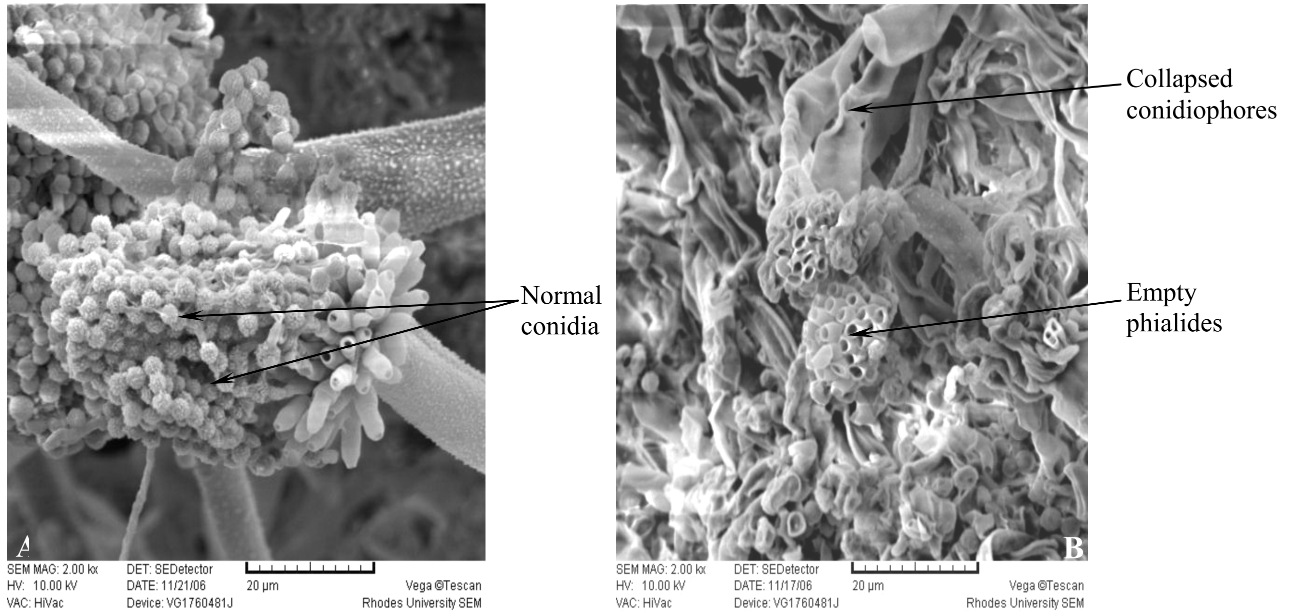

Figure 3.25: Scanning electron microscopy (SEM) of TpacCi87 grown on GMM + Arg buffered to pH 4.0 (A) and pH 8.0 (B) at 2000X magnification

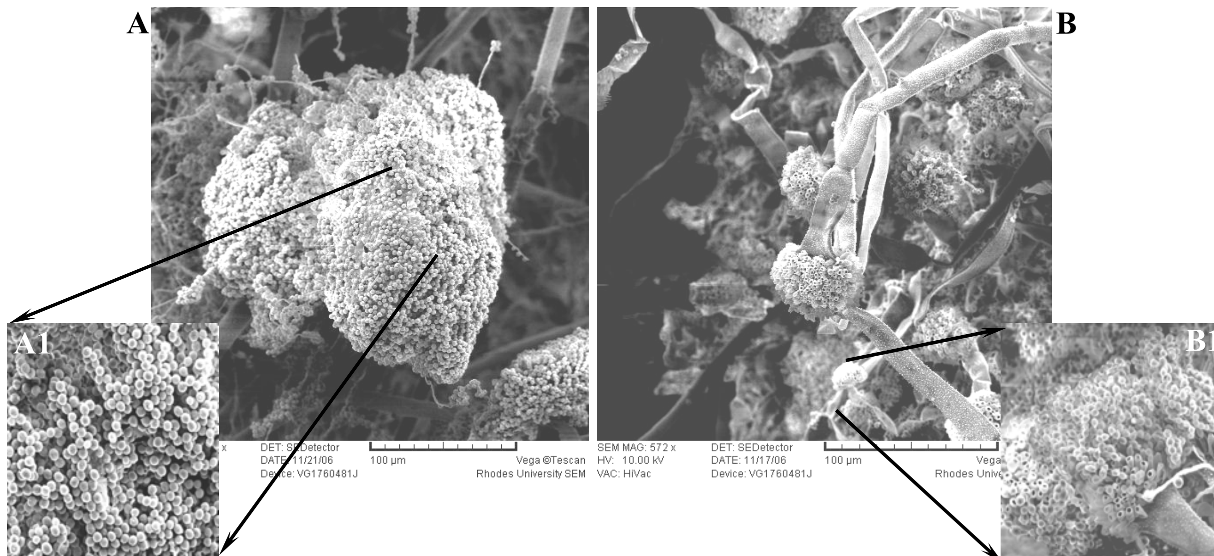

Figure 3.26: Scanning electron microscopy (SEM) of TpacCi88 GMM + Arg buffered to pH 4.0 at 500X (A) and 2000X (A1) magnification and pH 8.0 at 500X (B) and 2000X (B1) magnification.

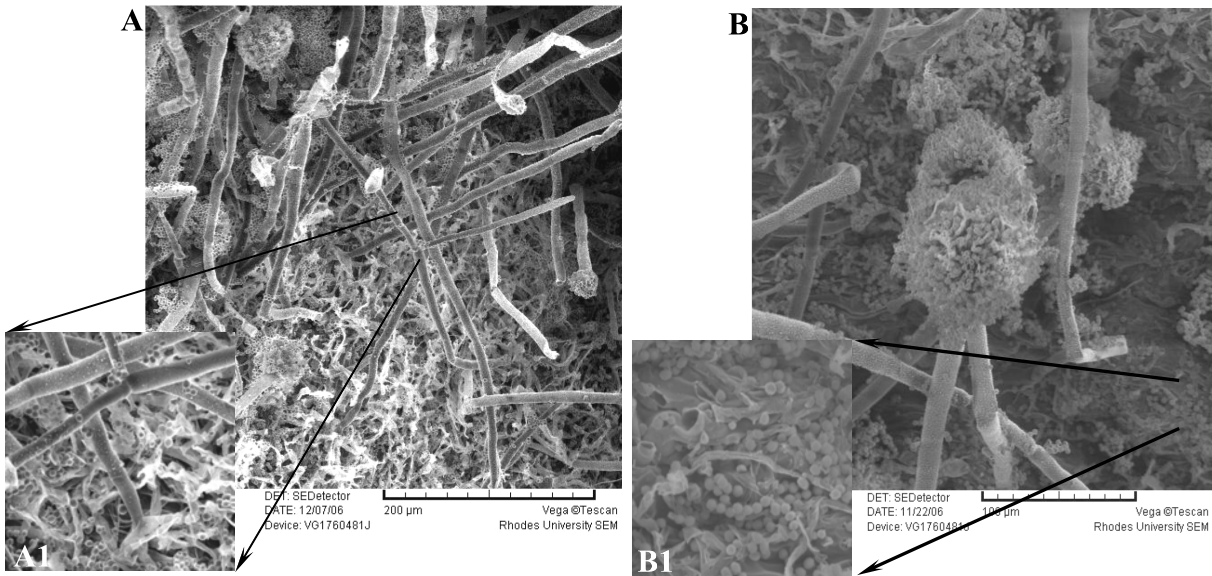

Figure 3.27: Scanning electron microscopy (SEM) of TpacCi96 grown on GMM + Arg buffered to pH 4.0 at 333X (A) and 1330X (A1) magnification and pH 8.0 at 500X (B) and 2000X (B1) magnification.

Silencing of the *pacC* gene adversely influences the morphology and development of *A. flavus* asexual reproductive structures such as conidia, conidiophores and phialides. Comparison of SEM micrographs obtained for *pacC* mutants in this study with that of strains mutated in genes involved in and regulating conidiation e.g. *abaA*, *alb1, apsA, brlA*, *medA* and *stuA* (Aguire *et al* 1993; Prade and Timberlake, 1993; Fischer and Timberlake, 1995; Adams *et al*, 1998; Tsai *et al*, 1998) indicated that the conidial morphology of *pacC* RNAi mutants (obtained in this study) did not resemble that of the above-mentioned mutants. When grown on media at alkaline pH conidia of the *pacC* RNAi mutants were deformed and there were large numbers of empty phialides implying that gene pathways prior to initiation of conidiation were affected. However, there are more than 1000 genes involved in conidiophore development and conidiation in *Aspergillus nidulans,* most of which are largely uncharacterised (Boylan *et al*, 1987).

At present it remains unknown whether or not *pacC* directly or indirectly regulates genes involved in *A. flavus* conidiation and development under alkaline conditions. Sequence analysis of some genes involved in conidiation such as *brlA*, *veA,* and *fluG* (Clutterbuck, 1969; Lee and Adams; 1994; Hicks *et al,* 1997; Calvo *et al*, 2002; Kato *et al*, 2003) were found (results not shown) to contain one or more PacC binding sites (GCCARG; Penalva and Arst, 2002). From the results shown here it is concluded that *pacC* is essential for regulating asexual reproduction in *A. flavus*. However, the exact mechanism of PacC regulation remains to be determined.

3.4 GENERAL DISCUSSION AND CONCLUSION

The physiological effects on *pacC* silencing on the growth, conidiation, pathogenicity and structural morphology of the *A. flavus* mutants were investigated. Radial growth analysis has shown that mutation of the *pacC* gene does not affect radial growth of the mutants when compared to untransformed *A. flavus* 8610 at either an acidic or an alkaline pH. This leads us to believe that *pacC* does not play a significant role in primary growth and development similar to that found by Lambert *et al* (1997).

However, there were large decreases in the conidial production of TpacCi87 and TpacCi88 mutants compared to that of untransformed *A. flavus* 8610 at both acidic and alkaline pH. A similar observation was made by Flaherty *et al* (2003) in *F. verticilliodes*. SEM results have indicated that these mutants have many "open ended" phialides and poorly developed conidiophores leading us to conclude that *pacC* activation of conidial production genes is also required. TpacCi96 is the only mutant that behaves differently from 8610 in that it produces more conidia than 8610. The reason for this is unknown and possible reasons have been suggested earlier (see Chapter 2). TpacCi96 needs to be investigated further before any conclusions can be drawn.

All mutants displayed a very slow initial germination rate (16-20 hours) on both acidic and alkaline media. However between 20 and 24 hours the germination rates increased rapidly with greater than 90% germination occurring on acidic media after 24 hours while more than 80% of spores germinated after 24 hours on alkaline media. Since *pacC* is also a positive regulator of many genes, silencing effectively decreased but did not halt the transcription of these genes. This in turn would explain the delay in spore germination. However, once transcript levels had reached sufficient levels the germination rate increased.

Evaluation of the pathogenicity of mutants (harvested after growth on acidic and alkaline media) on maize showed that the conidia harvested from acidic media were pathogenic on maize kernels since most of the seed surfaces were colonized by fungal growth. In contrast, infection of maize kernels with conidia harvested from alkaline media resulted in extremely low seed colonization and concomitant extremely low conidial production (>99% reduction) when compared to maize infected with conidia harvested from acidic media. Only TpacCi96 produced visible signs of pathogenicity on maize kernels after infection with spores harvested from both acidic and alkaline media although infection was reduced in maize kernels infected with spores harvested from alkaline media. The results obtained here are similar to that of *pacC* null mutants in *A. nidulans, C. albicans* and *F. oxysporum* which also exhibited low pathogenicity (Davis *et al*, 2000; Fonzi, 2002; Caracuel *et al*, 2003; Bignell *et al*, 2005; Cornet *et al*, 2005). Thus *pacC*

RNAi silencing severely impaired the ability of the *A. flavus* mutants to infect and cause damage on maize.

In TpacCi87 and TpacCi88 mutants silencing of the *pacC* gene resulted in poorly developed conidia and decreased pathogenicity. In contrast well developed conidia of TpacCi96 and 8610 were pathogenic on maize. This is supported by the observation of Tsai *et al* (1998) who found that disruption of the conidial pigmentation gene, *alb1* in *A. fumigatus* resulted in the production of smooth surfaced conidia that had a significant reduction in virulence. From the pathogenicity and SEM data it is concluded that pathogenicity of *A. flavus* on maize is directly related to the structural integrity of conidia, which in turn is greatly influenced by PacC.

In conclusion *pacC* silencing affects germination, conidiation, asexual reproduction, aflatoxin biosynthesis (Chapter 2) and pathogenicity of *A. flavus*. This gene is a global transcriptional regulator and may either repress or activate one or many genes in each of the above pathways (Penalva and Arst, 2002). Further study into the genetic mechanisms of *pacC* regulation on these pathways is needed to elucidate the mechanisms of activation or repression of these genes.

3.5 REFERENCES

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APPENDIX A

MEDIA AND REAGENTS

Aspergillus nidulans Defined Media

20X Minimal Nitrate Salts

$NaNO_3$	120g
KCl	10.4g
$MgSO_4.7H_2O$	10.4g
KH_2PO_4	30.4g

ddH_2O to 1 litre, store at room temperature
For minimal ammonium medium, substitute 74.5g of NH_4Cl for $NaNO_3$

Trace Elements:

$ZnSO_4.7H_2O$	2.2g
H_3BO_3 (Boric Acid)	1.1g
$MnCl_2.4H_2O$	0.5g
$FeSO_4.7H_2O$	0.5g
$CoCl_2.5H_2O$	0.16g
$CuSO_4.5H_2O$	0.16g
$(NH_4)6Mo_7O_{24}.4H_2O$	0.11g
Na_4EDTA ($C_{10}H_{12}N_2O_8Na_4.2H_2O$)	5.0g
Or	
Na_2EDTA	4.5g

Add the solids in order to 80ml of ddH_2O, dissolving each completely before adding the next. Heat the solution to boiling, cool to 60°C, adjust the pH to 6.5-6.8 with KOH pellets. Cool to room temperature and adjust volume to 100ml with ddH_2O.

Glucose Minimal Medium + Arg + Uridine + Uracil (pH 4.0):

20x Salt solution	50ml/l
Trace Elements (shake before using)	1ml
D-glucose	10g
L-Arginine	1.0g
Uridine	1.26g
Uracil	0.56g
Na_2HPO_4	19.3mM
Citric acid	15.35mM
Agar	15g

Adjust pH to 6.5
ddH_2O to 1 litre
autoclave for 15 mins at 121°C

To prepare GMM at pH 8.0 add 47.3mM/l Na_2HPO_4 and 2.7mM/l NaH_2PO_4 instead of 19.3mM/l Na_2HPO_4 and 15.35mM/l Citric acid.

LETS Buffer (for DNA Isolation:

0.1M LiCl
20mM EDTA (pH 8.0)
10mM Tris-HCl (pH 8.0)
0.5% SDS

Reagents for generation of Protoplasts

Mycelium Wash Solution

$MgSO_4$	147.9g
ddH_2O	1l

Osmotic Medium

$MgSO_4$	147.9g
10mM Sodium Phosphate buffer	
(Can be made from a 2M NaPB stock, which has 90.9g Na_2HPO_4 and 163.4g NaH_2PO_4 per liter)	
Adjust to pH 5.8 with 1M Na_2HPO_4	
ddH_2O	500ml

Trapping Buffer

Sorbitol (0.6M)	109.3g
Tris-HCl, pH 7.0	0.1M
ddH_20	to 1l

STC Buffer

Sorbitol (1.2M)	218.6g
$CaCl_2$ (10mM)	1.11g
Tris-HCl, pH 7.5	10mM
ddH_20	to 1l

PEG Solution

PEG4000	60%
$CaCl_2$ (50mM)	2.35g
Tris-HCl, pH 7.5	50mM
ddH_20	to 1l

BUFFERS FOR HYBRIDIZATIONS

Denaturation solution

NaCl	1.5 M
NaOH	0.4M

Pre-Hybridization Solution

Phosphate buffer[4] (pH 7.2);	0.5M
EDTA (pH 8.0)	1mM
SDS	7% (w/v)

[4] 0.5M phosphate buffer contains 80g Na_2HPO_4, 4ml of 85% H_3PO_4 and ddH_2O to 1liter.

APPENDIX B

BLAST ANALYSIS OF PARTIAL *pacC* ISOLATED FROM *A. flavus* MRC2527WT

BLASTN 2.2.15 [Oct-15-2006]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
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.

RID: 1165494262-30874-129409037017.BLASTQ4

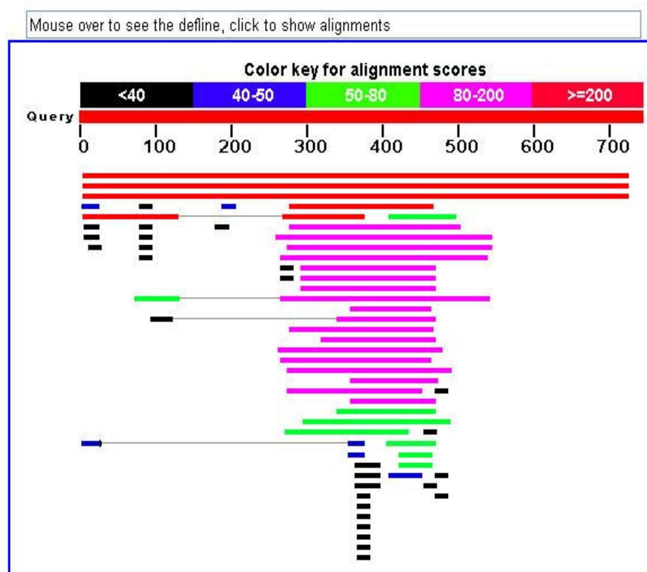
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,
GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
4,634,455 sequences; 18,617,716,848 total letters

If you have any problems or questions with the results of this search
please refer to the **BLAST FAQs**
Taxonomy reports

Query=
Length=745

Distribution of 66 Blast Hits on the Query Sequence

Mouse over to see the defline, click to show alignments



Sequences producing significant alignments:		Score (Bits)	E Value	
gi|83767101|dbj|AP007155.1|	Aspergillus oryzae RIB40 genomic DNA	1360	0.0	
gi|12082077|dbj|AB035899.1|	Aspergillus oryzae gene for pacC, co	1360	0.0	
gi|15431015|gb|AF408430.1|AF408430	Aspergillus parasiticus pu...	1249	0.0	G
gi|7839540|gb|AF260325.1|AF260325	Colletotrichum sublineolum ...	371	7e-101	
gi|84573035|dbj|AB225332.1|	Aspergillus oryzae cDNA, contig sequ	238	7e-61	
gi|1403129|emb|X98417.1|ANPACCGN	A.niger pacC gene	188	6e-46	
gi|695415|emb|Z47081.1|ANPACCGEN	A.nidulans pacC gene for DNA bi	178	6e-43	
gi|67524794|ref|XM_655367.1|	Aspergillus nidulans FGSC A4 hyp...	167	2e-39	G
gi|54067002|gb|AY763122.1|	Aspergillus giganteus putative tra...	163	3e-38	
gi|30142037|gb|AY125958.1|	Fusarium oxysporum pH transcriptio...	143	3e-32	
gi|24430364|emb|AJ514259.1|GFU514259	Gibberella fujikuroi pac...	143	3e-32	
gi|29150632|gb|AY216461.1|	Gibberella moniliformis pH regulat...	135	8e-30	
gi|115396557|ref|XM_001213918.1|	Aspergillus terreus NIH2624 ...	123	3e-26	G
gi|22770973|gb|AF536982.1|	Penicillium echinulatum putative t...	117	2e-24	
gi|55669160|gb|AY647218.1|	Botryotinia fuckeliana strain B05-...	111	1e-22	
gi|23267150|gb|AF539700.1|	Glomerella cingulata putative Zn f...	109	4e-22	

Figure B1: BLAST analysis of sequencing results obtained for the partial *pacC* fragment of *A. flavus* MRC2527WT.

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> gi|83767101|dbj|AP007155.1| D Aspergillus oryzae RIB40 genomic DNA, SC003
Length=4170692

 Features in this part of subject sequence:
   unnamed protein product

 Score = 1360 bits (686),  Expect = 0.0
 Identities = 711/721 (98%), Gaps = 1/721 (0%)
 Strand=Plus/Minus

Query  7        ACCGCAGCCGCAGCGACCGCAGCTGTGGCATCTCCCCCTGTTAATGGTGCTGCGCGCCCG  66
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2037276  ACCGCAGCCGCAGCGACCGCAGCTGTGGCATCTCCCCCTGTTAATGGTGCTGCGCGCCCG  2037217

Query  67       ACCGAAGAGTTGTCTTGTCTTTGGCAAGGATGTTCTGAGAAGTGTCCCACCCCAGAATCC  126
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2037216  ACCGAAGAGTTGTCTTGTCTTTGGCAAGGATGTTCTGAGAAGTGTCCCACCCCAGAATCC  2037157

Query  127      CTCTATGTAAGCTCACCCGTGATCCCATACTCCTGCGATCATCGTCACCCCACACAGCAG  186
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2037156  CTCTATGTAAGCTCACCCGTGATCCCATACTCCTGCGATCATCGTCACCCCACACAGCAG  2037097

Query  187      GCATTGTTGCCACTTGTCCTTTCGACATATCACATGGGAGCACCTCAATCGCTTACTTTT  246
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2037096  GCATTGTTGCCACTTGTCCTTTCGACATATCACATGGGAGCACCTCAATCGCTTACTTTT  2037037

Query  247      GAACGTAGCTGATCATGTTATAGGAGCACGTCTGCGAACGTCACGTAGGTCGCAAGAGCA  306
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2037036  GAACGTAGCTGATCATGTTATAGGAGCACGTCTGCGAACGTCACGTAGGTCGCAAGAGCA  2036977

Query  307      CAAATAATCTCAACTTGACTTGCCAATGGGGCAGTTGCCGAACTACTACTGTTAAGCGCG  366
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2036976  CAAATAATCTCAACTTGACTTGCCAATGGGGCAGTTGCCGAACTACTACTGTTAAGCGCG  2036917

Query  367      ACCATATCACCTCTCATATCCGAGTACATGTGCCTTTAAAACCTCACAAGTGTGACTTCT  426
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2036916  ACCATATCACCTCTCATATCCGAGTACATGTGCCTTTAAAACCTCACAAGTGTGACTTCT  2036857

Query  427      GTGGAAAGGCCTTCAAGCGCCCTCAGGACTTGAAGAAACATGTCAAGACACATGCAGATG  486
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2036856  GTGGAAAGGCCTTCAAGCGCCCTCAGGACTTGAAGAAACATGTCAAGACACATGCAGATG  2036797

Query  487      ATTCGGTTCTGGTTCGGTCACCTGAACCGGGTTCCCGGAATCCAGATATAATGTTTGGAG  546
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2036796  ATTCGGTTCTGGTTCGGTCACCTGAACCGGGTTCCCGGAATCCAGATATAATGTTTGGAG  2036737

Query  547      GTAACCCAGCAAAAGGTGTGTCCTGTGTCTCTAGCTGCATTCGCTGCGTAACTGACCCTA  606
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2036736  GTAACCCAGCAAAAGGTGTGTCCTGTGTCTCTAGCTGCATTCGCTGCGTAACTGACCCTA  2036677

Query  607      TGGAGCTCTAGGCTATGCTACCGCTACNCATTACTTCNAACCCGCCTTAAAACCCCGTCC  666
                ||||||||||||||||||||||||||| ||||||||| |||||||||| |||||||||||
Sbjct  2036676  TGGAGCTCTAGGCTATGCTACCGCTACGCATTACTTCGAACCCGCCTT-AAACCCCGTCC  2036618

Query  667      CCANCCAGGGTTATGCTCACGGANCTCCNCNGNACNATCAATCNCATCATCCACCTCAAC  726
                ||| ||||||||||||||||||| |||| | | || ||||||| ||||||||||||||||
Sbjct  2036617  CCAGCCAGGGTTATGCTCACGGAGCTCCTCAGTACTATCAATCTCATCATCCACCTCAAC  2036558

Query  727      C  727
                |
Sbjct  2036557  C  2036557
```

Figure B2: Sequencing homology of the partial *pacC* fragment of *A. flavus* MRC2527WT to *A. oryzae*.

APPENDIX C

SECONDARY STRUCTURE ANALYSIS OF 1.8kb INVERTED REPEAT TRANSGENE (IRT)

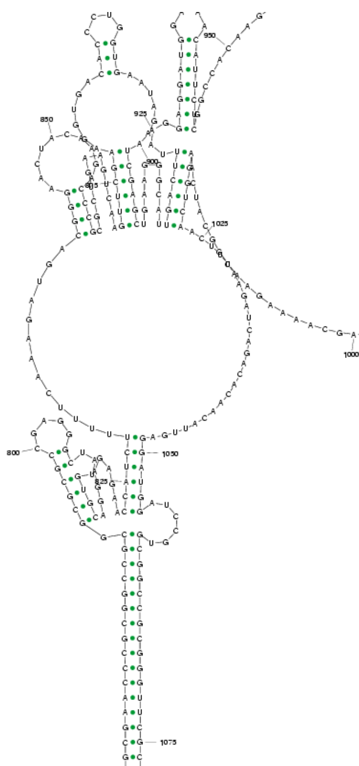

Figure C1: Predicted hairpin loop structure formation of the *pacC* IRT

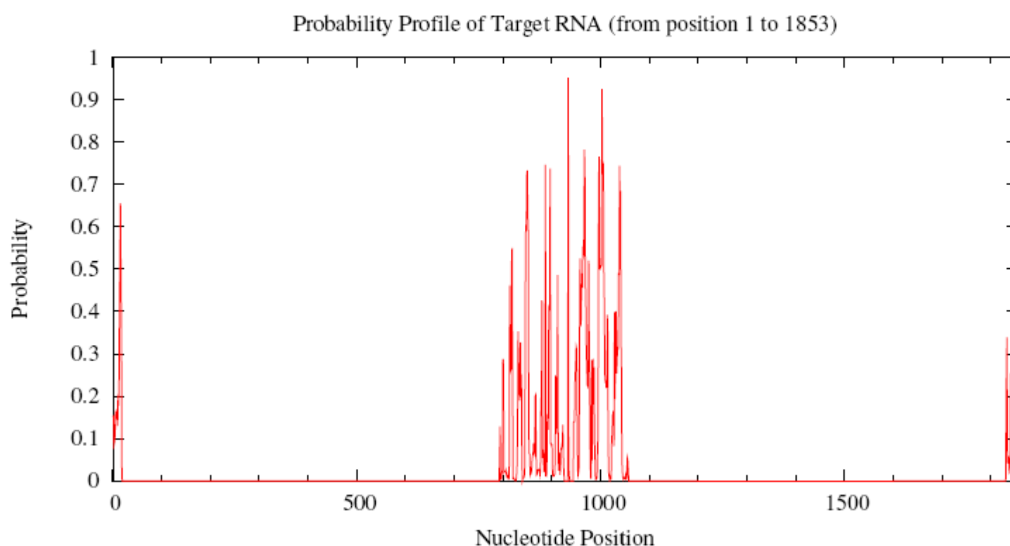

Figure B2: Probability Profile of the 1.8kb forming a hairpin loop secondary structure.
NB: from regions ~0-750bp and 1100bp-1.8kb the probability of secondary structure formation is low. However between 800-1100bp the probability of secondary hairpin-loop structure formation is very high. This implies that the 1.8kb IRT forms a very stable hairpin loop secondary structure.