Transcriptional regulation during appressorium formation and function in *Glomerella cingulata*

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Abstract

Glomerella cingulata, anamorph *Colletotrichum gloeosporioides*, causes bitter rot in apples and fruit rot in other subtropical fruits. In response to environmental cues such as contact with the host, *Glomerella cingulata* forms a special structure called an appressorium, which accumulates glycerol and thereby generates a sufficiently high turgor pressure to push an infection peg into the host tissue. It is known that the cAMP and MAPK signalling transduction pathways control appressorium formation and function in *Colletotrichum* species and other appressorium-forming fungi. This process is accompanied by a global change in gene expression. Little is known of transcriptional regulation during this process. The aim of this project was to study the transcriptional regulation of appressorium formation and function in *G. cingulata.*

The *G. cingulata SAP* gene had previously been shown to be expressed as a longer transcript during the early stage of appressorium differentiation. It was considered possible that the transcription factors that regulated expression of the longer transcript may be also involved in the regulation of appressorium differentiation. Identification of the transcription factor involved may help to understand the mechanisms that regulate appressorium differentiation. The plan was to use the yeast one-hybrid system to isolate the transcription factor. This required identification of the promoter regions responsible for expression of the longer *SAP* transcript. Therefore, the *G. cingulata SAP* promoter was characterized by mapping the transcription start point. Three transcription start points were determined by RLM-RACE. To further characterise the promoters, *SAP-GFP* reporter plasmids were constructed and transformed into *G. cingulata.* Even though a reasonable level of *GFP* expression was observed in RT-PCR experiments, however, no differences in fluorescence intensity were seen between the wild type and GFP reporter transformants. Therefore, no further attempts to study the *sap* promoter were made.

The candidate gene approach was chosen as an alternative way to study the transcriptional regulation of appressorium formation and function in *G. cingulata.* The

G. cingulata StuA gene was cloned using degenerate PCR, single specific primer PCR, subgenomic library screening and plasmid rescue from a disruption mutant. Targeted gene deletion of the *G. cingulata StuA* gene was successful. Deletion mutants display many phenotypic changes. Complementation mutants were constructed to confirm the function of this gene. A full length copy of this gene together with a second selection marker was reintroduced into the deletion mutant and the wild type phenotype was restored.

Deletion mutants form appressoria at the normal rate and with unaltered morphology. In comparison with the wild type, these appressoria did not generate high turgor pressure as shown by a cytorrhysis assay. This resulted in a defect in appressorium penetration of onion epidermal cells. Nor were these mutants able to invade unwounded apples. Therefore, the *G. cingulata StuA* gene is required for appressorium function. In addition, deletion mutants displayed stunted aerial hyphae, "wettable" mycelium, reduced conidia production, and a defect in conidiophore and perithecium formation. These results suggested that the *G. cingulata StuA* gene has multiple roles in fungal development.

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Abbreviations

bp	base pair
BSA	Bovine serum albumin
cAMP	cyclic AMP
cDNA	complementary deoxyribonucleic acid
CWDE	cell wall degradation enzyme
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dNTP	deoxynucleotide triphosphate
DHN	dihydroxynaphthalene
DTT	1,4-dithiothreitol
DIG	Digoxigenin
EDTA	Ethylenediamine tetraacetic acid
ECM	extracellular matrix
GFP	green fluorescence protein
kb	kilobase pair
MAPK	mitogen activated protein kinase
ORF	open reading frame
PCR	polymerase chain reaction
PEG	Polyethylene glycol
PKA	protein kinase A
5' UTR	5' un-translated region
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription PCR
RACE	Rapid amplification of cDNA ends
REMI	restriction enzyme mediated integration
Sec	Second(s)
SDS	Sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate (buffer)

Chapter one

Introduction

1.1 Plant Pathogenic Fungi

Fungi are one of the five kingdoms of life. They are small eukaryotic organisms. There are over one million species of fungi in this world. Many fungi are beneficial and useful while some cause diseases to humans, animals and plants. More than 8,000 species of fungi are known to cause plant diseases. All plants are attacked by some kind of fungus, and each pathogenic fungus can attack one or more kinds of plant (Agrios, 2005). Some fungi are biotrophs, they can grow and reproduce in nature only in living hosts, and they are called obligate parasites. Others can live on either living or dead hosts and on various nutrient media and they are therefore called nonobligate parasites. Many fungi exhibit a two-phase infection and colonisation process involving an initial symptomless or biotrophic phase, followed by a final visibly necrotic phase. During the symptomless biotrophic phase, the pathogen invades host cells without killing them and feeds on living cells. Subsequently, the pathogen switches to a necrotrophic mode of nutrition, feeding on dead host tissues. These fungi are described as hemibiotrophs. The hemibiotrophic fungi are readily grown in simple media and are consequently the best studied of the plant pathogenic fungi (Bailey *et al*., 1992).

Many plant pathogenic fungi belong to the Class Ascomycota. The Ascomycetes (the sac fungi) are characterised by their sexual process which involves the production of haploid ascospores through the meiosis of a diploid nucleus in an ascus. Most Ascomycetes also carry out asexual sporulation. Conidia are produced on specialized aerial hyphae, the conidiophores that rise above the substratum. The sexual phase of an Ascomycete is termed the teleomorph, and the asexual phase the anamorph. Different names are used for these two phases in a fungus, however, when both anamorphic and teleomorphic phases are present, the teleomorph name is used (Alexopoulos, 1979). There are some fungi in which the sexual stage has not been observed. The asexual stage dominates most infections, while the sexual stage is produced on infected leaves, fruits or stems only at the end of the growing season or when the nutrient supply is declining. The sexual stage can overwinter and ascospores act as the primary inoculum and cause the first infections in the following year. For most of the growing season the pathogens grow on their host and reproduce by means of asexual conidia that act as

secondary inoculum and cause all subsequent infections (Agrios, 2005). Such a life cycle can be demonstrated in the genus *Glomerella* (Fig 1.1).

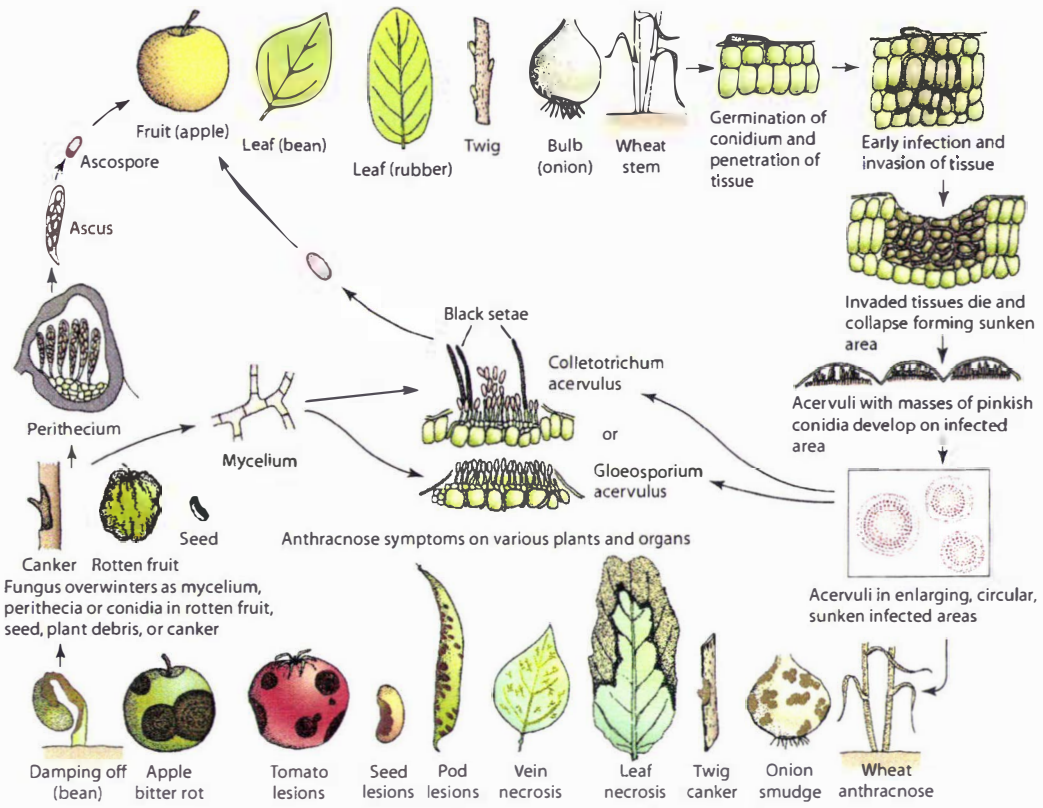


Figure 1.1 The life cycle of *Glomerella*

The life cycle of *Glomerella* begins with the breaking of dormancy of a spore (which is either a conidium from an acervulus or an ascospore from a perithecium), and is followed by spore germination, appressorium differentiation and penetration. Colonization is then established by growth of mycelium from the penetration hyphae. Acervuli or perithecia formed on the plant produce spores which then serve as the inoculum to cause another round of the life cycle. The figure was taken from Agrios (2005).

1.2 *Glomerella* and *Colletotrichum*

1.2.1 Genus *Glomerella*

Glomerella is a genus in the order Melanconiales of the Coelomycetes which is a small, acervuli-forming group of Ascomycete fungi (Sutton, 1992). Of the 80 species described in the genus *Glomerella*, a *Colletotrichum* anamorph has been reported for only 20 of these. Asexual stages, if they exist for the other *Glomerella* species, have not been found. On the other hand, some *Colletotrichum* species exist that are known only by their asexual stages. They have either lost the sexual stage as a result of their evolutionary development, or possess sexual stages as yet undiscovered (Sutton, 1992). The teleomorph name *Glomerella* and anamorph name *Colletotrichum* will be used interchangeably throughout this thesis depending upon which name was used in the literature cited.

Several species of *Glomerella* cause serious anthracnose diseases of cereals, grasses, legumes, vegetables, perennial crops and tree fruits. These are economically significant plants worldwide. Above–ground plant parts can be infected at all stages of maturity, from seedlings to mature plants and seeds (Bailey *et al.*, 1992). One species, *Glomerella cingulata* (Stoneman) Spaulding & von Schrenk (anamorph *Colletotrichum gloeosporioides* (Penzig) Penzig & Saccardo), also causes cankers and dieback of woody plants such as camellia and privet, bitter rot of apples, and ripe rot of grape, pears, peaches and other fruit (Agrios, 2005).

1.2.2 *Glomerella cingulata* and *Colletotrichum gloeosporioides*

Glomerella cingulata and its asexual state *Colletotrichum gloeosporioides* were first described in 1903 and 1884 respectively (Mordue, 1971). Their structures are shown in Fig 1.2. *G. cingulata* produces ascospores in a perithecium, which can be solitary or aggregated, partially or completely immersed in host tissue and on various parts of the plant but usually on dead leaves, twigs and mummified fruits. A perithecium is from 85-300 μm in diameter, dark brown to black in colour, globose to obpyriform in shape.

Asci are unitunicate, clavate to cylindrical, and contain eight ascospores. Ascospores are unicellular, hyaline, and narrowly oval to cylindrical (Mordue, 1971).

Colletotrichum gloeosporioides produces conidia at a hyphal tip or within an acervulus (fungal fruiting structure). Short, simple, colorless conidiophores produce abundant conidia. The disk or cushion shaped acervuli break through the surface of the host tissue. Long, black setae may or may not be produced among the conidiophores. Conidia are colorless when viewed alone, but may appear pink or salmon colored *en mass*. Conidia are short, ovoid to cylindrical and single celled (Mordue, 1971).

1.2.3 Bitter rot of apple in New Zealand

In New Zealand, *G. cingulata* has been found on apple, avocado, fig, grape, grapefruit, lemon, lime, mandarin, orange, pear, tomato and walnut (Atkinson, 1971). It causes bitter rot of apple in all major fruit growing areas nationwide. In a wet, warm autumn bitter rot may cause up to 10% loss at picking time, and up to 30% or more loss in storage (Atkinson, 1971). Bitter rot attacks fruit when they are full grown. Both types of spores can take part in disease initiation (Sutton, 1992). Infection can take place by direct penetration of germinating spores into the intact fruit or through a wounded part (Brook, 1977). Initiation of the rot is seen as minute, light brown spots which rapidly increase in size to become circular and slightly sunken in the centre. The rot spreads towards the apple core and forms a cone shaped lesion. In the early stages of development a lesion may show concentric zones of light and dark brown tissue, then small blisters occur near the centre either arranged in circles or scattered irregularly beneath the epidermis; these cover acervuli, which swell rapidly, rupturing the cuticle and exposing gelatinous pink spore masses. Under favourable conditions, this process may take only 3-6 days. As the rotted area increases more rings of spore masses appear (Fig 1.3). Spores may be carried to other fruits by rain, wind, insects or birds, to cause another infection cycle. Infected fruits may rot completely on the tree, forming shrivelled mummies which survive the winter and act as a source of spores the following year (Atkinson, 1971; Snowdon, 1988).

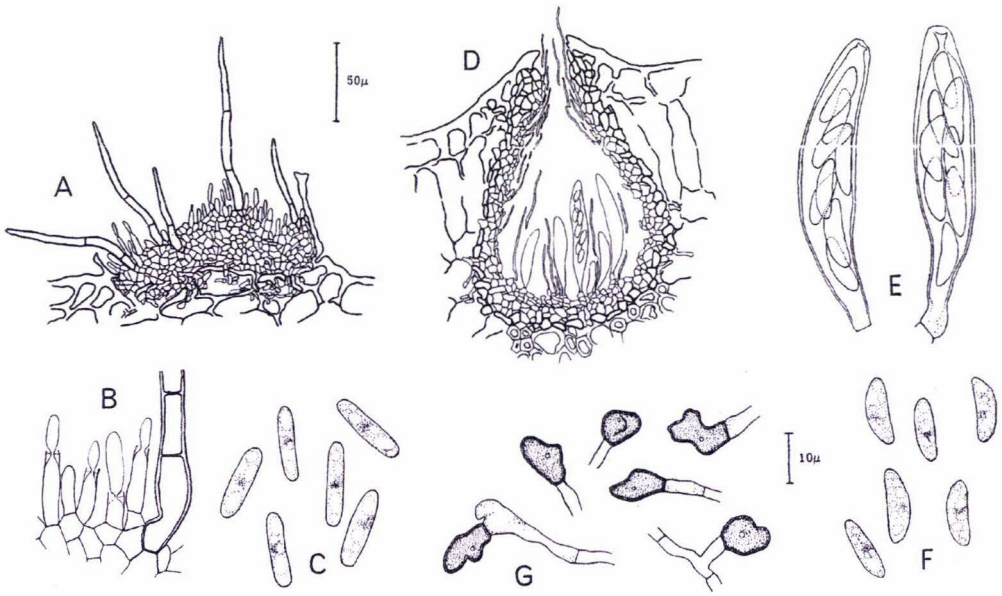


Figure 1.2 Description of *Glomerella cingulata* structures

A, acervulus; B, conidiophore; C, conidia; D, perithecium; E, asci; F, ascospores; G, appressorium formation from hyphae. The figure is taken from (Mordue, 1971).

Figure 1.3 An apple infected by *Glomerella cingulata*

A Granny Smith apple infected by *G. cingulata*. The rot is slightly sunken in the centre and spreads towards the apple core. Rings of spore masses are visible on the apple surface. The photo is taken from the website http://www.hortnet.co.nz.

1.2.4 Pathogenicity of *G. cingulata*

G. cingulata causes many plant diseases both nationwide and worldwide. Understanding the mechanism of pathogenicity of *G. cingulata* is essential for prevention and control of the diseases that it causes. This will also contribute to understanding pathogenicity in other fungi. As with the rice blast pathogen, *Magnaporthe grisea*, and some other fungi, pathogenicity of *Glomerella* species relies on their ability in spore dispersal, spore attachment, germination, appressorium formation and penetration, and the establishment of biotrophic and necrotrophic growth (Perfect *et al.*, 1999). With advances in molecular biology, the molecular mechanisms of pathogenicity have begun to be unravelled by cloning and characterisation of a number of genes in *Glomerella*. Studies of morphogenesis and pathogenicity in other model fungi also benefit from the knowledge obtained and guide further study of *Glomerella* species.

1.3 The infection process in *Colletotrichum* and other fungi

The life cycle of an Ascomycete fungus begins with the breaking of dormancy of a spore and its germination. A series of morphogenetic events governs a spore from its germination to hyphal growth and mycelium formation. For phytopathogenic fungi, nutrients for growth and development are obtained from plant tissue. Invasion of the plant tissue in order to access these nutrients requires overcoming the various barriers present at the plant surface (Mendgen *et al.*, 1996). Phytopathogenic fungi have evolved a range of strategies to penetrate the waxy cuticle and epidermal cell wall in order to gain access to the underlying tissues. A number of necrotrophic fungi secrete large amounts of cell wall degrading enzymes and toxins that kill host cells and degrade plant tissues. In this way they invade the host and obtain nutrients for growth (Kolattukudy, 1985; Schafer, 1994). On the other hand, many other fungi, like *Colletotrichum* species and *M. grisea*, enter the plant through the formation of specialized infection structure called an appressorium (Tucker and Talbot, 2001). The morphogenetic events leading to appressorium formation depend on specific signals provided by the plant surface. Although these signals and morphogenetic events are species-specific, the molecular mechanism underlying their development is well conserved.

The infection process for these fungi begins when fungal spores land on a susceptible host. Spores can adhere to the host surface tightly, allowing the physical or chemical signals from the plant surface to be sensed by the spore. The signals are transmitted via transmembrane proteins to trigger the endocellular signalling cascades, resulting in a series of physiological and morphological changes in the fungal cell, including spore germination, appressorium differentiation and penetration. Colonization is then established by mycelial growth from the penetration hyphae (Howard and Valent, 1996; Perfect *et al.*, 1999). A typical infection process for *G. cingulata* is summarized in Fig1.4. Infection structure formation is controlled by complex regulatory pathways and accompanied by regulated gene expression. This process and the molecular mechanisms that underlie it have been studied in *Colletotrichum* species, *M. grisea* and some other phytopathogenic fungi.

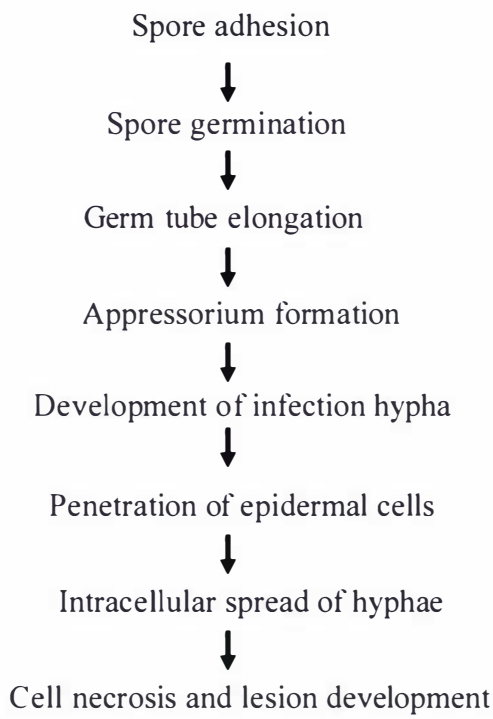


Figure 1.4 Outline of the infection process

The infection process is described in the text.

1.3.1 Spore adhesion

After fungal spores land on the plant surface, adhesion is the first step for establishment of pathogenesis. Successful adhesion allows the fungal spore to remain on the plant surface and perceive the plant signals for the initiation of appressorium development. Adhesion in many fungi includes an initial passive phase followed by a second active phase involving secretion of an extracellular matrix (Perfect *et al.*, 1999).

In the genus *Colletotrichum* (*Glomerella*), conidia and ascospores are produced and released from acervuli and perithecia, respectively. Spores can be carried to the host surface by rain, wind, insects or birds. Adhesion of ungerminated conidia of *Colletotrichum graminicola, Colletotrichum musae,* and *Colletotrichum lindemuthianum* is initiated by a passive hydrophobic interaction between the hydrophobic surfaces of the fungal spore and the plant surface (Mercure *et al.*, 1994a; Mercure *et al.*, 1994b; Sela-Buurlage *et al.*, 1991; Young and Kauss, 1984). For example, *C. musae* conidia were shown to adhere as well to a polystyrene surface as to the host surface (Sela-Buurlage *et al.*, 1991). In addition to passive hydrophobic interactions, which provide rapid adhesion to the host, spore adhesion in *C. graminicola, C. musae,* and *C. lindemuthianum* also requires active metabolism, including protein synthesis (Mercure *et al.*, 1994a; Mercure *et al.*, 1994b; Sela-Buurlage *et al.*, 1991; Young and Kauss, 1984). This second phase of adhesion may serve to consolidate the initial hydrophobic attachment. Release of an extracellular matrix (ECM), which spreads outward from the spore as a thin film over the substratum or plant surface, has been demonstrated in some *Colletotrichum* species as shown in Fig 1.5 (Kuo, 1999; Mercure *et al.*, 1994a). Treatment of conidia with either cycloheximide (a protein synthesis inhibitor) or brefeldin (a glycoprotein synthesis and transport inhibitor) significantly reduced adhesion (Mercure *et al.*, 1994a; Mercure *et al.*, 1994b). A monoclonal antibody which recognises a *C. lindemuthianum* spore surface glycoprotein can also inhibit spore adhesion (Hughes *et al.*, 1999).

In the case of *M. grisea*, attachment of the spore to a plant surface results in the release of mucilage from the spore tip (Hamer *et al.*, 1988). This mucilage, which contains

carbohydrates, protein and lipid components, is released immediately upon hydration and may serve as an adhesive. Several components have been shown to play a role in this process. The gene *EMP1*, encoding a putative glycoprotein in extracellular matrix, was shown to mediate spore adhesion on hydrophobic surfaces. Appressorium formation was also affected in a deletion mutant (Ahn *et al.*, 2004). A class of abundant cell wall proteins called hydrophobins mediates adhesion by forming an amphipathic protein layer between the polysaccharides in the hyphal wall and the hydrophobic plant cuticle (Reviewed by Wessels, 1997). Mutants of *M. grisea* with a disruption in the hydrophobin-encoding gene *MPG1* showed strongly impaired ability to form appressoria and to cause lesion development on rice leaves (Beckerman and Ebbole, 1996; Talbot *et al.*, 1996). Recently, a second *M. grisea* hydrophobin gene *MHP1* was identified (Kim *et al.*, 2005). *mhp1* mutants exhibited pleiotropic effects on fungal morphogenesis, including reduction in conidiation, conidial germination, appressorium development and infectious growth in host cells. As yet, no hydrophobin like protein has been reported in *Colletotrichum* species.

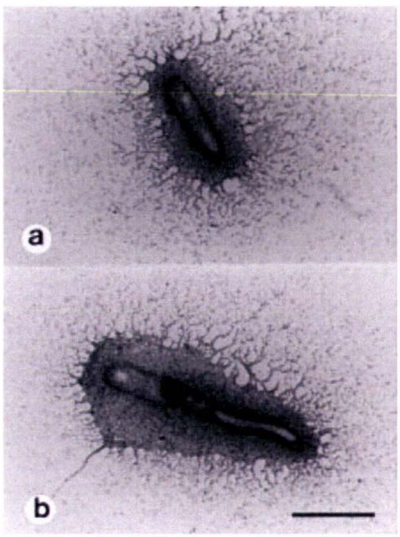


Figure 1.5 Extracellular matrix of a spore

The extracellular matrix of *C. gloeosporioides*. (a) An ungerminated conidium surrounded by the extracellular matrix it has secreted. (b) A germinated conidium. The extracellular matrix surrounds both the conidium and the germ tube. Bar= 10 μm. The image is taken from Kuo (1999).

1.3.2 Spore germination and appressorium formation

Following successful adhesion of a fungal spore to the host surface, a series of developmental events begin, which result in germ tube emergence, elongation and appressorium differentiation (Fig 1.6). Spore germination in most filamentous fungi requires the presence of low-molecular-mass nutrients such as sugars, amino acids, inorganic salts (Carlile, 1994) and other signals from the hosts. In *M. grisea* hydration alone is sufficient to induce spore germination (Tucker and Talbot, 2001). For *Blumeria graminis*, contact stimulation is the most important signal (Wright *et al.*, 2000). For *C. graminicola* conidia, both surface hydrophobicity and surface rigidity can induce germination, in the absence of nutrients. On the other hand, if a source of carbon is present, the conidia will germinate regardless of the surface characteristics (Chaky *et al.*, 2001). Although the stimuli may be diverse and specific to each species, the process of germination in all fungal spores requires reorganization of cell wall, mobilization of cell organelles and is accompanied with metabolic activities during germ tube extension (Tucker and Talbot, 2001).

The germ tube is a specialized structure, growing sometimes for only a very short distance (for example, 10-20 μm in *C. graminicola* and *B. graminis*) before differentiating into an appressorium (Mendgen *et al.*, 1996; Tucker and Talbot, 2001). The germ tube is the structure through which perception of the host surface occurs. If appropriate environmental signals are not perceived, the germ tube will continue to grow and will eventually arrest upon nutrient depletion. If appropriate physical and chemical signals are detected by the germ tube, then signal transduction pathways are triggered, resulting in appressorium formation (Mendgen *et al.*, 1996; Tucker and Talbot, 2001).

Environmental cues inducing germ-tube growth and appressorium formation are primarily physical and chemical factors derived from the host. Plant surfaces release a variety of chemical signals, a few of which have been characterised. Ethylene produced by ripening fruit (Kolattukudy *et al.*, 1995; Podila *et al.*, 1993), the fatty alcohol fraction of fruit wax (Kolattukudy *et al.*, 1995) and the cutin monomers from the plant cuticle

(Gilbert *et al.*, 1996) have been shown to induce germination and appressorium formation in *Colletotrichum* species and *M. grisea*. Specific physical signals and topography of the plant surface were also shown to have a role in signalling appressorium formation in *C. lindemuthianum* (Mercer *et al.*, 1975), *C. truncatum* (Staples *et al.*, 1976), *C. graminicola* (Lapp and Skoropad, 1978), and *C. lagenarium* (Suzuki *et al.*, 1982). A recent quantitative study showed that the *C. graminicola* germ tube required more than 4 μm of continuous contact with a hydrophobic substratum for induction of appressorium formation under low nutrient conditions (Apoga *et al.*, 2004).

Appressoria are usually differentiated from the tips of the germ tubes (Dean, 1997). Appressoria can also form from hyphae, either at the tips or along the hyphal elements. The tip swells to form a dome-shaped or multilobed appressorium. The young appressorium is lightly pigmented or hyaline. Appressoria often undergo a maturation process. For many fungi that directly penetrate the plant cuticle, the appressorium cell wall undergoes extensive modifications. Cell walls of appressoria produced by *Colletotrichum* species, *Magnaporthe grisea* and others become thicker, multilayered, and highly melanized, but where the appressorium is in contact with the plant interface the cell wall appears less modified and is thinner (Howard and Valent, 1996; Tucker and Talbot, 2001).

1.3.3 Appressorium development and penetration

Appressorium development and penetration is accompanied by a series of precisely regulated events, including nuclear division, reorganisation and melanisation of the cell wall, formation of a penetration hypha, secretion of extracellular enzymes and generation of physical force to bring about surface penetration.

1.3.3.1 Morphological development

Conidia of *Colletotrichum* species are usually elliptical cells which contain a single nucleus (Takano *et al.*, 2001b). During conidium germination, mitosis occurs before the formation of a septum. One nucleus moves into the germ tube where it undergoes a

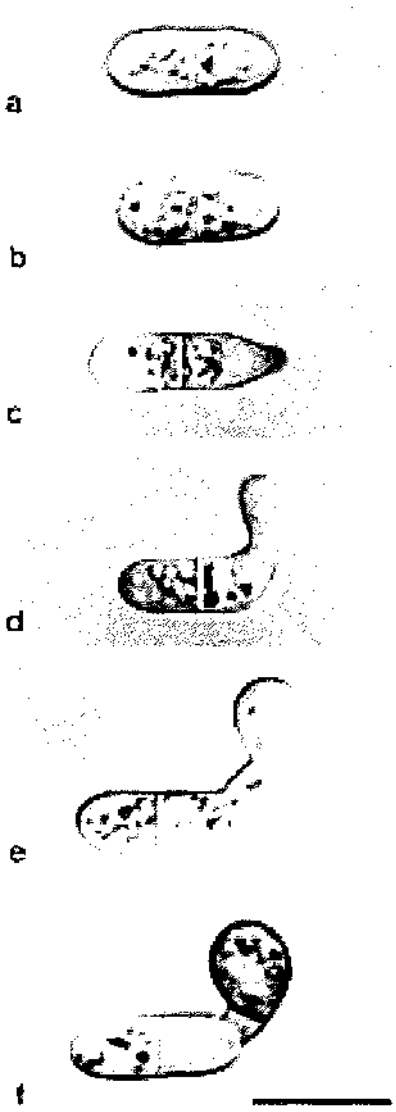


Figure 1.6 Process of spore germination and appressorium formation

Germination and appressorium formation in *Colletotrichum gloeosporioides*. (a) A conidium that contains many spherical bodies. (b) A septum forms in the center of the conidium and divides the conidium into two compartments. (c) A germling emerges from one compartment of the conidium. (d) The tip of the germ tube senses the differentiation signal and starts to swell. (e) The tip swelling continues to develop. (f) Tip swelling ceases and a melanized appressorium starts to form. Bar = 10 μm. The image is taken from Kuo (1999).

second mitosis and a second septum formed, so that only one single nucleus is in the cell that differentiates into an appressorium. A further mitosis results in a mature, binucleate appressorium. During infection one nucleus moves into the penetration peg, subsequent growth produces a multinucleate mycelium in the host.

In *M. grisea*, spores are three-celled. Under induction conditions, a nucleus migrates into the germ tube. One of the daughter nuclei migrates into the incipient appressorium after mitosis occurs. Another nucleus returns to the spore. Three nuclei undergo autophagic cell death and the spore collapses during appressorium formation (Veneault-Fourrey *et al.*, 2006).

Once formed, an appressorium adheres firmly to the plant surface. This ensures that the appressorium remains in contact with the host for penetration to occur. Adhesion has to be strong enough to withstand the invasive force applied by the fungus, which is demonstrated by the observation that fragments of the appressorial wall remain attached to the substrate after sonication (Pain *et al.*, 1996). The appressorium is usually covered by an extracellular matrix (ECM) similar to that covering the germ tube. In *C. lindemuthianum*, glycoproteins are found in this ECM and it has been suggested that they have a role in appressorium adhesion (Hutchison *et al.*, 2002; O'Connell *et al.*, 1996).

In *C. gloeosporioides*, appressorium ultrastructure was studied during the penetration of citrus fruit surface (Brown, 1977). The exposed upper surface of the appressorium is enclosed by double layered wall, which meets the slime layer at the juncture of the appressorium and the fruit cuticle. A narrow, threadlike infection hypha emerges through a pore in the lower portion of the appressorium. The appressorium wall surrounding the pore extends inward into the appressorium to form a funnel-shaped collar. The cone is formed upon the collar and extends from beyond the collar edge within the appressorium to the pore where it was continuous with the wall of the emerging infection hypha (Fig 1.7, panel B). During penetration of the cuticle, the infection hypha extends and enlarges to form larger hyphae which continue to grow throughout the peel and cause fruit decay. Similar appressorial ultrastructure and

infection hypha have been described in other *Colletotrichum* species, including *C. lindemuthianum* (Mercer *et al.*, 1975; O'Connell *et al.*, 1985), *C. lagenarium* (Xuei *et al.*, 1988) and *C. trifolii* (Mould *et al.*, 1991).

Appressorium structure of *M. grisea* is similar to that of *Colletotrichum*. There is no appressorial cone but the pore is surrounded by a ring of dense cell wall material that may act like an "O-ring" to help seal the appressorium to the plant surface (Fig 1.7, panel C and D). This enables pressure to be focused to the appressorial pore without detaching the appressorium from the plant surface (Deising *et al.*, 2000; Howard *et al.*, 1991).

1.3.3.2 Mechanism of appressorium penetration

The mechanism of penetration by appressorium of *Colletotrichum* species and *M. grisea* has been studied for many decades. For these fungi, that produce melanin-pigmented appressoria, the most important means of penetration seems to be direct penetration by physical force, that is by the turgor pressure generated in the appressorium. This force was measured in *C. graminicola* (Bechinger *et al.*, 1999), by using elastic optical waveguides. The force exerted by appressoria of *C. graminicola* was found to be about 17 micronewtons, this force is considered sufficient for breaching of most plant cuticles. In *M. grisea*, the turgor pressure in appressoria was measured using a cell collapse (cytorrhysis) assay. It was shown that appressoria can withstand pressures of up to 8 MPa (Howard *et al.*, 1991). This implies that within the appressorium there is a similarly high internal turgor pressure. Generation of turgor pressure in *M. grisea* is mediated by high concentrations of glycerol which draws water into the cell by osmosis to generate hydrostatic turgor. Glycerol concentrations in appressoria of *M. grisea* can be up to 3.2 M and the glycerol is retained in the cell by the presence of the melanin cell wall layer (Jong *et al.*, 1997).

Melanization is required for appressorial function in *M. grisea* or *Colletotrichum* species, although it is not required for appressorial development or adhesion (Bell and Wheeler, 1986). Melanization of the appressorial cell wall is essential for retaining the

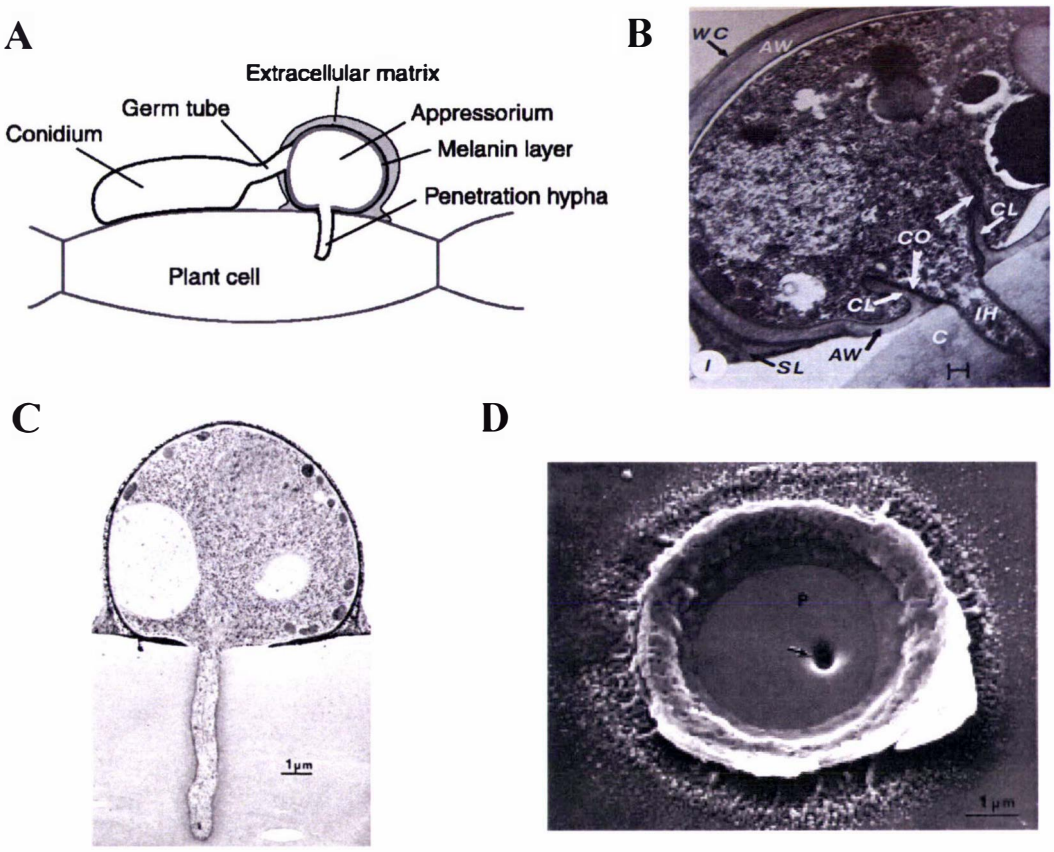


Figure 1.7 Appressorium ultrastructure

A: Schematic drawing of a cross section through a conidium, a germ tube, and an appressorium infecting a plant cell (not to scale). The drawing is taken from Berchinger (1999).

B: Cross-section of a *C. gloeosporioides* appressorium. WC= wall covering; AW= appressorial wall; SL= slime layer; CL= collar; CO= cone; C= cuticle; IH= infection hypha. The image is taken from Brown (1977).

C: An appressorium of *M. grisea* formed on cellophane membrane with the penetration peg invading the substratum (Deising *et al.*, 2000).

D: Remnant of an appressorium formed on Mylar after sonication has removed all but the lower, substrate-attached portions of the cell wall. A hole (*arrow*) left in the Mylar by a penetration peg can be seen within the appressorium pore (P). The image is taken from Howard *et al.* (1991).

glycerol. It has been estimated to reduce the porosity of appressorial cell walls of *M. grisea* from about 2 nm to less than 1 nm and is sufficient to block efflux of glycerol and other cytosolic solutes. Appressoria from mutants impaired in melanin biosynthesis allowed glycerol to pass freely across the cell wall (Howard *et al.*, 1991).

The process by which glycerol accumulates has not been studied in *Colletotrichum* species. In *S. cerevisiae*, glycerol accumulation is mediated by a MAP kinase, HOG1. Its homolog in *M. grisea* (OSM1) was found to be dispensable for appressorium turgor generation (Dixon *et al.*, 1999). Glycerol in *M. grisea* is mainly produced from the degradation of glycogen and lipid, and both lipid bodies and glycogen granules have been observed to mobilise to the appressorium. They are degraded before the onset of turgor generation. This is accompanied by the induction of triacylglycerol lipase activity (Thines *et al.*, 2000). Initial movement of lipid bodies and glycogen to the developing appressorium is regulated by the MAP kinase, PMK1. Mobilization does not occur in a *pmk1* mutant. On the other hand, the cAMP signal transduction pathway seems to control the degradation of lipids and glycogen, since this is significantly retarded in a c*PKA* mutant which lacks the catalytic subunit of cAMP-dependent protein kinase A (Thines *et al.*, 2000). The non-reducing disaccharide trehalose may also have a role in turgor generation (Foster *et al.*, 2003). Appressoria produced by a *TPS1* mutant (lacking the gene encoding trehalose-6-phosphate synthase) did not develop full turgor or elaborate penetration hyphae efficiently. It was suggested that trehalose is an additional means of turgor generation in appressorium.

1.3.3.3 The role of extracellular enzymes

The role of extracellular enzymes in appressorium penetration has been debated for a long time. Direct penetration by mechanical force alone may be possible for fungi with melanised appressoria, such as in *Colletotrichum* species and *M. grisea*, but for fungi that form small, non-melanized appressoria, secreted extracellular enzymes may be the major factor in appressorium penetration. Most likely, many fungi use a strategy combining both mechanical force and enzyme activity. Extracellular enzymes have been found to be secreted before and during appressorium development. Enzymes secreted

include cell wall degrading enzymes (CWDE) that may erode and soften the host surface to assist in penetration. Cuticular waxes on the plant surface are the first barrier encountered by fungal pathogens. Cutinases, that degrade cuticular materials, have been found in a number of species and have been suggested to be important for fungal pathogenicity and virulence in *C. gloeosporioides, C. lagenarium,* and *C. graminicola* (Bonnen and Hammerschmidt, 1989; Dickman *et al.*, 1982; Huang and Kuc, 1995; Pascholati *et al.*, 1993). Cutinase deficient mutants in *C. gloeosporioides* failed to infect intact pawpaw surfaces but produced normal lesions when the fruit surfaces were artificially wounded (Dickman and Patil, 1986). Furthermore, the cutin monomer released by cutinase activity contributed to the induction of appressorium formation (Kolattukudy *et al.*, 1995).

Another group of cell wall degrading enzymes is the pectin degrading enzymes. Pectinases are typically produced first, in the largest amounts, and are the only cell wall degrading enzymes (CWDE) capable of macerating plant tissue and killing plant cells on their own (Wijesundera *et al.*, 1989). The pectin matrix is found throughout the primary plant cell wall but is most concentrated in the middle lamella between cells (Carpita and Gibeaut, 1993). Fungi produce different types of pectinases that are classified by their substrates, type of lysis and mode of action on the pectin polymer. *C. gloeosporioides* has been found to produce endopolygalacturonase (Yakoby *et al.*, 2001a), pectin lyase A (*pnlA*) (Bowen *et al.*, 1995; Templeton *et al.*, 1994), and pectate lyase B (*pelB*) (Wattad *et al.*, 1997) during the colonization of infected tissue. Functional analysis by gene disruption often leads to no discernible phenotype, presumably because isoforms or several enzymes with similar functions are secreted by the fungus. For example, disruption of pectin lyase A in *C. gloeosporioides* did not reduce its virulence (Bowen *et al.*, 1995). However, disruption of pectate lyase B in *C. gloeosporioides* showed a reduced virulence (Yakoby *et al.*, 2001b). A reduction (36 to 45%) in the diameter of decaying tissue was observed. In addition, these *pelB* mutants induced a significantly higher host resistance to the fungus by an unknown mechanism.

1.3.4 Signal transduction during appressorium development

All eukaryotic cells, from yeasts to mammalian cells, respond to environmental cues and transduce these external signals to the interior of the cell where changes in gene expression are affected, resulting in altered patterns of protein expression. There are at least two conserved signal transduction pathways identified in yeasts and other filamentous fungi, that cooperate to regulate growth and development (Banuett, 1998; Kronstad *et al.*, 1998; Lengeler *et al.*, 2000). These are the cAMP-dependent protein kinase A (cAMP-PKA) signal transduction pathway and the mitogen activated protein kinase (MAPK) signal transduction pathway. Studies in the appressorium-forming fungi *Colletotrichum* species and *M. grisea* showed that these two signal transduction pathways govern appressorium development and function.

1.3.4.1 Sensing mechanism

Signals from environmental stimuli, whether they are chemical, physical, or otherwise, have to be transmitted through the cell membrane in order to trigger the downstream signal transduction cascades. Very little is known of this process for phytopathogenic fungi. An early study in *Uromyces appendiculatus* suggested that there is a mechanosensitive ion channel in the cell membrane. This channel could transduce the membrane stress induced by the leaf topography into an influx of a variety of ions, including Ca^{++}, that may trigger infection structure differentiation (Zhou *et al.*, 1991). Although it is not known whether this mechanosensitive ion channel is related to the calmodulin (CaM) signal transduction pathway, other studies demonstrated that the calmodulin signal transduction pathway is involved in appressorium formation. In *C. gloeosporioides,* CaM is induced by hard-surface contact and antagonists of both CaM and CaM kinase inhibited conidial germination and appressorium formation, implying that CaM is involved in this process (Kim *et al.*, 1998; Liu and Kolattukudy, 1999). This is supported with a similar study from *M. grisea* (Lee and Lee, 1998).

Transmission of the environmental stimulus is also mediated by other transmembrane proteins. A hard surface induced gene from *C. gloeosporioides*, *chip3*, was shown to be

a nine-transmembrane-domain-containing protein possibly involved in signal transduction. However, its function is uncertain because a *chip3* mutant showed no effect on appressorium formation or virulence. A transmembrane protein, encoded by the *PTH11* gene, has been identified in *M. grisea*, that appears to respond to the presence of hydrophobic surfaces, transmitting a cue for appressorium formation (DeZwaan *et al.*, 1999). Localization of the Pth11 protein to the cell membrane and vacuoles was demonstrated using a green fluorescent fusion protein. The *pth11* mutants failed to form appressoria efficiently on hydrophobic surfaces. This was due to the failure of the surface recognition step. Since the *pth11* mutant could be complemented by addition of exogenous cAMP, the most likely intermediate for transmission of a PTH11-perceived surface signal to the cAMP-PKA pathway is a GTP-binding protein. Another recently cloned gene, *CBP1* from *M. grisea,* encodes a putative extracellular chitin-binding protein (Kamakura *et al.*, 2002). It may also play an important role in hydrophobic surface recognition, since *CBP1* deletion mutants are defective in appressorium differentiation on hydrophobic surfaces and this can be bypassed by using a chemical inducer.

1.3.4.2 cAMP-PKA signal transduction patheway

Our current knowledge of cAMP signaling is based largely on work done with mammalian cells and *S. cerevisiae.* The cAMP signaling pathway is involved in nutrient sensing in *S. cerevisiae* and regulates pseudohyphal differentiation in response to nitrogen-limiting conditions (D'Souza and Heitman, 2001; Palecek *et al.*, 2002). In model filamentous fungi such as *Neurospora crassa* and *Aspergillus nidulans*, cAMP regulates hyphal growth, polarity and morphogenesis, conidiation, and spore germination (Bruno *et al.*, 1996; Fillinger *et al.*, 2002).

In a classical cAMP signaling pathway, a transmembrane cell surface receptor Gpr1 senses a specific extracellular signal that is transmitted into cells via heterotrimeric G-proteins composed of α, β and γ subunits. Binding of an inducing ligand to the receptor activates the G-proteins in a process that involves GDP-to-GTP exchange of the guanine nucleotide bound to the Gα subunit, followed by release of the Gα subunit (Gpa2) from

the Gβγ dimer (Gilman, 1987; Simon *et al.*, 1991). Effectors such as adenylyl cyclase can then be stimulated by either Gα or Gβγ depending on the system (Gilman, 1984; Sternweis, 1994), and the cellular cAMP level is dependent upon the relative activities of adenylyl cyclase and degradative enzymes (phosphodiesterases). The cAMP-dependent protein kinase (PKA) mediates the physiological effects of increased cAMP levels in fungi and other multicellular eukaryotes (Taylor *et al.*, 1990). PKA can either activate or inhibit transcriptional activators or repressors to achieve specific control of downstream events. Different outputs may result from cooperation with different partners.

A gene encoding a Gα subunit, *CTG-1*, was isolated and characterized in *C. trifolii* which causes alfalfa anthracnose (Truesdell *et al.*, 2000). This gene was present as a single copy in the genome. Transcripts of this gene accumulated on germination. Deletion of *CTG-1* decreased both growth rate and spore germination (only 5% spores germinated in comparison with 80% for the wild-type strain). About half of the germinated conidia developed appressoria. Germination frequency was not increased by exogenous addition of cAMP. When mutant conidia were inoculated on detached leaves, a few (<5%) germinated and formed appressoria, but these were capable of penetrating and causing lesions on intact alfalfa leaves.

In *M. grisea*, three G protein α subunit genes, *MAGA*, *MAGB*, and *MAGC*, have been cloned and characterized by gene disruption and site-directed mutagenesis (Fang and Dean, 2000; Liu and Dean, 1997). Disruption of *MAGB* significantly reduced vegetative growth, conidiation, appressorium formation and decreased infection and colonisation on either intact or wounded host tissue. Unlike the *ctg-1* mutant of *C. trifolii*, the defect in appressorium formation of *magB* mutant strains could be restored by the addition of cAMP or 1,16-hexadecanediol.

Measurement of PKA enzyme activity and cAMP levels showed that they were developmentally regulated in *C. trifolii*. Pharmacological agents that elevated endogenous cAMP levels induced appressorial differentiation on a non-inductive surface and specific inhibitors of PKA impaired it, suggesting a role for cAMP signal

transduction in appressorium formation in *C. trifolii* (Yang and Dickman, 1997). This work was followed by characterization of the genes encoding both the regulatory (Yang and Dickman, 1999b) and catalytic subunits (Yang and Dickman, 1999a) of PKA in *C. trifolii*. Based on hybridization analysis, both are single copy genes and both genes showed developmental regulation at the level of transcript accumulation. Insertional inactivation of *Ct-PKAC* (encoding the PKA catalytic subunit) resulted in negligible PKA activity, a small reduction in growth rate, and early conidiation. However, the total number of conidia produced was similar in both mutant and wild type. When conidia were inoculated onto intact host plants, conidium germination and appressorium formation were indistinguishable between mutant and wild type, but lesions were only observed on the host inoculated with wild type conidia, not with the mutant. However, characteristic lesions and acervuli production on the plant surface were found when the leaves were wounded prior to inoculation with either the wild-type strain or the mutants. This fact suggested that the loss of pathogenicity was due to a defect in appressorial penetration. The functions of the regulatory subunit gene of the *C. trifolii* PKA remain to be elucidated, although it was shown to complement the *Neurospora crassa mcb* mutant carrying a temperature-sensitive mutation in the gene encoding the regulatory subunit of PKA (Yang and Dickman, 1999b).

The *RPK1* gene encoding the PKA regulatory subunit has been isolated from *C. lagenarium*, the pathogen of cucumber anthracnose (Takano *et al.*, 2001a). The *rpk1* deletion mutant showed a severe reduction in growth rate and conidiation on nutrient agar, indicating that *RPK1* was required for these processes in *C. lagenarium*. PKA assays demonstrated that the *rpk1* mutants have much higher PKA activity than the wild type during vegetative growth. Again, the *rpk1* mutant could form appressoria which were not able to penetrate the host tissue and thus infection could only occur in wounded plants. In subsequent work, these investigators isolated genes of the PKA catalytic subunit *CPK1* and the adenylate cyclase *CAC1* (Yamauchi *et al.*, 2004). The *cpk1* and *cac1* gene deletion mutants showed a slight reduction in vegetative growth and conidiation, as well as a very low spore germination rate. Appressoria could still differentiate from germinated spores, however they also failed to penetrate host tissue. Cytological analysis indicated that the appressoria of the *cpk1* mutant contained larger

numbers of lipid bodies compared with the wild type, suggesting that cAMP-mediated regulation of lipid metabolism was important for appressorium function. Furthermore, both the *cpk1* and *cac1* genes were required for infectious growth *in planta*. The adenylate cyclase encoding gene *mac1* in *M. grisea* displayed similar function (Choi and Dean, 1997). Mutants in *mac1* showed a decrease in growth rate, conidiation, spore germination and appressorium formation. They were not able to infect intact host tissue and growth on wounded host tissue was also limited.

The gene encoding the catalytic subunit of the cAMP-dependent protein kinase (*CPKA*) in *M. grisea* was identified independently by two groups (Adachi and Hamer, 1998; Mitchell and Dean, 1995; Xu *et al.*, 1997). In contrast to the growth and sporulation phenotypes observed for G protein α subunit and adenylyl cyclase defects, mutants defective in PKA activity displayed normal vegetative growth, and unimpaired sexual and asexual development. Different results for appressorium formation and infectivity were observed by these two groups. However, the current consensus is that *cpkA* mutants are delayed in appressorium formation and have reduced pathogenicity toward healthy plants. Differences in the timing used in the assays of appressorium formation probably account for the different findings originally reported by these groups (Lee *et al.*, 2003). Appressoria of *cpkA* mutants were smaller than those of wild-type germlings and although they are fully melanized, the appressoria appear to be defective in plant penetration. *cpkA* mutants exhibited invasive growth *in planta* when inoculated onto wounded tissue. These results suggest that in *M. grisea* cAMP signaling plays a role in appressorial penetration, similar to that in *C. trifolii*.

In other pathogenic fungi, inactivation of the components of the cAMP signal transduction pathway also resulted in changes in mating, hyphal morphogenesis, sporulation, spore germination, infection structure formation and pathogenicity. Examples include the study of gene deletion mutants of the *Ustilago maydis* adenylyl cyclase gene (*UAC1*) (Barrett *et al.*, 1993) and G-protein α-subunit encoding gene (*GPA3*) (Regenfelder *et al.*, 1997) and PKA catalytic subunit encoding gene (*ADR1*) (Durrenberger *et al.*, 1998), the *Botrytis cinerea* G-protein α-subunit encoding gene

(*BCG1*) (Gronover *et al.*, 2001), the *Cryphonectria parasitica* (*CPG-1*) G-protein α-subunit encoding gene (Gao and Nuss, 1996).

1.3.4.3 MAP kinase pathway

MAPK signal transduction pathways generally contain three protein kinases that act in series: a MAP kinase kinase kinase (MAPKKK or MEKK), a MAP kinase kinase (MAPKK or MEK), and a MAP kinase (MAPK) (Marshall, 1994). Thus, when the cascade is activated, the MAPKKK phosphorylates the MAPKK, which in turn phosphorylates the MAPK. These MAPK cascades often regulate transcription factors by MAPK-mediated phosphorylation. Many extracellular and intracellular signals modulate transcription of specific genes through activation or inhibition of MAPK cascades. For example, *S. cerevisiae* contains five MAPKs belonging to five functionally distinct cascades (Gustin *et al.*, 1998).

Among the plant fungal pathogens, the MAPK signal transduction pathway has been best studied in *M. grisea*. Three MAP kinase genes, A MAPKK (*mst7*) and a MAPKKK (*mst11*) (Zhao *et al.*, 2005), *PMK1*, *MPS1* and *OSM1*, that are homologous to *FUS3/KSS1*, *SLT2*, and *HOG1*, respectively, in three of the *S. cerevisiae* MAPK signal transduction pathways, were isolated and characterised (Dixon *et al.*, 1999; Xu and Hamer, 1996; Xu *et al.*, 1998). All of them were shown to be single-copy genes and were dispensable for fungal growth. Their deletion mutants were all viable and had pleiotropic phenotypes.

The *mst7* and *mst11* deletion mutants have similar growth rate to that of wild type, but produced much less aerial hyphae and conidia. Conidia from these mutants failed to form appressorium on hydrophobic surfaces and failed to infect unwounded plants. Inoculation of the deletion mutant conidia on wounded plants only caused restricted necrotic lesions (Zhao *et al.*, 2005).

Expression analysis using a GFP-PMK1 fusion protein showed that pmk1 expression was increased in developing conidia and appressoria, suggesting a role in appressorium

formation (Bruno *et al.*, 2004). *M. grisea* mutants carrying a *pmk1* deletion produced normal mycelia and conidia. Conidia from these mutants could attach to and germinate on artificial surfaces as efficiently as wild type conidia. The germ tubes of these mutants still recognised hydrophobic surfaces and formed subapical swollen bodies, but failed to arrest germ tube tip growth or form appressoria on either Teflon membranes or rice plants. Therefore, the mutant was nonpathogenic. In addition, even when inoculated through wound sites, *pmk1* deletion mutants failed to grow invasively in rice plants (Xu and Hamer, 1996).

M. grisea mps1 deletion mutants displayed normal vegetative growth but the central part of *mps1* colonies underwent autolysis under normal incubation conditions, and mycelium from liquid cultures was hypersensitive to cell wall-degrading enzymes, indicating a weaker cell wall structure. The *mps1* mutants also showed a great reduction in conidiation and aerial hyphae development. Conidia could germinate and form melanized appressoria on Teflon membranes or hydrophilic surfaces. Appressoria formed by *mps1* mutants failed to penetrate the host surface, however, they were able to infect plant tissue that had been wounded and were still able to trigger plant-cell defence responses (Xu *et al.*, 1998).

In *M. grisea, OSM1* controlled the accumulation of arabitol in addition to glycerol in mycelia. Gene deletion mutants of *OSM1* grew at normal rates and produced normal conidia and hyphal cells but were sensitive to osmotic stresses. However, glycerol accumulation and turgor generation in appressoria were not affected in the *osm1* mutants. The *osm1* mutants still formed normal functional appressoria and exhibited unaltered virulence. *OSM1* may be critical for *M. grisea* to survive under field conditions because the *osm1* mutants have increased sensitivity to desiccation, UV light, and osmotic stresses (Dixon *et al.*, 1999).

Less is known about the MAPK pathways in *Colletotrichum* species. A MAPKK gene, *CgMEK1*, was cloned from *C. gloeosporioides* (Kim *et al.*, 2000a). Deletion of this gene resulted in conidia that failed to germinate and form appressorium due to a defect in polarized growth and septum formation. Host signals (e.g., ethylene) or addition of

nutrients could rescue conidial germination but not appressorium formation. The *C. lagenarium* homolog of the *Magnaporthe grisea PMK1* gene, *CMK1*, has been cloned and characterised (Takano *et al.*, 2000). In contrast to *M. grisea pmk1* mutants, conidia of *cmk1* mutants failed to germinate on both host plant and glass surfaces, but could be rescued by addition of yeast extract. Germinating conidia of *cmk1* mutants failed to form appressoria and the mutants were unable to grow invasively in the host plant. This phenotype resembled that of *CgMEK1* mutants, suggesting that the *CMK1* gene product is a MAP kinase that acts downstream of a MAPKK. Furthermore, three melanin genes showed no or reduced expression in the *cmk1* mutant when conidia failed to germinate, suggesting that *CMK1* has a role in gene expression required for appressorial melanization.

The *C. lagenarium MAF1* gene, a MAP kinase and homolog of the *S. cerevisiae MPK1* and *M.grisea MPS1* genes, has been isolated and functionally characterized (Kojima *et al.*, 2002). Deletion mutants of *MAF1* displayed normal vegetative growth but conidiation was reduced to 10% of wild type levels, as seen for the *M. grisea mps1* mutants (Xu *et al.*, 1998). In both mutants, infective growth only occured when conidia were inoculated onto wounded plants. However, the phenotype of the *MAF1* mutants was different from that of *M. grisea mps1* mutant in other respects. The *maf1* mutant failed to form appessoria and the cell wall was resistant to degradative enzymes. In *C. lagenarium*, *MAF1* seems to be required for the early phase of appressorium formation, whereas *CMK1* is involved in maturation of the appressorium.

Components of MAPK signal transduction pathways have also been isolated in other phytopathogenic fungi, including the *Ustilago maydis* MAPK and MAPKK encoding genes (*UBC4* and *UBC5*) (Andrews *et al.*, 2000; Smith *et al.*, 2004), the *Fusarium oxysporum* MAPK (*FMK1*) (Di Pietro *et al.*, 2001), the *Pyrenophora teres* MAPK (*PTK1*) (Ruiz-Roldan *et al.*, 2001), the *Claviceps purpurea* MAPK (*CPMK2*) (Mey *et al.*, 2002), and the *Fusarium graminearum* MAPK (*GPMK1*) (Jenczmionka *et al.*, 2003). These genes were also found to have a role in fungal morphology and pathogenicity. In another appressorium-forming fungus, *Cochliobolus heterostrophus*, the *CHK1* gene (homolog of the *M. grisea PMK1*) was also shown to have a role in appressorium

formation similar to that in *M. grisea* and *Colletotrichum* species (Lev and Horwitz, 2003).

Both MAPK and cAMP signal transduction pathways are involved in surface recognition, spore germination, appressorium formation and function, and infective growth. The homologous genes in these pathways have conserved functions and also display some different roles in different fungi, which may represent the different life and/or disease cycles of individual organisms. The genes involved in these two signal transduction pathways and the two target transcription factors (described in the next section) are summarised in Table 1.1 and Table 1.2 for *Colletotrichum* species and *M. grisea*. The functions of these two pathways could overlap each other and the crosstalk between the MAPK and cAMP signal transduction pathways may have a role in these processes as it does in *S. cerevisiae* and *C. albicans* (Mosch *et al.*, 1999). Although a direct interaction between components of these pathways has yet to be investigated, that cAMP is able to restore appressorium formation in the *M. grisea pmk1* mutant (Xu and Hamer 1996), suggests a possible connection between a MAPK cascade (represented by *PMK1*) and the cAMP pathway that influences infection structure formation.

1.3.5 Transcriptional regulation

Little is known about transcriptional regulation during the infection process of phytopathogenic fungi. Transcription factors that directly regulate gene expression are usually the downstream targets of protein kinases in the signal transduction pathways. Two transcription factors downstream of the MAP kinase pathway and their role in appressorium development have been studied in *M. grisea* and *C. lagenarium*. Transcription factors downstream of the cAMP protein kinase pathway have not been reported. However, a possible candidate for this role will be discussed. In addition, studies on the regulation of nitrogen metabolism have suggested that this pathway may be involved in the regulation of appressorium development.

Table 1.1 Summary of genes encoding components of the cAMP signal transduction pathway and the phenotypes of the corresponding deletion mutants.

Gene	Protein	Organism	Phenotype in gene disruption mutant	Reference
CTG-1	G-protein α subunit	*C. trifolii*	Slow growth, deficient in spore germination, appressorium formation and penetration	Truesdell *et al.*, 2000
MAGB	G-protein α subunit	*M. grisea*	Reduction in growth, conidiation, appressorium formation; autolysis of colony; deficient in invasive growth on either intact or wounded host; failed to form perithecia	Fang and Dean, 2000; Liu and Dean, 1997
CAC1	adenylate cyclase	*C. lagenarium*	slight reduction in growth and conidiation, very low spore germination rate	Yamauchi *et al.*, 2004
MAC1	adenylate cyclase	*M. grisea*	decrease in growth, conidiation, spore germination, appressorium formation and deficient in invasive growth	Choi and Dean, 1997
Ct-PKAC	PKA catalytic subunit	*C. trifolii*	deficient in growth, and appressorium penetration	Yang and Dickman, 1999b
CPK1	PKA catalytic subunit	*C. lagenarium*	slight reduction in growth and conidiation, very low spore germination rate, deficient in lipid metabolism and appressorium penetration	Yamauchi *et al.*, 2004
CPKA	PKA catalytic subunit	*M. grisea*	normal growth, unimpaired sexual and asexual development. deficient in appressorium penetration	Adachi and Hamer, 1998; Mitchell and Dean, 1995; Xu *et al.*, 1997
RPK1	PKA regulatory subunit	*C. lagenarium*	deficient in growth, conidiation and appressorium penetration	Takano *et al.*, 2001

Table 1.2 Summary of genes encoding components of the MAPK signal transduction pathway and the phenotypes of the corresponding deletion mutants.

Gene	Protein	Organism	Phenotype in gene disruption mutant	Reference
MST11	MAPKKK	*M. grisea*	Normal growth rate, reduction in aerial hyphae development and conidiation, fail to form appressoria, fail to infect host and deficient in invasive growth on host	Zhao *et al.*, 2005
MST7	MAPKK	*M. grisea*	Normal growth rate, reduction in aerial hyphae development and conidiation, fail to form appressoria, fail to infect host and deficient in invasive growth on host	Zhao *et al.*, 2005
CgMEK1	MAPKK	*C.gloeosporioides*	deficient in conidia germination and appressorium formation	Kim *et al.*, 2000
PMK1	MAPK	*M. grisea*	normal growth, conidiation, spore germination, deficient in lipid and glycogen mobilisation, appressorium formation and invasive growth,	Bruno *et al.*, 2004; Xu and Hamer, 1996
MPS1	MAPK	*M. grisea*	normal growth, reduction in conidiation and aerial hyphae development, autolysis of colony; normal germination and appressorium formation, deficient in appressorium penetration.	Xu *et al.*, 1998
OSM1	MAPK	*M. grisea*	Normal growth, spore germination, appressorium formation and function.	Dixon *et al.*, 1999
CMK1	MAPK	*C. lagenarium*	deficient in conidia germination, appressorium formation, appressorial melanization and invasive growth	Takano *et al.*, 2000
MAF1	MAPK	*C. lagenarium*	Decrease in conidiation, deficient in appressorium formation	Kojima *et al.*, 2002
MST12	Transcription factor	*M. grisea*	deficient in appressorium penetration, possibly due to the defective in microtubule reorganization; defective in invasive growth	Xu *et al.*, 1998
CST1	Transcription factor	*C. lagenarium*	deficient in appressorium penetration, possibly due to the low level of lipid droplets	Park *et al.*, 2004

1.3.5.1 Transcription factors linked to the MAP kinase pathway

In *M. grisea*, the transcription factor *MST12* (the homolog of *Ste12* in *S. cerevisiae*) was shown to function downstream of the MAP kinase *PMK1*, and its function was studied in gene deletion mutants (Park *et al.*, 2002). The mutants showed no difference in vegetative growth, conidiation, conidia germination or appressorium formation. Appressoria developed normal turgor pressure and ultrastructure. However, the appressoria formed by the *mst12* mutants failed to penetrate onion epidermal cells or other host surfaces. This was possibly due to a defect in microtubule reorganization associated with penetration peg formation (Park *et al.*, 2004). When inoculated through wounded sites, the *mst12* mutants also failed to cause spreading lesions and were defective in invasive growth. This indicated that other transcription factors must exist in *M. grisea* that function downstream of *PMK1* for appressorium formation.

In *C. lagenarium*, the gene *CST1*, downstream of the MAP kinase *CMK1* (the homolog of *MST12* in *M. grisea* and *Ste12* in *S. cerevisiae*) has been cloned and characterised by deletion of this gene (Tsuji *et al.*, 2003). Conidia of the *cst1* mutants could germinate and form melanized appressoria on both the host leaf surface and artificial cellulose membranes, but could not produce infectious hyphae, suggesting that *CST1* is essential for appressorium penetration. The *cst1* mutants were nonpathogenic on intact host leaves, but in contrast to the *M. grisea mst12* mutant, were able to form lesions when inoculated on to wounded leaves. In addition, mature appressoria of the *cst1* mutants contained an extremely low level of lipid droplets compared with the wild-type strain. Whether this was related to the generation of appressorial turgor pressure and appressorium penetration remains to be answered.

1.3.5.2 The APSES transcription factor family

A member of the APSES family may be the downstream target of the cAMP-PKA signal transduction pathway in *M. grisea* and *Glomerella cingulata.* This hypothesis is based on the conserved function of this gene family in filamentous fungi and the evidence that one member, the *Candida albicans EFG1* gene product, was the downstream target of the cAMP-PKA signal transduction pathway. The APSES family

consist of five transcription factors from different fungi. The name is derived from the names of the five genes encoding these proteins. The family consists of *PHD1* and *SOK2* from *Saccharomyces cerevisiae* (Gimeno and Fink, 1994; Pan and Heitman, 2000; Ward *et al.*, 1995), *EFG1* from *Candida albicans* (Stoldt *et al.*, 1997), *StuA* from *Aspergillus nidulans* (Miller *et al.*, 1991; Miller *et al.*, 1992), and *ASM1* from *Neurospora crassa* (Aramayo *et al.*, 1996). Two additional members have recently been added in this family. They are *StuA* from *Penicillium marneffei* (Borneman *et al.*, 2002) and *FoSTUA* from *Fusarium oxysporum* (Ohara and Tsuge, 2004). These proteins share a conserved bHLH region which is called the APSES domain (*Asm-1p, Phd1p, StuAp, Efg1p* and *Sok2p*). They are regulatory proteins with a role in fungal development.

In yeast the APSES proteins have been shown to regulate dimorphic growth. *S. cerevisiae* grows mitotically by budding as oval cells or as filamentous chains of elongated cells termed pseudohyphae. The switch from unicellular to filamentous growth is induced by nitrogen starvation (Gimeno and Fink, 1994). Regulation of this dimorphism involves MAPK and cAMP dependent pathways that function in parallel (Mosch *et al.*, 1999). The APSES protein encoding gene *PHD1* was identified in a screen for mutants that failed to display pseudohyphal growth. Subsequently, overexpression of *PHD1* was shown to induce pseudohyphal growth on rich medium and enhance pseudohyphal growth on nitrogen starvation medium (Gimeno and Fink, 1994). A second protein with significant homology to Phd1p in its putative DNA-binding region, Sok2p, was found to play an opposite role in pseudohyphal growth since *sok2/sok2* diploid strains form pseudohyphae at an accelerated rate (Ward *et al.*, 1995). Sok2p appears to act downstream of PKA but also in a separate, PKA-independent pathway (Pan and Heitman, 2000). Like *S. cerevisiae*, *C. albicans* can grow as either yeast or pseudohypha. Under certain conditions, it can also form true hyphae. A member of the APSES family, encoded by *EFG1*, plays a major role in regulating hyphal development in *C. albicans*. Reduced expression or deletion of *EFG1* results in rod-like, elongated cells. The *efg1/efg1* null mutant failed to produce true hyphae under all conditions tested (Stoldt *et al.*, 1997) and is avirulent (Lo *et al.*, 1997). Overexpression of *EFG1* in *C. albicans* leads to enhanced filamentous growth (Stoldt *et al.*, 1997). In the *efg1/efg1* null mutant, expression of the secreted aspartic proteinase genes *SAP1, SAP3, SAP4, SAP5* and *SAP6* is reduced or eliminated (Felk *et al.*, 2002;

Korting *et al.*, 2003; Staib *et al.*, 2002). Efg1p was shown to act downstream of the cAMP/PKA signaling pathway and to work in parallel with the *Cph1*-mediated MAP kinase pathway (Stoldt *et al.*, 1997). In addition, genome-scale gene expression profiles revealed that *EFG1* has a global role in gene expression. Efg1p regulates expression of a few hundred genes (Doedt *et al.*, 2004; Kim *et al.*, 2004a; Lane *et al.*, 2001). Notably, many genes encoding cell wall proteins were up- or down-regulated, consistent with a role for Efg1p in the transition from yeast to hyphal growth.

The Stunted protein (StuAp) from *A. nidulans* was the first protein characterised in the APSES family (Miller *et al.*, 1991). StuAp is required for correct cell pattern formation during asexual reproduction (conidiation) and for initiation of the sexual reproductive cycle in *A. nidulans*. The *stuA* null mutants form extremely shortened conidiophores that lack metulae and phialides and instead produce conidia directly from buds formed on the conidiophore vesicle. The *stuA* mutants lack intermediate cell types, the metulae and phialides. The *stuA* mutants are also sexually sterile, failing to form Hulle cells, cleistothecia, or ascospores (Wu and Miller, 1997). Characterisation of the *Asm-1* gene in *N. crassa* and the *StuA* from the human pathogen *P. marneffei* (Borneman *et al.*, 2002) suggested that these genes also have a similar role in mycelium morphology, asexual and sexual development.

F. oxysporum was the first plant pathogen in which a APSES gene (*FoSTUA*) was characterised (Ohara and Tsuge, 2004). *F. oxysporum* lacks sexual reproduction and produces three kinds of asexual spores, macroconidia, microconidia, and chlamydospores. *FoStuA* deletion mutants exhibited normal microconidium formation. However, the mutants lacked conidiophores and produced macroconidia at a low frequency only from intercalary phialides. Thus, *FoSTUA* appears to be necessary for conidiophore differentiation. In contrast, chlamydospore formation was dramatically promoted in the mutants. These data demonstrate that *FoStuA* is a positive regulator for the development of macroconidia and a negative regulator for the development of chlamydospores. It is dispensable for microconidium formation. The disease-causing ability of *F. oxysporum* was not affected by mutations in *FoSTUA*.

Taken together, all members of the APSES family function as regulatory genes during fungal development. Although it appears that functions of APSES proteins in the filamentous fungi *A. nidulans*, *Neurospora crassa, P. marneffei* and *F. oxysporum* are distinct from those in the yeasts *S. cerevisiae* and *C. albicans*, they do share a common role in regulating morphological transitions. In filamentous fungi, this transition is from the filamentous growth state of the vegetative hyphae and conidiophore stalk to the uninucleate, yeast-like morphology of the sterigmata and conidia. The elongated shape and polar budding pattern of metulae and phialides cells strongly resemble that of pseudohyphal cell division in *S. cerevisiae*. In the yeasts, the opposite occurs with the transition being from a unicellular mode of yeast growth to either a pseudohyphal or true hyphal form. (The exception is the transition from hyphal to unicellular chlamydospores in *C. albicans*). Each APSES protein also has additional roles that differ from species to species, possibly due to their coordination with different partners in each species.

Among the fungi in which a member of the APSES family has been identified, only one is a plant pathogen and it is not an appressorium-forming fungus. A hypothetical protein with high homology to the APSES proteins is found in a recently published sequence database for *M. grisea* (http://www.broad.mit.edu/annotation/fungi/ magnaporthe/), but nothing is known about its function. Although appressorium development is a distinct process and no counterpart can be identified in the fungi described above, all share some features in common. Firstly, they are developmental transitions involving morphological changes. Secondly, these morphological changes occur in response to environmental cues and at least one cue is common to them all, i.e. nitrogen starvation. Furthermore they all involve the reorganisation of the cell wall. In addition, this gene family may be the downstream target of the cAMP-PKA signal transduction pathway, although direct evidence has only been obtained for the *C. albicans EFG1* gene. As yet no transcription factors in this family have been studied in *Colletotrichum* species, *M. grisea* or other appressorium-forming fungi. Thus it would be of considerable interest to examine the role that an APSES protein might play in appressorium formation and function.

1.3.5.3 Transcriptional regulation of nitrogen metabolism

As already discussed, *Colletotrichum* species and other plant fungal pathogens develop special infection structures (e.g appressorium) for invasion of their plant hosts. This is mainly regulated by MAPK and cAMP signal transduction pathways. Gene expression profiles reveal a global change in cellular metabolism, which may require other regulatory pathways acting alongside these two for completion of the infection process. These additional pathways may be activated by the same environmental cues that activate appressorium formation, or by other environmental cues that have not yet been clearly demonstrated. One such environmental cue may be nitrogen starvation.

A relationship between nitrogen regulation and pathogenicity was demonstrated in the rice blast fungus *M. grisea*. A *M. grisea* homolog of the *AreA/NIT2* genes, *NUT1*, was cloned and its function studied in a deletion mutant (Froeliger and Carpenter, 1996). This gene was required for full expression of the hydrophobin gene *MPG1* in response to nitrogen starvation. The infection efficiency of *nut1* mutants on susceptible rice plants was similar to that of the parental strain. However, it was also suggested that in this mutant, nitrogen starvation and inhibition of fungal growth might occur sooner than in the wild type strains because *nut1* mutants failed to grow on a variety of nitrogen sources (Froeliger and Carpenter, 1996). Likewise, a mutant stain carrying an inactivated form of the *NRF1* gene, the *AreA/NIT2* orthologue in *C. fulvum*, is as virulent as wild-type strains (Perez-Garcia *et al.*, 2001). During an analysis of *MPG1* expression, two genetic loci, *NPR1* and *NPR2* that are unlinked to *NUT1*, were found (Lau and Hamer, 1996). These two genetic loci are required for *MPG1* expression during nitrogen limitation, and the expression of a number of genes whose products are involved in utilisation of secondary nitrogen sources. *Npr1* and *Npr2* mutants are severely defective in pathogenicity. *Npr2* mutants were also deficient in appressorium formation. Both the MAP kinase and cAMP signal transduction pathways were found to regulate *MPG1* expression (Soanes *et al.*, 2002). The *PMK1* gene, encoding a MAP kinase, was required for full expression of *MPG1* in response to nitrogen starvation. The *CPKA* gene, encoding the catalytic subunit of protein kinase A, was shown to be required for repression of *MPG1* during growth in rich medium. During appressorium formation, both the *CPKA* and the *NPR1* genes were required for high level *MPG1*

expression. In a gene expression study, by Northern blot analysis with a set of cDNA probes in *M. grisea* (Talbot *et al.*, 1997), it was found that gene expression patterns under nitrogen limitation were very similar to those obtained in the conditions encountered by the fungus *in planta*, particularly during symptom outbreak. In addition, a senescence-inducing activity was greatly induced in cultures starved for nitrogen, similar to the host response caused by the fungus itself. This was not observed in *nut1*, *npr1* or *npr2* mutants, and indicates a correlation between nitrogen regulation and the fungal-host interaction. Taken together, these results suggest that a nutritionally linked regulatory mechanism may be involved in the expression of *MPG1* and possibly additional genes critical for appressorium formation and pathogenesis.

Isolation of an *AreA*-like gene, *CLNR1*, was recently reported in *C. lindemuthianum* (Pellier *et al.*, 2003). In contrast to the *AreA/NIT2* deletion mutants in *A. nidulans* and *N. crassa*, deletion of the *clnr1* gene reduced growth of *C. lindemuthianum* on ammonia and glutamine, the preferred nitrogen sources for most fungi. This suggests that inactivation of the *clnr1* gene affects uptake of ammonia and glutamine in *C. lindemuthianum* as observed previously with the inactivation of the *AreA/NIT2* homologous gene from *Aspergillus oryzae* (Christensen *et al.*, 1998), *Aspergillus niger* (Lenouvel *et al.*, 2001) and *C. fulvum* (Perez-Garcia *et al.*, 2001). The *clnr1* mutants do not display any effect during the early phases of the infection process. The *clnr1* mutants are able to differentiate the infection structures required for the penetration stage (the appressorium), the biotrophic phase (the infection vesicle and primary hyphae) and progress through these first two steps without delay when compared with the wild-type strain. However, establishment of the necrotrophic phase was impaired in the *clnr1* mutants. The *clnr1* mutants were able to enter the necrotrophic phase, and they produced few secondary hyphae compared with the wild-type strain, leading mostly to abortive lesion development. Accordingly, the *clnr1* mutants are non-pathogenic in contrast to the *nut1* mutants in *M. grisea*. The authors suggested that the nitrogen source for germination and appressorium development could be stored in the conidia, whereas the nitrogen sources used for the development of infection vesicles and primary hyphae in the biotrophic phase might be the glutamine, glutamate, aspartate and asparagines present in the chlorophyll-containing organs of most leguminous plants. The *clnr1* mutants could utilise these amino acids as a nitrogen source. After the switch

to the necrotrophic phase, development of secondary hyphae needs a large nitrogen source which would require the expression of genes controlled by CLNR1. The inactivation of this gene thus results in a defect in necrotrophy and the loss of pathogenicity.

Proteolytic activity has been detected in sections of apple fruit rotted by *G. cingulata* and a secreted aspartic proteinase gene (*SAP*) has been isolated from the fungus (Clark *et al.*, 1997). The secretion of SAP was induced by exogenous protein and repressed by ammonium salts. This is consistent with the pattern of proteinase expression observed under these conditions. However, gene disruption analysis shows that *SAP* is dispensable for pathogenicity in this fungus, possibly because *G. cingulata* secretes a previously undetected peptidase (Plummer *et al.*, 2004). Interestingly, the *SAP* gene was shown to be expressed as two transcripts. A shorter transcript was present during the late stages (from 4 hours onwards) of appressorium formation, whereas the longer transcript was thought to only be expressed during the early stages of appressorium formation. The shorter transcript was also detected in mycelium incubated in the absence of a nitrogen source. This suggested that the transcription factor(s) that regulate the *SAP* gene long transcript may also regulate other genes critical for appressorium formation. On the other hand, genes like *avr9* in *C. fulvum* and *MPG1* in *M. grisea* are not involved in nitrogen metabolism but regulated by *AreA*-like transcription factors. Furthermore, *MPG1* is regulated not only by *AreA*-like transcription factors but coordinatedly with other transcription factors. Bioinformatic analysis of the *SAP* promoter region revealed the presence of motifs for binding of the *AreA* and APSES family of transcription factors. This may result from an evolutionary adaption that allows fungi to coordinate several different pathways and functions to maximise their ability to invade their host.

1.3.6 Gene expression during appressorium formation

In *C. gloeosporioides*, genes specifically expressed during appressorium formation have been studied by differential screening of a cDNA library prepared from the RNA of early stage appressoria. The first set of genes reported was *cap3*, *cap5*, *cap20*, and *cap22* (Hwang and Kolattukudy, 1995). CAP20 and CAP22 proteins have no homology

to any known protein. However, the *cap20* deletion mutant showed a drastically decreased virulence on avocado and tomato fruits (Hwang *et al.*, 1995). The *cap3* and *cap5* genes showed homology to metallothionein encoding genes but their function has not yet been determined. The second set of genes identified as expressed during appressorium formation included *chip1, chip2, chip3* and *chip6*. These were identified using differential display (Liu and Kolattukudy, 1998). The *chip1* gene encodes a ubiquitin-conjugating enzyme whose expression is induced by hard-surface contact or by ethylene treatment. This gene can complement the proteolysis deficiency of the *S. cerevisiae ubc4 ubc5* mutant, indicating that ubiquitin-dependent protein degradation is involved in conidial germination and appressorial differentiation (Liu and Kolattukudy, 1998). The *chip2* and *chip3* gene products have no homology to any known protein. The *chip2* gene product is predicted to contain a nuclear localization signal, a leucine zipper motif, and a heptad repeat region which might dimerize into a coiled-coil structure. The *chip3* gene product is predicted to be a nine-transmembrane-domain-containing protein, possibly involved in signal transduction. However, disruption of these genes had no effect on appressorium formation or virulence. This suggested that either *C. gloeosporioides* might have genes functionally redundant to *chip2* and *chip3* or that these genes control processes not directly involved in pathogenesis (Kim *et al.*, 2000b). The *chip6* gene encodes a protein with homology to sterol glycosyl transferases. The deletion mutant of this gene can still form appressoria, but showed a reduction in virulence on the host accompanied by decreased transferase activity (Kim *et al.*, 2002). However, the requirement for a sterol glycosyl transferase and its function in either appressorium formation or pathogenicity is not clear.

The *MPG1* gene is known to be expressed during appressorium formation in *M. grisea* (Talbot *et al.*, 1996). A few other genes expressed during appressorium formation have also been identified. One of them is *PLS1*, which encodes a transmembrane protein expressed only in appressoria, as determined by GFP-tagging assays (Clergeot *et al.*, 2001). The *pls1* deletion mutant failed to form penetration pegs and was not pathogenic on rice. By screening a subtraction library enriched for genes regulated by *PMK1*, two additional genes were identified. These genes, *GAS1* and *GAS2*, encode small proteins with unknown homology (Xue *et al.*, 2002). Both *GAS1* and *GAS2* are dispensable for

mycelial growth, conidiation, and sexual reproduction, but they are required for appressorial penetration and lesion development.

Melanization is required for appressorial function in *M. grisea* or *Colletotrichum* species (Bell and Wheeler, 1986). Melanin deficient mutants are avirulent in both these species (Tsuji *et al.*, 2000). In fungi several melanin biosynthesis pathways are known. The best studied is the DHN (dihydroxynaphthalene) pathway. The DHN melanin synthesis pathway begins with the head-to-tail conjoining catalysed by polyketide synthase, of five ketide subunits obtained from five acetate precursor molecules. The polyketide undergoes cyclization which is also catalyzed by polyketide synthase (Takano *et al.*, 1995). This produces 1,3,6,8-tetrahydroxynapthalene (T4HN) which is reduced to produce scytalone. From scytalone, there are two dehydration steps and one reduction step, i.e., dehydration of scytalone to 1,3,8-trihydroxynaphthalene (T3HN), reduction of 1,3,8-THN to vermelone, and dehydration of vermelone to 1,8-dihydroxynaphthalene. The 1,8-dihydroxynaphthalene is then polymerized and oxidized to yield melanin. Three key structural genes involved in melanin biosynthesis in *C. lagenarium* have been cloned. The first of these, *PKS1*, encodes a polyketide synthase (Takano *et al.*, 1995). The *THR1* gene encodes T3HN reductase (Perpetua *et al.*, 1996) and the *SCD1* gene encodes scytalone dehydratase (Kubo *et al.*, 1996). During appressorium differentiation, transcripts of these three melanin biosynthesis genes accumulated 1 to 2 h after conidia were placed on an inductive surface. The level of transcript began to decrease after 6 hours (Takano *et al.*, 1997). The *SCD1* and *THR1* genes have also been studied in *M. grisea* (Motoyama *et al.*, 1998; Thompson *et al.*, 1997). Transcription factors Cmr1p in *C. lagenarium* and Pig1p in *M. grisea*, that regulate the expression of these melanin synthesis genes during mycelium melanization but not in appressorium development, have recently been identified (Tsuji *et al.*, 2000).

Many investigators have used large scale analysis methods to identify the genes that are expressed at the different stages of infection or by different cell types in *Colletotrichum* species and *M. grisea*. Examples of these techniques include large scale sequencing of expressed sequence tags (ESTs) (Inagaki *et al.*, 2000; Jantasuriyarat *et al.*, 2005), yeast two-hybrid system (Kulkarni and Dean, 2004), screening a subtractive suppressive library (Lu *et al.*, 2005), Serial Analysis of Gene Expression (SAGE) (Irie *et al.*, 2003)

and proteome analysis (Kim *et al.*, 2004b). The published genome sequence of *M. grisea* allowed an analysis of gene expression using microarray technology (Takano *et al.*, 2003). These studies showed that appressorium formation is a complex process that involves the expression of thousands of genes. Although a large amount of gene sequence information has been generated, the functional analysis on each gene will be more informative to our understanding of appressorium formation.

1.3.7 Colonisation

The early stages of fungal development on the plant surface are essentially the same for all *Colletotrichum* species. Major differences only become apparent after host invasion. Fungi employ different strategies to get access to host nutrients at this stage. *Colletotrichum* species utilise two main infection strategies: intracellular colonization or subcuticular intramural colonization.

Following penetration, subcuticular intramural pathogens do not immediately enter into the cell lumen, but initially grow between the cell walls of host epidermal cells. Intramural development is associated with extensive swelling and dissolution of plant cell walls. Subsequently, these fungi proliferate rapidly through the tissue both intra-and intercellularly, killing plant cells and dissolving cell walls ahead of the infection. There is no detectable biotrophic stage in these interactions. Examples of subcuticular intramural pathogens include *C. capsici* on cowpea (Pring *et al.*, 1995) and cotton (Roberts and Snow, 1984; Roberts and Snow, 1990), *C. gloeosporioides* on papaya and Stylosanthes species (Irwin *et al.*, 1984), and *C. circinans* on onion (Perfect *et al.*, 1999).

Other *Colletotrichum* species exhibit the second type of infection strategy, i.e., intracellular colonization. They initially establish infection through a brief biotrophic phase, associated with large intracellular primary hyphae. The length of the biotrophic stage is variable and is absent in many interactions. The biotrophic phase is characterised by the following properties: (1) highly developed infection structures; (2) limited secretory activity, especially of lytic enzymes; (3) carbohydrate-rich and protein-containing interfacial layers that separate fungal and plant plasma membranes; (4) long-term suppression of the host defense (Mendgen and Hahn, 2002). Later

intracellular colonization pathogens switch to a destructive, necrotrophic phase, associated with narrower secondary hyphae which spread throughout the plant tissue. Those fungi which employ such a strategy are considered to be hemibiotrophic or facultative biotrophs. This transient biotrophic phase is significant for colonization establishment because the host plant does not appear to recognize the pathogen and there is no specific resistance response. Examples where distinct biotrophic stages are well-characterized include the interactions between *C. lindemuthianum* and bean (O'Connell *et al.*, 1985; O'Connell *et al.*, 1996), *C. truncatum* and pea (O'Connell *et al.*, 1993), *C. destructivum* and cowpea (Latunde-Dada, 2001) and *C. sublineolum* and sorghum (Wharton and Julian, 1996). *C. gloeosporioides* follows both strategies, depending on the host plant (Perfect and Green, 2001). In addition, several *Colletotrichum* species are known to form long-term quiescent infections (Beno-Moualem and Prusky, 2000).

The best studied example of intracellular colonization is the infection of *C. lindemuthianum* on bean (O'Connell, 1991; O'Connell *et al.*, 1985; O'Connell *et al.*, 1996; Perfect and Green, 2001). After an infection hypha has penetrated the cuticle and epidermal cell wall, it expands within the epidermal cell lumen to form a globose infection vesicle, from which intracellular infection hyphae develop. The intracellular infection vesicle and the primary hyphae colonize only a few host cells. Both are surrounded by a matrix that separates the fungal cell wall from the invaginated host plasma membrane. This separation allows fungal colonization either without triggering, or by suppressing, host defence responses such as hypersensitive cell death and callose deposition. The matrix is extracytoplasmic and connected to the plant apoplast. Within the interfacial matrix, a fungal glycoprotein, encoded by *CIH1*, was identified (Perfect *et al.*, 1998). The protein was shown to be present uniquely at this interface in the biotrophic stage of hemibiotrophic *Colletotrichum* species. Its expression was switched off at the onset of necrotrophic development. The host cell remains alive during this phase which can vary from less than 24 hours to over 3 days, but then the cytoplasm gradually degenerates and the cell dies, indicating the completion of the biotrophic phase and the beginning of the necrotrophic phase. As the lesion spreads, narrower secondary hyphae grow within the cell wall and death of host cells and wall dissolution

occurs rapidly in the region ahead of the advancing fungal colony. This is accompanied with the secretion of cell-wall-macerating enzymes (Perfect *et al.*, 2000).

In recent years, some studies have been conducted to understand the molecular mechanism of the switch and development of biotrophic and necrotrophic phases. In *C. gloeosporioides*, screening for nitrogen-starvation-induced genes resulted in the cloning of *CgDN3* (Stephenson *et al.*, 2000). *CgDN3* is specifically expressed during the early biotrophic growth phase and might be required to maintain the initial stage of intracellular development. A mutant disrupted in this gene provoked a hypersensitive-like response on intact leaves of the host plant, *Stylosanthes guianensis*. In *C. lindemuthianum*, a nonpathogenic mutant generated by random mutagenesis was able to induce necrotic spots similar to those observed during the hypersensitive reaction (Dufresne *et al.*, 2000). Cytological observations showed that it could produce normal primary hyphae, but no secondary hyphae were observed. It was suggested that the mutant was blocked in the switch from biotrophic to necrotrophic growth. It was shown that the gene *CLTA1*, which appears to be a GAL4-like transcriptional activator belonging to the zinc cluster family, is responsible for this phenotype. But how it functions requires further study. Another gene *CLNR1*, the *AreA*-like transcription factor, showed a similar phenotype in the development of necrotrophic growth (Pellier *et al.*, 2003). It has been clearly shown that the *clnr1* mutant failed to use the host nitrogen sources for fungal development. In *C. graminicola*, REMI mutagenesis (restriction enzyme mediated integration mutagenesis) identified an insertion mutant showing a reduction in transcript levels of a gene (*CPR1*) encoding a putative signal peptidase (Thon *et al.*, 2002). This mutant was defective in pathogenesis. Cytological studies indicated that the mutant continued to form primary hyphae to a limited extent but was unable to switch to necrotrophic growth. It was speculated that the *cpr1* mutant may be unable to secrete sufficient quantities of degradative enzymes to support that transition.

Necrotrophy is clearly linked to the increased expression of plant cell wall degrading enzymes such as endo-polygalacturonases (endo-PG) and pectin lyases. The secretion of these enzymes by *C. lindemuthianum* both in culture and during pathogenesis has been investigated by several groups. Wijesundera *et al.* (1984, 1989) showed that endo-PG,

two forms of pectin lyase, galactopyranosidase, arabinofuranosidase, and a protease are secreted into culture medium containing polypectate or bean cell walls. Pectin lyase activity was first observable 4 days after inoculation of beans with *C. lindemuthianum*, rising to maximum activity at 7 days, after which activity declined (Wijesundera *et al.*, 1989). Thus, the expression of pectin lyase activity correlated well with the onset of necrotrophy and the subsequent development of lesions.

1.4 Aim of this study

When this project began, no transcription factor involved in appressorium formation and function had been identified in any appressorium forming-fungus. The aim of this study was to identify a transcription factor(s) that is involved in appressorium formation or function.

The first strategy adopted was to characterise the *G. cingulata SAP* promoter with the intention of using the information obtained in the yeast one-hybrid system for isolation of the transcription factors involved in the *G. cingulata SAP* expression. The alternative strategy was to use a candidate gene approach. Two genes were of interest. One was an *AREA*-like gene, because it is required for regulation of nitrogen metabolism and possibly also for the *G. cingulata SAP* gene expression and appressorium differentiation. However, the *AREA*-like gene *CLNR1* in *C. lindemuthianum* was shown by others (Pellier *et al.*, 2003) to be dispensible for appressorium formation and function. The second candidate gene was a member of the APSES family of transcription factors. The reasons for choosing this gene have been given in section 1.3.5.2. In fungi, members of the APSES family are involved in regulation of morphological changes in response to environmental cues. Furthermore, the *C. albicans* APSES transcription factor is the downstream target of the cAMP-PKA signal transduction pathway. No transcription factors in this family have been studied in *Colletotrichum* species, *M. grisea* or other appressorium-forming fungi, although the cAMP pathway is known to be involved in signalling of appressorium formation and function. The candidate gene selected was, therefore, a member of the *G. cingulata* APSES family. Its role in appressorium formation and function was determined.

Chapter two

Materials and methods

2.1 Suppliers

2.1.1 Enzymes

Restriction endonucleases and DNA modifying enzymes were obtained from the following companies: Invitrogen Life Technologies Inc., MD, USA; Promega Corporation, WI, USA; Roche Molecular Biochemicals, Germany; Stratagene, La Jolla, CA, USA; New England Biolabs Inc., MA, USA. *Taq* DNA polymerase was purchased from Roche Molecular Biochemicals, Germany; Glucanex was purchased from Novozymes, Denmark.

2.1.2 Vectors

The cloning vector pGEM-T Easy was purchased from Promega Corporation, Madison, USA and pBlueScriptII KS (+) from Stratagene, La Jolla, CA, USA. The plasmid pBC-phleo was obtained from Dr Rosie E. Bradshaw, Massey University.

2.1.3 Laboratory chemicals

Oligonucleotides were manufactured by Sigma Chemical Company, St. Louis, MO, USA or Invitrogen Life Technologies Inc., MD, USA. Radioisotopes were purchased from Amersham Pharmacia Biotech (UK). Ready-To-Go DNA Labelling Beads and MicroSpin^{TM} G-50 Columns were purchased from Amersham Pharmacia Biotech (UK). X-ray film was obtained from Eastman Kodak, NY, USA. Photographic developer and fixer were purchased from Eastman Kodak, NY, USA. Hygromycin B was supplied by Roche Molecular Biochemicals, Germany. Phleomycin was purchased from Apollo Scientific Ltd, UK. TRI Reagent was from MRCgene company, USA. QIAEX II Gel Extraction Kit and QIAquick PCR Purification Kit were from QIAGEN Pty Ltd, Australia. General laboratory chemicals were purchased from Sigma Chemical Company (WI, USA), Merck & Co., Inc (NJ, USA), Amresco (USA) or BDH (WI, USA).

2.1.4 Buffers and Solutions

Buffers and solutions are listed in Appendix 1.

2.2 Fungal and bacterial strains, plasmids

Fungal and bacterial strains, and plasmids used in this study are listed in Tables 2.1 and 2.2 respectively.

Table 2.1 Fungal and bacterial strains used in this study

Strains	Relevant Characteristics	Reference
Fungi		
WT	*Glomerella cingulata* ICMP 11061	(Bowen *et al.*, 1995; Rikkerink *et al.*, 1994)
BHG1	p(*gpd*)*GFP;HPH*, reporter transformant	This study
BHG2	p(*gpd*)*GFP;HPH*, reporter transformant	This study
BHG3	p(*gpd*)*GFP;HPH*, reporter transformant	This study
BHG4	p(*gpd*)*GFP;HPH*, reporter transformant	This study
BHG5	p(*gpd*)*GFP;HPH*, reporter transformant	This study
BHG6	p(*gpd*)*GFP;HPH*, reporter transformant	This study
BHG7	p(*gpd*)*GFP;HPH*, reporter transformant	This study
BHG8	p(*gpd*)*GFP;HPH*, reporter transformant	This study
BHS551	p(*sap*)*GFP;HPH*, reporter transformant	This study
BHS552	p(*sap*)*GFP;HPH*, reporter transformant	This study
BHS553	p(*sap*)*GFP;HPH*, reporter transformant	This study
BHS554	p(*sap*)*GFP;HPH*, reporter transformant	This study
SC1	*stuA::hph*, disruption mutant	This study
SC2	*stuA::hph*, disruption mutant	This study
SC3	*stuA::hph*, disruption mutant	This study
SC4	*stuA::hph*, disruption mutant	This study
SC5	*stuA::hph*, disruption mutant	This study
SC6	*stuA::hph*, disruption mutant	This study

SC7	*stuA::hph,* disruption mutant	This study
SC8	*stuA::hph*, disruption mutant	This study
SC9	*stuA::hph,* disruption mutant	This study
SC10	*stuA::hph,* disruption mutant	This study
SC11	*stuA::hph,* disruption mutant	This study
SC12	*stuA::hph*, disruption mutant	This study
SC13	*stuA::hph,* disruption mutant	This study
SC14	*stuA::hph,* disruption mutant	This study
SC15	*stuA::hph,* disruption mutant	This study
SC16	*stuA::hph*, disruption mutant	This study
SC17	*stuA::hph,* disruption mutant	This study
SC18	*stuA::hph,* disruption mutant	This study
SC19	*stuA::hph,* disruption mutant	This study
SC31	*stuA::hph,* disruption mutant	This study
SC40	*stuA::hph,* disruption mutant	This study
DC42	*ΔstuA::hph*, deletion mutant	This study
DC43	*ΔstuA::hph,* deletion mutant	This study
Complementation mutants		
R532-2	*ΔstuA::(STUA,BLE^R,HPH)*	This study
R532-9	*ΔstuA::(STUA,BLE^R,HPH)*	This study
R532-10	*ΔstuA::(STUA,BLE^R,HPH)*	This study
R532-11	*ΔstuA::(STUA,BLE^R,HPH)*	This study
R532-4	*ΔstuA::(STUA,BLE^R,HPH)*	This study
R532-6	*ΔstuA::(STUA,BLE^R,HPH)*	This study
R532-8	*ΔstuA::(STUA,BLE^R,HPH)*	This study
NR5	*ΔstuA::STUA,BLE^R*	This study
NR13	*ΔstuA::STUA,BLE^R*	This study
Escherichia coli		
DH5α	*end*A1 *hsd*R17 *suppE*44ΔlacU169 *thi*-1 *rec*A1 *gyr*A96 *rel*A1(80*lac*ZΔM15)	Invitrogen Corp.

Table 2.2 Plasmids used in this study

Strains	Relevant Characteristics	Reference
pBlueScript (pBS)	Cloning vector,	Stratagene Corp
pGEM-T Easy	Cloning vector,	Promega Corp
pAN7-1	Transformation vector	(Punt *et al.*, 1987)
pBC-phleo	Transformation vector	(Silar, P., 1995)
pFAT-3	Binary cloning vector	(Fitzgerald *et al.*, 2003)
pFAT-3gfp	GFP reporter construct	(Fitzgerald *et al.*, 2003)
pBH5	HPH cassette in pBS	This study
pFSG	GFP reporter construct in pFAT-3	This study
pBHG	GFP reporter construct in pBS	This study
pBHS	GFP reporter construct in pBS	This study
pGT595	StuA595 fragment in pGEM-T Easy	This study
pXho41	5' region of *StuA* in pBS	This study
pAN595	Disruption vector	This study
pERV3	Plasmid from plasmid rescue	This study
pHE40	3' region of *StuA* in pBS	This study
pXHE	The entire *StuA* sequence in pBS	This study
pSXH	Deletion vector	This study
pBPS53	Complementation vector	This study
pXHE-phleo-R	Complementation vector	This study

2.3 Growth and maintenance of organisms

2.3.1 Growth and maintenance of *Escherichia coli*

Escherichia coli strains were grown at 37^0C overnight (~16 hours) on Luria-Bertini (LB) agar plates or in LB broth (Appendix 1.1) with shaking at 200 rpm. The medium was supplemented with antibiotics as appropriate. For long-term storage, cultures were stored at -80^0C in 15% (v/v) glycerol.

2.3.2 Growth and maintenance of *G. cingulata*

Unless otherwise specified, strains of *G. cingulata* and its derivatives were routinely grown on potato dextrose agar (PDA) plates (Appendix 1.1) for one or more weeks or in potato dextrose broth (PDB, Appendix 1.1) at 25^0C with shaking at 150 rpm for 3-5 days depending on the growth rate and size of the inoculum. Mycelium for DNA and RNA extraction, or for inoculating liquid medium, was grown on PDA plates overlaid with a cellophane membrane (Waugh Rubber Bands, Wellington). Sub-culturing on PDA plates was performed by transferring a small piece of mycelium cut from the edge of a colony to a fresh plate. For sub-culturing into liquid medium, mycelia, collected by scraping the colony using a scalpel, was ground to a fine suspension in 1-2 ml of PDB and transferred to PDB (50-100 ml). Inocula from silica gel stocks were prepared by vortexing a few beads of the silica gel in 0.5-1.0 ml of MQ water. Aliquots of 50 µl were used to inoculate plates or liquid medium (50-100 ml). When fresh conidia were used to inoculate liquid medium, the final concentration was adjusted to no more than 10^6/ml. The concentration and volume was not critical for inoculating fresh conidia to PDA plates. For mycelium grown in SAP induction medium or pectin lyase A induction medium (Appendix 1.1), mycelium was first grown in minimal medium (Appendix 1.1) or PDB for 3-5 days, harvested by filtration through 8 layers of nappy liner (Arico Wipeese, New Zealand), washed with MQ water and transferred into the medium for subsequent growth.

For short to medium-term storage (< 3 months), cultures were maintained on PDA plates at 4^0C. For long-term storage, fungal conidia were kept at -80^0C in 20% (v/v) glycerol. For medium term storage (3 months to 1 year), conidia were maintained at 4^0C on silica gel. To prepare silica gel stocks, conidia were harvested and suspended in 2 ml of a 20% (w/v) skim milk powder solution, which had been previously sterilised by autoclaving at 10 psi for 10 min. This suspension of conidia was transferred to a universal bottle one third full with silica gel beads (3 mm beads, no indicator) which had been sterilised in a 160^0C oven for 2 hours. The beads were dried in a dessicator for 1-3 days and then stored at 4^0C (Clark, 1998).

2.4 DNA isolation, purification and quantification

2.4.1 Isolation of *G. cingulata* DNA

G. cingulata genomic DNA was isolated using the method described by Al-Samarrai and Schmid (2000). Freeze-dried mycelia (10 to 30 mg) were ground to a fine powder in liquid nitrogen with a sterile plastic pestle in a microcentrifuge tube, and re-suspended in 500 µl of freshly prepared cell lysis buffer (40 mM Tris-acetate; 20 mM sodium acetate; 1 mM EDTA and 1% (w/v) SDS; pH 7.8). The suspension was mixed vigorously by pipetting about 50 times with a Gilson P1000 pipetman until lots of froth formed. Then 165 µl of 5 M NaCl solution was added, and the solution was mixed, by briefly vortexing it and centrifuged at 18,000 g for 10 min to precipitate polysaccharides, proteins and cellular debris. The supernatant was recovered and extracted with phenol/chloroform (Section 2.4.5). The DNA in the aqueous phase was precipitated with 1 volume of isopropanol and centrifuged at 18,000 g for 10 min. The DNA pellet was washed three times with 70% ethanol, air dried then re-suspended in 50 µl 1x TE buffer. The DNA obtained by this small scale preparation was used for PCR analysis.

The DNA used for Southern blot analysis was prepared on a larger scale. The fungal material and buffer volumes were scaled up ten fold. Centrifugation was performed in an Eppendorf Centrifuge 5810 with an A-4-62 rotor at 4,000 rpm (3,220 g). In addition, the DNA pellet obtained after isopropanol precipitation was dissolved in 500 µl TE buffer and incubated at 65^0C for 20 min to denature DNase. RNase (Appendix 1.3) was then added at a final concentration of 100 ng/ml and incubated at 37^0C for 60 min. Finally, DNA was purified by phenol/chloroform extraction as described in Section 2.4.5.

2.4.2 Plasmid DNA isolation

Plasmid DNA was isolated from *E. coli* cells using the alkaline lysis method (Sambrook and Russell, 2001). An overnight culture (1.5 ml) was centrifuged at 13,000 g for 30

seconds and the pellet re-suspended in 100 µl of ice-cold solution I (Appendix 1.3). To the suspension, 200 µl of freshly prepared solution II (Appendix 1.3) was added and mixed thoroughly by inversion. An aliquot of 150 µl of solution III (Appendix 1.3) was added to the solution, which was inverted several times to mix it thoroughly. The mixture was incubated on ice for 3-5 min, and then centrifuged at 13,000 g for 5 min. The supernatant was recovered and extracted with phenol/chloroform (Section 2.4.5). DNA in the supernatant was precipitated by the addition of two volumes of 95% ethanol, and centrifuged for 5 min. The DNA pellet was washed with 70% ethanol, air dried and re-suspended in 1x TE buffer. DNA was treated with RNase as described in Section 2.4.1 when necessary.

2.4.3 Isolation of DNA from agarose gels

DNA containing the fragment to be purified was analysed by electrophoresis in a 0.7-1.5% agarose gel in 1x TAE buffer. After staining in ethidium bromide (1 mg/l), the DNA was visualised under UV light, and the gel block containing the appropriate band was excised using a clean scalpel blade. The DNA was extracted from the agarose using the QIAEX II Gel extraction kit (QIAGEN) according to the manufacturer's instructions.

2.4.4 Purification of PCR products

PCR products were purified either by agarose gel electrophoresis (Section 2.4.3), or using the QIAquick PCR purification Kit (QIAGEN) by following the manufacturer's instructions.

2.4.5 Purification of DNA by phenol/chloroform extraction

Phenol/chloroform (500 µl) was added to an equal volume of DNA in TE buffer and mixed by inversion. The mixture was centrifuged for 5 minutes at 13,000 g in a desktop centrifuge. The aqueous phase was transferred to a fresh microcentrifuge tube and 500 µl of chloroform was added, mixed and centrifuged at 13,000 g for 5 minutes. The upper phase was transferred to another fresh microcentrifuge tube. DNA was

precipitated by adding 1/10 volume of 3 M sodium acetate, and 1 volume of isopropanol. The mixture was incubated at room temperature for 2 minutes, and then centrifuged at 13,000 g for 5 minutes. The pellet was washed once with 70% ethanol, left to air-dry and then the DNA was dissolved in TE buffer.

2.4.6 Agarose gel electrophoresis of DNA

DNA samples mixed with gel loading buffer were size-fractionated by electrophoresis through 0.7%-2.0% agarose gels in 1 x TAE buffer (Appendix 1.3). Electrophoresis was carried out at 80 to 120 V for 1-2 hour, or at 20 V overnight for gels to be used for Southern blot analysis. An aliquot of 1 kb plus DNA ladder (Invitrogen) was run alongside the samples. After electrophoresis, gels were stained in 1 mg/l ethidium bromide for 10 to 20 min for a small gel or 30 min for a large gel, then destained in MQ water. Bands on the gel were visualised on a UV transilluminator and the image captured with an Alphamager 2000 system (Alpha Innotech Corporation, USA). DNA fragment sizes were determined by comparison to the 1 kb plus DNA ladder or the *Hin*dIII digested λ standard (Invitrogen).

2.4.7 Determination of DNA concentration

An estimation of the quantity and purity of the DNA was made by using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). DNA was quantified on the basis that a solution containing 50 μg/ml of double-stranded DNA has an absorbance of 1.0 at 260 nm (Sambrook and Russell, 2001). Samples were usually diluted so that the absorbance reading was between 0.1 to 1.0. The purity was estimated from the ratio of the absorbencies at 260 and 280 nm.

Alternatively, an aliquot of DNA was analysed by agarose gel electrophoresis alongside quantification standards (10-100 ng), prepared by digesting a known amount of vector with a restriction endonuclease. Fragments were visualised under UV illumination, and

the concentration was estimated by comparing the intensity of ethidium bromide fluorescence to that of the quantification standards.

2.5 DNA digestion, ligation, cloning and subcloning

2.5.1 Restriction endonuclease digestion of genomic DNA

Digestion of *G. cingulata* genomic DNA was carried out in a reaction mixture (100 µl) containing 5-10 µg DNA, 1 x buffer and an excess of enzyme (about 10 to 20 units depending on the efficiency of the enzyme). The digestion was performed in a water bath at temperatures recommended by the manufacturer (usually 37^0C) overnight. A small aliquot of digested DNA was checked by agarose gel electrophoresis to ensure complete digestion. If digestion was incomplete as indicated by intensive staining in the high molecular size region, another aliquot of enzyme was added and the incubation continued for a further period. If the digest failed to go to completion, the DNA was purified by phenol/chloroform extraction (Section 2.4.5) and re-digested. For Southern blotting, the digested DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol, incubated at room temperature for 5 min, and centrifuged for 10 min at 13,000 g in a desktop centrifuge. The DNA pellet was washed with 70% ethanol, air-dried and re-suspended in 20 µl 1x TE buffer.

2.5.2 Restriction endonuclease digestion of plasmid DNA

Digestions of plasmid DNA or purified PCR products were carried out in a similar manner to that described for digestion of genomic DNA (Section 2.5.1), except that the volume was smaller (usually 20 µl) and contained 500-1000 ng DNA, 1 x buffer and 5 units of enzyme. Incubation time was one to two hours.

2.5.3 Exonuclease III digestion of DNA

Exonuclease III digestion was performed by a modification of the method described by Sambrook and Russell (2001). Plasmid DNA (10 μg) was digested with two restriction enzymes to generate a recessed 3′ terminus and a protruding 3′ terminus. DNA was purified by extraction with phenol:chloroform and precipitation with ethanol. The DNA pellet was dissolved in 60 μl of 1x exonuclease III buffer and a 1.5 μl aliquot of this solution was transferred to 24 microcentrifuge tubes on ice. Exonuclease III (5 units/μl) was added to each tube and tubes were incubated at 37^0C. Tubes were placed on ice at 30 seconds intervals and the nuclease S1 reaction mixture (7.5 μl) was added to each tube, which were then incubated at 30^0C for 30 minutes. The nuclease S1 stop mixture (1 μl) was added to each of the microcentrifuge tubes and incubated for 10 minutes at 70^0C. An aliquot (5 μl) from each tube was analysed on agarose gel electrophoresis. The samples containing DNA fragments of the desired size were added with Klenow mixture (0.5 μl) and incubated for 5 minutes at 37^0C. dNTPs (0.5 μl of 0.5 mM) were successively added and incubation was continued for 15 minutes at room temperature. T4 bacteriophage ligase mixture was mixed with the reactions and incubation continued for 2 hours at room temperature. These final reactions were used to transformed *E. coli* and the desired plasmids were screened as described in Section 2.5.6 and Section 2.5.7

2.5.4 Alkaline phosphatase treatment of vector DNA

Digested vector DNA was dephosphorylated using calf alkaline phosphatase (CAP, Invitrogen) to prevent self-ligation. Vector DNA (5 μg) was digested with the appropriate restriction enzyme (Section 2.5.2) and an aliquot was checked by gel electrophoresis for completion of the digestion. The restriction enzyme was inactivated by heating for 15 min at 65°C. To this reaction, CAP was added to a final concentration of 0.1 unit/μl and the reaction incubated at 37^0C for 30 min. CAP was inactivated by incubating the reaction at 65^0C for 15 min. The DNA was purified by phenol/chloroform extraction and precipitated with ethanol if the digestion was complete (Section 2.4.5). Otherwise the DNA was separated by gel electrophoresis and the desired band was recovered from the gel (Section 2.4.4).

2.5.5 Ligation of DNA fragments

Ligation of DNA fragments into digested vectors (usually CAP-treated) was performed in a reaction mixture (10 or 20 µl) containing 30 to 60 ng insert DNA fragments, 20 ng vector DNA, ligation buffer and 2 units of T4 DNA ligase (Invitrogen), and incubated at 22^0C for 3 hours or at 4^0C overnight.

Ligation of PCR products into pGEM-T Easy vector was carried out using a pGEM-T Easy ligation Kit (Promega). The reaction mixture (20 µl) contained 10 to 50 ng PCR products, 20 ng pGEM-T vector, 1 x ligation buffer supplied with the kit and 2 units of ligase, and was incubated at 22°C for 3 hours or at 4^0C overnight.

2.5.6 Preparation and transformation of competent *E.coli* cells

To prepare competent *E. coli* cells, one litre of SOB broth (appendix 1.1) was inoculated with 10 ml of an overnight culture of *E. coli* strain DH5α and incubated at 20^0C with vigorous shaking at 200 rpm until the OD_{600} of the culture reached to 0.50-0.70. The cells were chilled on ice for 10 minutes and collected by centrifugation at 8,000 g for 10 minutes at 4^0C. The pellet was re-suspended in 48 ml of ice-cold TB buffer (appendix 1.1) using an autopipette fitted with a cut off 5 ml tip, and recovered by centrifugation at 8,000 g for 10 minutes at 4^0C. The cells were washed once more and then resuspended in 8-16 ml of TB buffer. Cold DMSO (0.6-1.2 ml, 3/40 volume) was added drop by drop to the cell suspension. Cells were aliquoted into the ice cold 0.5 ml microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80^0C.

For transformation, 100 µl of competent *E. coli* cells were mixed with 5 µl of the ligation reaction and the mixture was left on ice for 30 minutes. The mixture was heat shocked at 42^0C for 1 minute, placed on ice for 2 minutes and then aliquots were plated on LB Agar plates containing the appropriate antibiotic and the plates were incubated overnight at 37^0C.

2.5.7 Screening for transformants

Transformants were usually screened by bacterial colony PCR (Section 2.7.7) with a primer pair that binds to the insert fragment, or one primer binds on the cloning vector and another binds on the inserted fragment, to check the presence of the insert and orientation of the insert in the plasmid.

Alternatively, transformants were also screened by restriction endonuclease digestion. The colonies (about 2 mm in diameter) were transferred to 10 μl of digestion buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% TritonX-100, 1x restriction digestion buffer). The reaction mixtures were incubated at 95^0C for 5 min and then cooled on ice. Restriction enzyme (2 units) was added to each reaction, which were incubated for 30 min at the appropriate temperature for the restriction enzyme used. The products were analysed by agarose gel electrophoresis.

2.5.8 Subgenomic library construction and screening

Genomic DNA was digested with restriction enzymes as described in Section 2.5.1 and size-fractionised by agarose gel electrophoresis as described in Section 2.4.6. Bands of the desired size were recovered from the gel and purified as described in Section 2.4.3. The purified digests were ligated into the cloning vector pBlueScript II KS that had been cut with the same restriction enzyme (Section 2.5.5). The ligation mixture was used to transform *E. coli* DH5α as described in Section 2.5.6 and the colonies were screened by PCR as described in Section 2.5.7.

2.6 DNA sequencing and sequence analysis

2.6.1 DNA sequencing

DNA sequencing was carried out by the DNA Analysis Service at the Allan Wilson Centre, IMBS, Massey University. DNA was sequenced by the dideoxynucleotide chain

termination method (Sanger *et al.*, 1977) using Big-Dye chemistry (PE Applied Biosystems, Foster City, CA). The products were separated on an ABI Prism 377 sequencer (Perkin-Elmer).

2.6.2 Bioinformatic analysis

Sequence data were assembled and analysed using the Vector NTI programme (Invitrogen). Homologs in the GenBank database were identified using the BLAST and FASTA programmes (Altschul *et al.*, 1997) at the National Centre for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed using CLUSTALW (http://www.ebi.ac.uk/clustalw). The promoter sequence was analysed using the software Vector NTI or on the TRANSFAC database (http://www.generegulation.com).

2.7 Southern blotting and hybridisation

2.7.1 Southern blotting (Capillary)

The blotting method used was based on that described by Southern (1975). DNA was digested as described in Section 2.4.1, resolved by electrophoresis (Section 2.4.6) in a 0.7% agarose gel at 20 V (1-2 volts/cm) overnight, stained in ethidium bromide (1 mg/l) and photographed with a ruler alongside the gel. The gel was gently agitated in 250 mM HCl for 15 minutes to depurinate the DNA in order to transfer high molecular weight DNA efficiently. The gel was rinsed with MQ water and transferred into denaturing solution (Appendix 1.5) for 45 minutes, followed by two changes of neutralising solution (Appendix 1.5) for 20 minutes each. Finally, the gel was soaked in 20 x SSC (Appendix 1.5) for 10 minutes.

The blotting was performed as follows (Sambrook and Russell, 2001). A sheet of 3MM Whatman paper on a glass plate with both ends in 20 x SSC was used as a wick. A film of gladwrap with a rectangular slot the size of the gel in it was used to cover the paper.

The gel was placed on the paper in slot and a piece of positively charged nylon membrane (Biodyne plus membrane, Gelman laboratory, USA) slightly larger in size than the gel was placed on top of the gel. Another two pieces of 3 MM paper soaked in 2 x SSC (Appendix 1.5) were placed on the nylon membrane, followed by a stack of paper towels and a weight. After overnight transfer of DNA, the apparatus was disassembled. The membrane was labelled, and washed in 2 x SSC for 5 min and cross-linked for 1.2 min at 1.2 J/cm^2 by using a UV Stratalinker 2400 (Stratagene, USA).

2.7.2 Hybridisation with Digoxigenin (DIG) labelled probe

Specific DNA fragments on the membranes were detected using a Digoxigenin-11-dUTP labelled probe according to the manufacturer's instructions (Roche).

DNA probes were labelled with DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) according to the manufacturer's instructions. The efficiency of the probe labelling was determined using autoradiography by comparing serial dilutions of the DIG-labelled probe with standards (supplied by the manufacturer) spotted on a positively charged nylon membrane.

The membranes were pre-hybridised in a rotary oven with DIG Easy Hyb buffer (Appendix 1.5) at 42^{0}C for 2 h. The probe was denatured by boiling for 5 min and then plunged into an ice water mixture for 2 min, and finally added to the hybridisation buffer at a concentration of 2.5 ng/ml in DIG Easy Hyb buffer. Hybridisations were carried out at 42^{0}C for 16 to 24 h. After hybridisation, excess probe was washed off the blot as follows. The membrane was taken out of the hybridisation tube with forceps, and washed twice in 100 ml of 2 x SSC, 0.1% SDS with shaking at room temperature for 5 minutes, followed by washing twice with 100 ml of pre-warmed 0.5 x SSC, 0.1% SDS at 68^{0}C for 15 minutes with constant shaking.

2.7.3 Chemiluminescent detection of DIG-labelled probes

Chemiluminescent detection was carried out at room temperature. The membrane was kept wet at all times. The membrane was washed in DIG washing buffer (Appendix 1.5) for one min, then soaked in blocking solution (Appendix 1.5) for 30 min. Anti-Digoxigenin-AP antibody (Roche) was diluted 1:20,000 into the blocking buffer and incubated with the membrane for 30 min. The membrane was washed twice in washing buffer for 15 min, equilibrated in detection buffer (Appendix 1.5) for 2 min, drained and placed in a plastic bag with the DNA face up. One millilitre of CSPD (Roche) in detection buffer was distributed evenly over the membrane. The bag was closed and bubbles on the membrane were removed. The bag was then incubated at 37^0C for 15 min and exposed to X-ray film for 20 min and the film developed in an automated developer (100 Plus Automatic X-ray Processor, All Pro Imaging). When necessary, a second image was collected for a longer or shorter exposure depending on the intensity of the signals obtained with the first exposure.

2.7.4 Hybridisation with ^{32}P labelled probe

Southern blots were also hybridised to ^{32}P labelled probes. The DNA probe was labelled with [α-^{32}P] dCTP using Ready-To-Go DNA Labelling Beads (Amersham Pharmacia Biotech, UK) and purified with a Probe-Quant™ G-50 Nficro Column (Amersham Pharmacia Biotech, UK) by following the manufacturer's instructions. Hybridisation buffers and procedures were the same as described in Section 2.9.9.

2.8 Polymerase chain reaction (PCR) amplification

PCR reactions were carried out in a PTC-200 Peltier Thermal cycler (MJ Research, USA) or a Mastercycler® gradient thermocycler (Eppendorf). To optimise PCR reaction conditions, annealing temperatures were varied depending on the melting temperature (quoted by suppliers) of the primers. Extension times of 1 min/kb were used. Sometimes, Mg^{2+} concentration, primer and DNA template concentrations were

adjusted as required. PCR products were analysed by gel electrophoresis and visualised as described in section 2.4.6.

2.8.1 Oligonucleotide primers

Primers were ordered from Sigma Corporation or Invitrogen Life Technologies. Each primer was dissolved in 1x TE buffer to a concentration of 100 μM and stored at -20^{0}C. The primers were diluted to a working stock concentration of 10 μM for PCR reactions and 3.2 μM for sequencing reactions. The primers used in this study are listed in Appendix 3.

2.8.2 Routine PCR

Routine PCR reactions (25 μl in a 0.2 ml tube) consisted of 1x *Taq* DNA polymerase buffer (Roche), 200 μM of each dNTP, 500 nM of each primer, 0.02 unit/μl of *Taq* DNA polymerase (Roche). DNA used as the template in a 20 μl PCR reaction was 20-100 ng for fungal genomic DNA or 20-100 pg for plasmid DNA amplification. The thermocycler conditions were 1 cycle of 2 min at 94^{0}C, 34 cycles of 10 sec at 94^{0}C, 30 sec at 60^{0}C (an indicative annealing temperature that may be adjusted depending on the melting temperature of the primers) and 1-2 min (1 min/1 kb) at 72^{0}C, and a final incubation for 5 min at 72^{0}C.

2.8.3 Degenerate PCR

Degenerate PCR was performed using the reaction and PCR cycle conditions described for routine PCR except that a higher primer concentration of 2.5 μM of each primer was used.

2.8.4 Bacterial colony PCR

Bacterial colony PCR was used for identifying bacterial transformants using bacterial cells from the colony as the template. A sterilised P10 pipette tip or toothpick was used

to gently touch the colony and the cells were suspended in the reaction mixture used for a routine PCR. The thermocycler conditions were as for routine PCR except the first cycle was 3 min at 96^0C

2.8.5 Single specific primer PCR

Single specific primer PCR was performed by a modification of the method described by Shyamala and Ames (1989). Genomic DNA (5 μg) was digested with 10 units of restriction enzyme in a 100 μl reaction containing 1 x buffer as described in Section 2.5.1. The digests were purified by Phenol/chloroform as described in Section 2.4.5. The purified digests were ligated into pBlueScript II KS that had been cut with the same restriction enzyme (Section 2.5.5). The ligation mixture was used as template for PCR containing one gene specific primer designed to anneal to the desired insert and one of two vector specific primers, either the T7 or T3 primer. Both vector specific primers were used because the inserted restriction fragment could be ligated in either orientation with respect to the vector sequence.

2.9 RNA manipulations

2.9.1 Isolation of total RNA

Total RNA was extracted from fungal conidia, mycelia, and appressoria using different methods. Conidia for RNA isolation were collected from PDA plates or liquid medium as described in Section 2.10.1. Mycelium from PDA plates was collected using a scalpel. Mycelium in liquid culture was harvested by filtration through eight layers of nappy liner (Arico Wipeese, New Zealand) by gravity flow. The mycelia were washed with MQ H_2O and press-dried with filter paper.

Fungal mycelium (about 1 g fresh weight) was ground to a fine powder in liquid nitrogen, and immediately transferred to a 15 ml Falcon tube containing 10 ml of TRI® Reagent (MRCgene). The contents were mixed well, stood at room temperature for 10

min, and centrifuged in an Eppendorf Centrifuge 5810 with an A-4-62 rotor at 4,000 rpm (3,220 g). The supernatant was transferred to a fresh Falcon tube, 2 ml of chloroform added, the contents mixed vigorously, and then centrifuged for 10 min at 3,220 g. The RNA in the aqueous phase was precipitated by adding 5 ml of isopropanol and recovered by centrifugation as above for 10 min. The RNA pellet was washed with 10 ml of 75 % ethanol, air-dried, and dissolved in 200 µl DEPC-treated water.

RNA extraction from *G. cingulata* appressoria was performed at room temperature as described by Clark (1998). Appressoria were induced as described in Section 2.11.4. After an appropriate period to allow appressorium induction, the MQ water was removed and the plate rinsed once with MQ water at room temperature. RNA extraction buffer was added to the plate and incubated for 2-4 min. Buffer was removed and ~40 ml liquid nitrogen was added to the plate. Once the liquid nitrogen had evaporated, the surface was immediately scraped with a razor blade and the wax-buffer mixture was transferred to a microcentrifuge tube. About 350 µl of material was obtained from one plate. This was mixed with 250 µl phenol equilibrated with DEPC treated water, 100 µl chloroform, 50 µl of 2 M sodium acetate (pH 4.0) and 200 µl glass beads (425-600 microns, Sigma). The tube was vortexed for 2 min and centrifuged at 13,000 rpm for 5 min in a desktop microcentrifuge. The aqueous phase was extracted with phenol/chloroform twice and chloroform once, followed by isopropanol precipitation. The pellet was washed with 70% ethanol, air dried, and dissolved in DEPC treated MQ water. Alternatively, the appressorial RNA could be extracted with TRI reagent (MRCgene). After the water was removed and the plate rinsed, the wax from one plate was scraped with a razor blade and transferred to a microcentrifuge tube containing 1.0 ml of TRI reagent and 0.4 ml chloroform. The chloroform was included to detach the appressoria from the wax. The tube was vortexed for 2 min and centrifuged at 13,000 rpm in a desktop microcentrifuge. The aqueous phase was precipitated with isopropanol. The pellet was washed with 70% ethanol, air dried, and dissolved in DEPC treated MQ water.

2.9.2 Quantification of RNA

An estimation of the quantity and purity of the RNA was made by using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). RNA was quantified on the basis that a solution containing 40 μg/ml of RNA has an absorbance of 1.0 at 260 nm (Sambrook and Russell, 2001). Samples were usually diluted so that the absorbance reading was between 0.1 to 1.0. The purity was estimated from the ratio of the absorbencies at 260 and 280 nm. Alternatively, the RNA concentration was measured in a ND-1000 Spectrophotometer (Nanodrop technologies, USA) which required only 2 μl of sample and displayed the concentration directly.

2.9.3 DNase I treatment of RNA

RNA samples used in RT-PCR and RACE experiments were treated with DNase I to remove residual DNA. Total RNA (10 μg) was incubated in a 20 μl reaction mixture containing 2 units of DNase I (Promega), 1 unit of RNase inhibitor (Invitrogen), 10 mM 1,4-dithiothreitol (DTT) in 1 x DNase I buffer (Appendix 1.4), at 37^0C for 45 min. Another 2 units of DNase I was added and the mixture was incubated for a further 45 min. The reaction was stopped by adding Stop mix (Appendix 1.4) and purified using phenol/chloroform as described in Section 2.4.5. The purified RNA was dissolved in DEPC-treated water and kept at -20^0C.

2.9.4 Reverse transcription of RNA into cDNA and amplification

RNA was reverse transcribed into first strand cDNA using Expand Reverse transcriptase (Roche) by following the manufacturer's instructions. Expand Reverse Transcriptase is a recombinant version of MMLV reverse transcriptase. Random hexamer primers (Invitrogen), or an oligo-dT primer (Invitrogen) (Appendix 3) were used depending on the downstream application. An aliquot of primer was mixed with DNase I treated RNA (1 μg), incubated at 70^0C for 5 min, and immediately cooled on ice. Then a 'cocktail' of reagents was prepared and added to the denatured RNA. The final reaction mixture (20 μl) contained 1 x Expand RT buffer, 10 mM DTT, 50 μM dNTPs, 1.0 unit/μl RNase inhibitor and 2.5 units/μl Expand Reverse Transcriptase. The

reaction was incubated at 42^0C for 45 min and then at 70^0C for 10 min. An aliquot (0.2-1.0 µl) of the reaction mixture was used as template for PCR amplification of the desired cDNA (Section 2.8.2).

In order to confirm removal of genomic DNA following DNase treatment, a duplicate reaction without addition of Reverse Transcriptase was prepared, and PCR amplification was carried out on this "no-Reverse Transcriptase" (mock cDNA reaction) control. Absence of PCR product in this control was taken to confirm the absence of genomic DNA.

2.9.5 RACE (Rapid Amplification of cDNA Ends)

3' RACE was used to determine the 3'end of an mRNA molecule. The reverse transcription reaction (Section 2.9.4) was carried out using the oligo dT-anchor primer (Appendix 3). The resulting cDNA served as template for PCR amplification (Section 2.8.2) using a gene-specific primer complementary to the first strand cDNA and a primer identical to the oligo dT-anchor primer except without the oligo-dT sequence. A pair of nested primers was used in a second PCR reaction if necessary to obtain specific products. PCR products were analysed by agarose gel electrophoresis and cloned into the pGEM-T Easy vector (Section 2.5.5) for DNA sequencing.

5' RACE was used to determine the 5' end of an mRNA molecule. Two different strategies were used in this study. The first strategy, the RLM-RACE (RNA ligase mediated RACE), was performed using a commercial kit (Ambion). RNA was treated with calf intestinal phosphatase (CIP) to remove free 5' phosphates from molecules by incubating 10 µg of total RNA with 2 µl of CIP in a 20 µl reaction for one hour at 37^0C. The reaction was terminated by adding ammonium acetate solution (3M) and purified by phenol-chloroform extraction followed by ethanol precipitation. The RNA pellet was dissolved in DEPC treated water. Tobacco acid pyrophosphatase (TAP, 1 µl in a 10 µl reaction) was added and incubated for 1 hour at 37^0C to remove the cap structure, exposing a 5' monophosphate. An aliquot of TAP reaction (1 µl) was used to ligate a 45 nucleotide long RNA adapter oligonucleotide using T4 RNA ligase in a 10 µl reaction

at 37^{0}C for 1 hour. The ligation reaction (1 μl) was used in a reverse transcription reaction followed by nested PCR amplification as described in Section 2.9.4. Sequencing these PCR products revealed the transcription start point.

The second 5' RACE strategy was based on the property of terminal transferase to add an oligonucleotide tail to the cDNA end, and thereby provided an artificial primer binding site. The first strand cDNA was synthesised with a gene specific primer using Expand reverse transcriptase (Roche) as described in Section 2.9.4. The cDNA product was purified with a QIAquick PCR purification Kit (QIAGEN). Purified cDNA (19 μl) was mixed with 2.5 μl of reaction buffer and 2.5 μl of 2 mM dATP. The mixture was incubated at 94^{0}C for 3 minutes and chilled on ice. Terminal transferase (1 μl, 80 units) was added and the reaction was incubated at 37^{0}C for 20 minutes. The reaction was terminated by heat inactivation of the terminal transferase at 70^{0}C for 10 minutes. This A-tailed cDNA (1 μl as template in a 20 μl PCR reaction) was amplified by PCR using a gene specific primer and an oligo dT-anchor primer. The oligo dT-anchor primer was a mixture of oligonucleotides carrying a non-T nucleotide (*i.e.* A, C or G) at their 3' ends. The PCR product was analysed by agarose gel electrophoresis. The desired band was recovered from the gel and cloned into the plasmid pGEM-T Easy vector (Section 2.5.4-2.5.6). Sequencing of the insert from these PCR products revealed the transcription start point.

2.9.6 Formaldehyde gel electrophoresis of RNA

RNA samples were resolved on a formaldehyde gel. Agarose (0.8 g) was melted in 50 ml DEPC-treated MQ water in a microwave oven and cooled to 60^{0}C. 5 x MOPS buffer (16 ml) and 37 % formaldehyde (14 ml) were added and the gel poured. RNA samples (11.25 μl) were mixed with RNA denaturation buffer (Appendix 1.4) and incubated at 65^{0}C for 15 min. The denatured sample was placed on ice for 2 min, mixed with 4 μl loading buffer and loaded onto the gel. Gel electrophoresis was carried out in RNA gel tank buffer (Appendix 1.4) at 5-8 volts/cm. When the bromophenol blue dye had migrated two thirds the length of the gel, the RNA was visualised on a UV

transilluminator and the image captured with an Alphamager 2000 system (Alpha Innotech Corporation, USA).

2.9.7 Northern blotting

RNA samples for northern hybridization analysis were first transferred to a nylon membrane from the formaldehyde gel (Section 2.9.6). After electrophoresis, the gel was rinsed with DEPC-treated MQ, soaked in 0.01 N NaOH/3 M NaCl for 20 min, and the RNA transferred in 0.01 N NaOH/3 M NaCl. The transfer procedures were the same as for Southern blotting (Section 2.7.1).

2.9.8 Probe labeling

The DNA probe was labelled with [α-^{32}P] dCTP using Ready-To-Go DNA Labelling Beads (Amersham Pharmacia Biotech, UK) and purified with a Probe-Quant^{TM} G-50 Nficro Column (Amersham Pharmacia Biotech, UK) by following the manufacturer's instructions.

2.9.9 Northern hybridization

For northern hybridisation, the nylon membrane was pre-hybridised with 20 ml of hybridization solution (Appendix 1.5) at 65^{0}C for at least 2 hours in a rotary oven. After pre-hybridisation, 100 ng of radio-labelled probe, denatured by boiling, was added to the solution and hybridisation was carried out at 65^{0}C overnight. The membrane was removed and pre-washed three times in 100 ml of 2 x SSC, 0.1% SDS for 20 minutes each at room temperature and in 0.5 x SSC, 0.1% SDS for 20 minutes at 65°C. The washed membrane was exposed to X-ray film (Kodak) at room temperature overnight and developed in an automatic developer (100 Plus Automatic X-ray Processor, All Pro Imaging). Alternatively, the membrane was exposed in a BAS Imaging Plate (Fujifilm) overnight and the plate was scanned in a FLA-500 Phosphorimager. The resulting image was analysed using the software Image Gauge ver.4.4 (Fujifilm).

2.10 *G. cingulata* transformation

2.10.1 Conidium induction, collection and quantification

A PDA plate overlaid with a cellophane membrane (Waugh Rubber Bands, Wellington) was inoculated with mycelium on an agar block or with conidia. The plate was incubated at 22^0C under a black light for 12 hours per day. Conidia were harvested after 5-7 days as follows. The cellophane membrane with mycelium on it was peeled off the agar with forceps, and put in a plastic bottle which contained 50 ml sterile MQ water and a magnetic stirring bar. The cellophane membrane was placed upside down in the bottle so that the mycelium was facing the MQ water. The water was mixed using a magnetic stirrer (Barnstead International) to wash conidia off the mycelial mat. The suspension of conidia was filtered through 8 layers of nappy liner (Arico Wipeese, New Zealand) and centrifuged (3,220 g) for 5 minutes to harvest the conidia. The pellet was washed with MQ water at least three times and the final suspension of conidia was serially diluted and counted using a haemocytometer.

Conidia were also induced in liquid medium by a modification of the method used for induction of *Penicillium* spores (Roncal and Ugalde, 2003). Mycelium, growing in PDB culture at 25^0C for 5 days, was harvested by filtration, fractionated with a Waring blender (Waring Laboratory, Connecticut, USA) and transferred to the conidium induction medium (Appendix 1.1). The culture was incubated at 25^0C with shaking at 200 rpm for another 3 days. The conidia were filtered through 8 layers of nappy linen and washed as described above.

2.10.2 Protoplast preparation

Protoplasts of *G. cingulata* were prepared using a modification of the method described by Yelton *et al* (1983). Both mycelium and conidia were tested for protoplast preparation. Mycelium for protoplast preparation was harvested from PDB culture and washed with MQ water followed by ST buffer (Appendix 1.6). Conidia for protoplast

preparation were harvested and washed as described in Section 2.10.1. It was found that conidia were the better starting material for protoplast preparation because the protoplasts were clean and uniform in size. Most transformations in this study were performed using protoplasts freshly prepared from conidia.

Mycelium (2 g in wet weight) or conidia (10^8-10^9) were suspended in 20 ml of cell wall digesting enzyme solution (Appendix 1.6) containing 5% (w/v) Glucanex, and shaken at 100 rpm at 25°C for 16-20 hours. Protoplast production was monitored microscopically by examining a 5 µl sample for spherical protoplasts as opposed to oval conidia. An overnight digestion would usually produce protoplasts from >80% of the conidia. The digest was transferred into two 15 ml Falcon tubes, overlaid drop by drop with 2 ml of ST buffer and centrifuged for 10 min in an Eppendorf Centrifuge 5810 with an A-4-62 rotor at 4,000 rpm (3,220 g). The protoplasts that formed a pink layer at the interface of the two solutions were transferred to a fresh Falcon tube, washed 3 times with 5 ml of STC buffer and collected by centrifugation at 3,220 g for 5 minutes. Finally, the protoplast pellet was re-suspended in STC buffer (Appendix 1.6) and stored on ice.

2.10.3 *G. cingulata* transformation

Protoplasts prepared as described above were transformed with DNA by a modification of the method described by Vollmer and Yanofsky (Vollmer and Yanofsky, 1986). In each transformation reaction, DNA (5-10 µg, 1.0 µg/µl) was mixed with 80 µl of protoplasts (1.25 x 10^8 protoplast/ml), and 20 µl of a 40% PEG solution (Appendix 1.6). The mixture was vortexed gently and put on ice for 30 min. A further 900 µl of 40% PEG solution was added and mixed gently by inversion. The transformation mixture was incubated at room temperature for 15-20 min. An aliquot of 100 µl of the transformation mixture was mixed with 5 ml of molten regeneration top agar (50^{0}C) (Appendix 1.6) and spread onto a plate containing 5 ml of regeneration base agar (Appendix 1.6). The plates were incubated at 25^{0}C overnight, and then overlaid with 15 ml of PDA containing the appropriate antibiotic to achieve a final concentration of 200 µg/ml of hygromycin B or 8 µg/ml of phleomycin. The plates were incubated at 25^{0}C for a further 3-5 days. Transformant colonies that emerged on the agar surface were

transferred to fresh selective PDA plates for further growth. In each transformation experiment, a sample of protoplasts without DNA was processed alongside as a negative control. Aliquots (100 µl) of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of the protoplasts were grown on regeneration plates to determine the number of viable protoplasts after transformation, and an aliquot (100 µl) of the negative control protoplasts was grown on regeneration plates containing antibiotic to confirm the sensitivity of the untransformed protoplasts.

2.10.4 Single spore isolation

Isolation of single spores of *G. cingulata* and mutants was performed to obtain genetically pure colonies. A hundred microliters of MQ water was applied to the mycelium surface and sucked up and down for a few times to wash off the conidia. This suspension of conidia was serially diluted and an aliquot (50 µl) was spread on a thin PDA plate (5 ml of agar in a 7 cm Petri dish). The plates were incubated at 25^0C for 24 hours. Single spore derived colonies were identified with the aid of a microscope and transferred to a fresh PDA plate.

2.10.5 Plasmid rescue

Plasmid rescue was performed to isolate the DNA flanking the disrupted vector previously integrated into the *G. cingulata* genome. Genomic DNA from a transformant was isolated (Section 2.4.1) and digested with an appropriate restriction enzyme (Section 2.5.1) which would not digest the disruption vector. The digests were purified by Phenol/chloroform as described in Section 2.4.5. An aliquot (1 µl) was added in to a ligation reaction mixture (9 µl), incubated at 4^0C overnight and transformed into *E. coli* (Section 2.5.6). Transformants were screened by restriction digestion (Section 2.5.7).

2.11 Phenotypic characterisation of *G. cingulata* mutants

2.11.1 Radial growth rate

Radial growth rate was measured following transfer of agar plugs (5 mm in diameter) from the growing edge of 5 days old PDA culture to the centre of a fresh PDA or BSA agar plate. At least six replicates were inoculated for each strain tested. The plates were incubated at 25^0C for 7-10 days and the diameter of each colony was measured daily along two axes intersecting at right angles. The average of these two measurements was taken as the diameter of the colony. The average and standard deviation was calculated using Microsoft Excel program (Microsoft Corp, USA). The growth rate was calculated by the slopes of linear regression data. The growth rates between strains were compared by Student's t-test analysis.

2.11.2 Mycelium morphology observation

Mycelium morphology was observed on PDA plates macroscopically or microscopically under a light microscope and photos were taken using a MagnaFire S99802 camera (Olympus, Japan). For observation of asci, perithecia from 14 day old PDA plates were collected using a scalpel into a microcentrifuge tube containing 0.1% methylene blue and briefly vortexed. An aliquot was transferred to a glass slide. A cover slip was put on, gently squashed and the sample was examined under a light microscope.

2.11.3 Conidia production

The production of conidia on PDA plates inoculated as described above was determined after 7 days incubation at 22^0C under a 12 hour light/dark cycle. At least six replicates were inoculated for each strain. Conidia were harvested and counted as described in Section 2.10.1.

2.11.4 Induction of germination and appressorium formation

Apple wax was extracted as described by Clark (1998). Apple wax at a concentration of 0.13 g/ml in chloroform was smeared onto the surface of a glass slide with a cotton bud. Surfaces were left to dry for 30 min to allow the chloroform to evaporate, and wiped with a fresh cotton bud to make the wax distribution even. The slide was left for one more hour before the conidia were applied. Conidia were collected and washed as described in Section 2.10.1. Washing with MQ water was carried out at least three times to remove the inhibitors for conidia germination. Twenty to fifty microlitre of conidia at a concentration of 10^5/ml were applied to the wax coated glass slide. These glass slides were placed in plastic boxes lined with wet paper towels to provide a moist atmosphere.

Induction of appressorium formation was scaled up for RNA extraction. Apple wax was applied to a glass plate (15 cm in diameter), dried and wiped as above. Freshly harvested conidia suspension (70 ml containing 1x 10^6/ml in MQ water) was applied to the wax coated plates and incubated at 25^0C for 4 or 16 hours. RNA was extracted as described in Section 2.9.1.

Conidia germination and appressorium formation was also performed on polystyrene Petri dishes (Epstein *et al.*, 1998).

2.11.5 Conidium adhesion assay

Conidia were harvested from the 7 day old culture on PDA plates and 100 µl of suspension of conidia in MQ water at 10^5/ml was inoculated on the surface of a polystyrene Petri dish. After incubation at 25^0C for 2 hours, conidia in marked areas were counted using a light microscope. The Petri dish with 25 ml water added was shaken with a reciprocal shaker at 50 rpm for 5 min. Water was then removed after shaking and 100 µl of water was applied to the marked areas. Conidia in marked areas were counted again and the percentage of adhered conidia was calculated.

2.11.6 Mobilisation of glycogen and lipid

Mobilisation of glycogen and lipid during appressorium development was observed by staining the cells with KI/I_2 and Nile Red (Thines *et al.*, 2000; Greenspan *et al.*, 1985; Weber *et al.*, 1999). Conidia suspension (50 µl) in water at a concentration of 10^5/ml was applied on a polystyrene Petri dish. After incubation at 22^0C for 6, 12, 24 hours, the water in suspension was replaced with the KI/I_2 or Nile red staining solution (Appendix 1.6). The stained cells were examined by bright-field microscopy for the presence of glycogen. The Nile red fluorescence of stained lipid was observed using an Olympus BX50 epifluorescent microscope (excitation at 450 to 490 nm, 505 nm dichroic mirror, 520 nm barrier filter). Photos were taken using a MagnaFire S99802 camera (Olympus, Japan).

2.11.7 Appressorium penetration assay

Appressorium penetration of onion epidermal peels was adapted from a procedure used in *Magnaporthe grisea* (Xu *et al.*, 1997). Onion epidermal peels were cut in ~1.0 cm x 1.0 cm square and rinsed with water. Conidia suspended (50 µl) in water at a concentration of 10^5/ml was applied onto onion epidermal peels and kept in a humid Petri dish at 22^0C for 24-72 hours. The peels were transferred to a glass slide and examined under a light microscope at the 24, 48 and 72 hours. Fungal structures above or below the onion epidermal cells were observed by adjusting the plane of focus. Photos were taken using a MagnaFire S99802 camera (Olympus, Japan).

2.11.8 Appressorium cytorrhysis assay

Conidia were harvested from the 7 day old culture on PDA plates and 100 µl of suspension of conidia in water at 10^5/ml was inoculated on the surface of a polystyrene Petri dish. After incubation at 25^0C for 12 or 24 hours, water was replaced with different concentrations of glycerol (1, 2, 3, and 4 M). The percentage of the collapsed appressoria was counted from at least three areas. Photos were taken using a MagnaFire S99802 camera (Olympus, Japan).

2.11.9 Pathogenicity Assay

Pathogenicity of *G. cingulata* and its derivatives was assayed on apple fruit using the method described by Brook (1977). Apples were first sterilised by soaking in 5% sodium hyperchloride for 10 min and rinsed with water. Conidia suspension (0.5 ml of 10^5/ml, from 7 day old cultures) was applied to the centre of a water agar plate. A Granny Smith apple was placed on the conidia and fixed in place with masking tape. This was then put into a plastic bag, sealed, and incubated at 25^0C for 72 hours. The apple was then removed and placed on a polystyrene block in a loosely closed screw cap plastic container containing sufficient water to cover the base of the container. The apple was not in contact with the water. The apples were incubated at 25^0C and the presence and number of lesions were recorded 12 days after inoculation.

Alternatively, apples were wounded by puncturing a hole (0.3 cm into the apple tissue) with a yellow micropipette tip. Conidia suspension (50 µl at a concentration of 10^6/ml) was applied on the wounded spot of the apple and incubated in a plastic container as described above. The apple surface was examined using a Leica MZ12 dissecting microscope and photos were taken using a Leica DFC 280 camera (Leica, Germany).

2.11.10 Firmness of lesion

Firmness of lesions on apples was assayed by an adapted method which was used for measurement of tomato fruit firmness (Jackman *et al.*, 1990). Conidia were inoculated on wounded apple and incubated in a plastic container as described above. After 14 days incubation, the diameter of lesions on apples was measured and the firmness of the lesions was assayed using a penetrometer. The spot for probe penetration was chosen at the centre point between the wounded site and the edge of the lesion. The forces required for probe penetration into the lesions was recorded by the penetrometer.

2.11.11 Statistics analysis

All statistical analysis was performed using the Student's t-test program on the website http://www.graphpad.com/quickcalcs/ContMenu.cfm.

Chapter three

Characterisation of the *SAP* promoter

3.1 Determination of the transcription start points of the *SAP* gene

When this study began, the *G. cingulata* secreted aspartic peptidase (*SAP*) gene was thought to be specifically expressed during the early stage of appressorium differentiation from a more upstream transcription start point than the previously characterized transcription start point (TSP) located 104 nucleotides upstream of the ATG start codon (Clark *et al.*, 1997). Transcription of the *SAP* gene from the TSP at -104 was observed later during appressorium formation and during growth of mycelium on medium containing protein as the nitrogen source. It was, therefore, considered possible that at least one of the transcription factors that regulated expression of the longer transcript may be also involved in the regulation of appressorium differentiation. Identification of that transcription factor may help to understand the mechanisms that regulate appressorium differentiation. The plan was to use the yeast one-hybrid system to isolate the transcription factor (Sieweke, 2000). This required identification of the promoter regions responsible for expression of the longer *SAP* transcript. Therefore, the *G. cingulata SAP* promoter was characterized by mapping the transcription start point. An attempt was also made to use a deletion series of promoter-green fluorescent protein reporter constructs to identify the region of the promoter that was active during the early stage of appressorium formation.

3.1.1 Mapping the most upstream transcription start point by RT-PCR

RT-PCR was performed to determine the approximate location of the most upstream transcription start point (TSP). RNA was extracted from mycelium grown on medium containing bovine serum albumin (BSA) as the sole nitrogen source, and from appressoria induced by incubation of conidia on apple wax coated glass Petri dishes for 2 or 8 hours. No significant degradation of the RNA was evident on a denaturing gel (not shown). cDNA was prepared using the reverse primer C847 (Appendix 3), designed to anneal to the *SAP* ORF at the 5' end of exon 2 (Appendix 3). The *SAP* transcripts were then amplified by PCR using the same reverse primer and a series of forward primers, designed to anneal at different positions 5' to the start of the *SAP* ORF

(Fig 3.1, panel A). As shown in Fig 3.1 (panel B), no PCR products were obtained using mock cDNA reactions as template, indicating that there was no genomic DNA contamination in the RNA samples. With the primer pair C847/S1723, the product obtained with cDNA as template was, as expected, slightly smaller in size than that obtained with genomic DNA as template (the primers span an 82 bp intron), indicating that the PCR products arose from amplification of cDNA. PCR products were obtained with all the primer pairs except the most upstream forward primer (L2801). A product of the expected size could be amplified from genomic DNA with the primer pair L2801/C847 (data not shown). Two additional forward primers (sap1861F and sap2019F, Appendix 3) which would anneal to the genomic DNA on either side of the primer L2801 binding site also failed to yield products in the RT-PCR with cDNA from mycelial and appressorial RNA. Taken together, these results indicated that the TSP of the longest transcript was in the region between the binding sites for primers L2242 and L2801. Unexpectedly, the same pattern of RT-PCR products was obtained with RNA from appressoria harvested at both 2 and 8 hours as well as mycelium, suggesting that the longer transcript of the *SAP* gene was produced under all three conditions.

3.1.2 Determination of transcription start points by RLM-RACE

RLM-RACE (RNA Ligase Mediated Rapid Amplification of cDNA Ends) is designed to amplify cDNA from only full-length, capped mRNA (Maruyama and Sugano, 1994; Shaefer, 1995). The procedure for determining the transcription start point of a gene is outlined in Fig 3.2. Total RNA from mycelia or appressoria harvested at 2 hours was treated with DNase I and checked by RT-PCR for any contaminating DNA by observation of the size difference between the PCR products from cDNA and genomic DNA template which has an intron present. If necessary the DNase treatment was repeated until no DNA was detected by RT-PCR. The RNA was then treated sequentially with calf intestinal phosphatase (CIP), tobacco acid pyrophosphatase (TAP), and RNA ligase to incorporate a 5'adapter oligonucleotide to the decapped mRNA. This was followed by RT-PCR in which cDNA was obtained using random decamers and amplified by PCR using a primer annealing to the 5'adaptor (5'RACE outer primer) and a gene specific primer (sap4b). The entire PCR reaction was purified

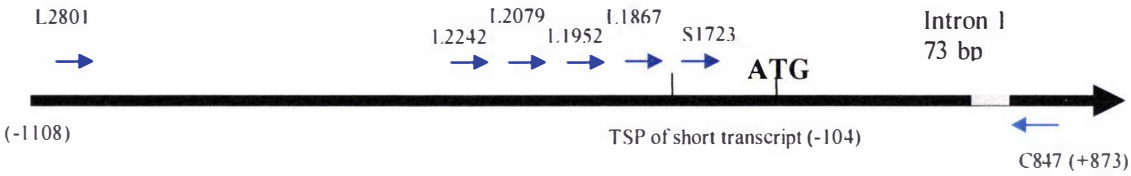
Figure 3.1 RT-PCR to map the most distant transcription start point of the *SAP* gene

A: The ATG start codon, approximate TSP for the short transcript and the single intron within the *SAP* gene are shown. Also shown are the position and orientation of the primers used in the RT-PCR. The sequence of the primers is given in Appendix 3. The drawing is not to scale.

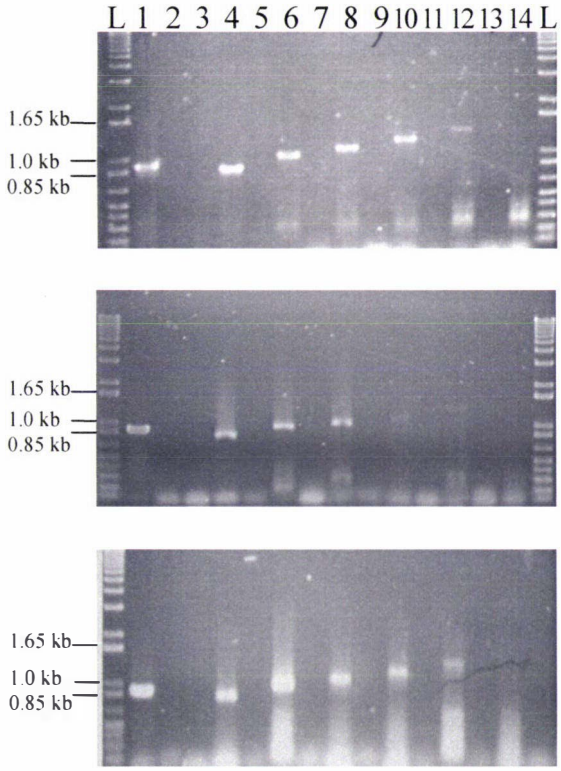
B: Gel Electrophoresis analysis of the RT-PCR products from 2 hour appressorial RNA (top), 8 hour appressorial RNA (middle) and mycelial RNA (bottom). Genomic DNA was used as a positive control (lane1) and RNA as a negative control (lane 2) with the primer pair C847/S1723. Mock cDNA was also used as a negative control in PCR reactions with the reverse primer C847 and the following forward primers: S1723 (lane 3), L1867 (lane 5), L1952 (lane 7), L2079 (lane 9), L2242 (lane 11) and L2801 (lane 13). PCR products were obtained in reactions with cDNA as template, the reverse primer C847 and forward primer S1723 (lane 4), L1867 (lane 6), L1952 (lane 8), L2079 (lane 10) and L2242 (lane 12), but not L2801 (lane 14). Faint bands for products of the expected size were visible in lane 10 and lane 12 of the gel shown in the middle panel. RT reactions were performed with 1.0 μg of total RNA using Expand reverse transcriptase (Roche) following the manufactures' instructions. PCR reactions consisted of 1x *Taq* DNA polymerase buffer (Roche), 200 μM of each dNTP, 500 nM of each primer, 0.02 unit/μl of *Taq* DNA polymerase (Roche). The thermocycler conditions were 2 min at 94^0C followed by 34 cycles of 10 sec at 94^0C, 30 sec at 60^0C, 90 sec at 72^0C, and a final incubation for 5 min at 72^0C.

Forward primers were not designed in the region between the primer L2801 and L2242. Instead, reverse primers were designed in this region for the subsequent RACE experiment to determine the putative transcription start point.

A


L2801
L2079
L1867
L2242
L1952
S1723
ATG
Intron 1
73 bp
(-1108)
TSP of short transcript (-104)
C847 (+873)

B


L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 L
1.65 kb
1.0 kb
0.85 kb
1.65 kb
1.0 kb
0.85 kb
1.65 kb
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0.85 kb

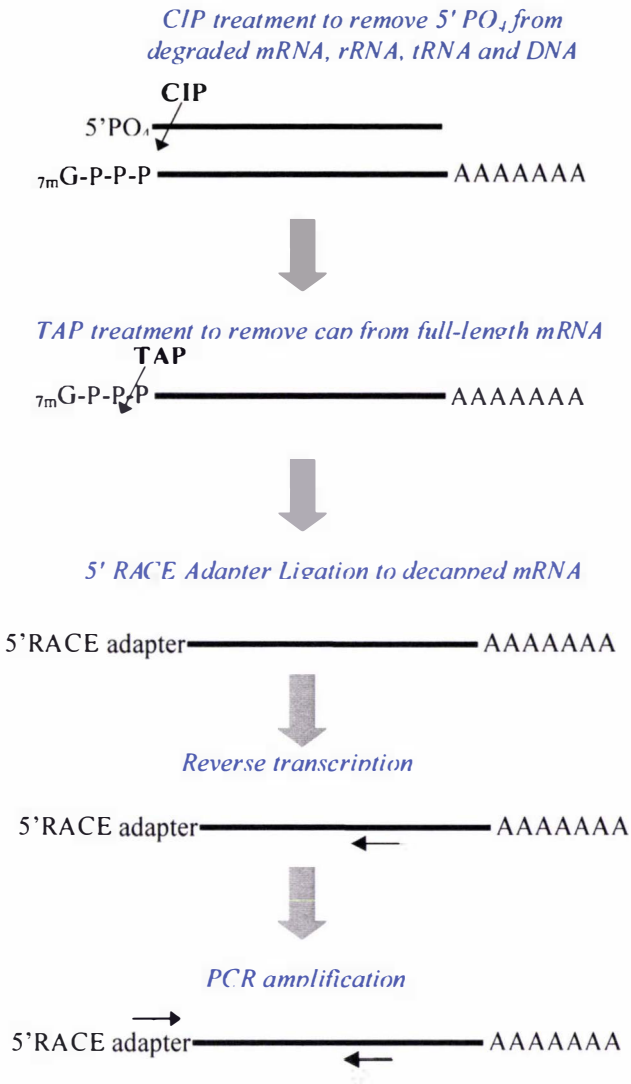


Figure 3.2 Outline of the RLM-RACE procedure

Total RNA was treated with calf intestinal phosphatase (CIP) to remove free 5' phosphates from molecules such as rRNA, tRNA, fragmented mRNA, and if present any contaminating genomic DNA. The cap structure found on intact 5' ends of mRNA is not removed by CIP. The RNA was then treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, exposing a 5' monophosphate. A 45 nucleotide long RNA adapter oligonucleotide was ligated to the 5' end of full length decapped mRNA using T4 RNA ligase. The adapter cannot be ligated to the previously dephosphorylated RNA molecules. A random primed reverse transcription reaction, and nested PCRs with primers to the adaptor oligonucleotide and gene specific primers was then used to amplify the 5' end of a specific transcript. Sequencing these PCR products revealed the transcription start point.

with a chromatography column and diluted for a second PCR using a nested forward primer annealing to the 5' adaptor (5' RACE inner primer) and a nested gene specific primer (sap4a). The primer sequences are shown in Appendix 3. The same products were obtained with both appressorial RNA and mycelial RNA in the second PCR (Fig 3.3). The sequencing results indicated the transcription start point of the *SAP* short transcript was at -110 relative to the translation start codon for both appressorial RNA and mycelial RNA, rather than at -104 as previously reported (Clark *et al.*, 1997). This was possibly due to the slight RNA degradation in their experiment. Using the 5'RACE inner primer and other gene specific reverse primers, additional PCR products were obtained. Sequencing of these PCR products revealed the presence of two additional TSP, at -496 and -700 for mycelial RNA (Fig 3.4). The latter TSP was within the region between the binding site for primer L2242 and L2801, and may therefore represent the TSP for the longer *SAP* transcript. A PCR product indicative of the TSP at -496 was also obtained with appressorial RNA. Although the RT-PCR analysis suggested that a longer transcript from a more upstream TSP was present in appressorial RNA, this was never detected by RLM-RACE. Failure to detect the TSP for the longer transcript may be due to a low abundance of full length transcripts from this TSP in the appressorial RNA samples. This could result from a lower frequency of transcript initiation from the -700 TSP in appressoria than during mycelial growth, or from RNA degradation during the multiple enzymatic reactions in the RLM-RACE procedure.

Other methods that can be employed to determine a TSP are primer extension and ribonuclease protection assays. However, neither would be more sensitive than the PCR based RLM-RACE procedure for detecting a rarely used TSP. Although the TSP for the longest transcript in appressorial RNA could not be determined directly, it was decided to continue with the reporter gene assay.

3.2 GFP-reporter gene assay

The gene encoding the jellyfish *Aequorea victoria* green fluorescence protein (GFP) has been used as a reporter gene for many years. A modified version with enhanced

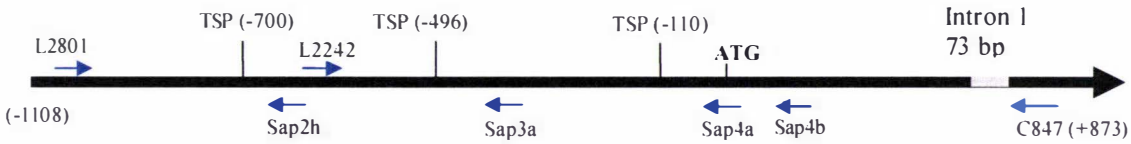
Figure 3.3 Determination of a transcription start point (TSP) by RLM-RACE

A: The ATG start codon, and the single intron within the *SAP* gene are shown. Also shown are the positions and orientation of the gene specific primers used in the RLM-RACE and three of the primers used for mapping the TSP (Fig 3.1). Three TSPs determined in this study are indicated. The sequence of the primers is given in Table 2.3. The drawing is not to scale.

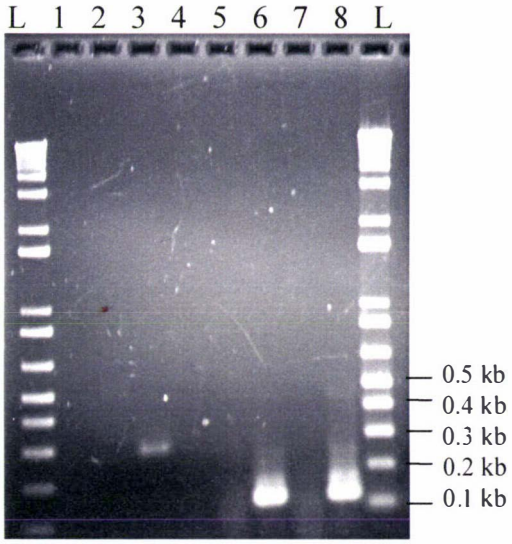
B: Electrophoretic analysis of the PCR products from RLM-RACE using appressorial RNA (lane 6) and mycelial RNA (lane 8). PCR products obtained with the 5'RACE outer primer and the primer sap4b were column purified, and reamplified with the 5'RACE inner primer and the primer sap4a. Negative controls included a no template control (lane 1), genomic DNA (lane 2), appressorial RNA (lane 3) or mycelial RNA (lane 4) treated with just CIP, appressorial RNA (lane 5) or mycelial RNA (lane 7) treated with only CIP and TAP. The size of the product in lane3 suggested that it was a non-specific product. RT reactions were performed at 42^0C for 45 min using MMLV reverse transcriptase from the RLM-RACE kit (Ambion). PCR reactions consisted of 1x *Taq* DNA polymerase buffer (Roche), 200 μM of each dNTP, 500 nM of each primer, 0.02 unit/μl of *Taq* DNA polymerase (Roche). The thermocycle conditions were 2 min at 94^0C followed by 34 cycles of 10 sec at 94^0C, 30 sec at 60^0C, 90 sec at 72^0C, and a final incubation for 5 min at 72^0C.

C: Sequence across the cDNA/adaptor oligonucleotide boundary for the PCR product shown in lane 8 (from mycelial RNA). The TSP is indicated by the vertical arrow. The sequence of the cDNA product (not shown in full) exactly matched the *SAP* promoter sequence downstream of the TSP. Sequencing result of the PCR product from lane 6 (from appressorial RNA) gave the same result (data not shown).

A


TSP (-700)
TSP (-496)
TSP (-110)
Intron 1
73 bp
L2801
L2242
ATG
(-1108)
Sap2h
Sap3a
Sap4a
Sap4b
C847 (+873)

B


L 1 2 3 4 5 6 7 8 L
0.5 kb
0.4 kb
0.3 kb
0.2 kb
0.1 kb

C

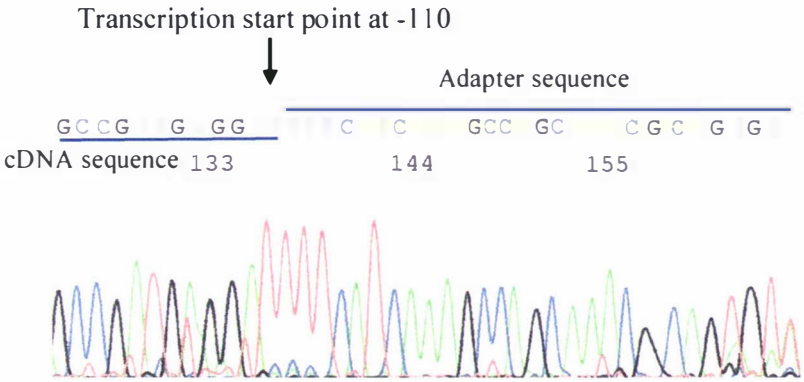

Transcription start point at -110
Adapter sequence
GCCG G GG C C GCC GC CGC G G
cDNA sequence 133
144
155

Figure 3.4 Determination of two further transcription start points (TSP) by RLM-RACE

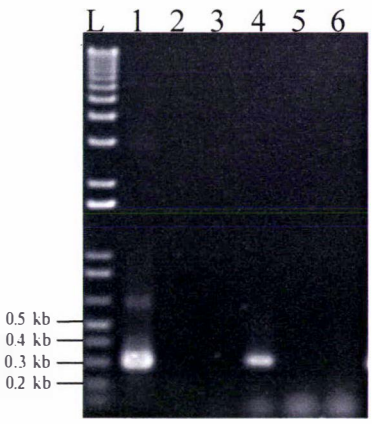
A: Electrophoretic analysis of the PCR products from RLM-RACE using appressorial RNA (lane 1) and mycelial RNA (lane 4). PCR products obtained with the 5'RACE outer primer and the primer sap4b were column-purified, and reamplified with the 5'RACE inner primer and the primer sap3a. Negative controls included a no template control (lanes 2 and 5) and genomic DNA (lanes 3 and 6). A faint band (0.65 kb) on lane 1 may be a non-specific product and was not isolated for further analysis. If it was from a true upstream TSP, it would be amplified easily when using other upstream primers, eg the primer sap2h used in panel B. RT reactions were performed at 42^0C for 45 min using MMLV reverse transcriptase from the RLM-RACE kit (Ambion). PCR reactions consisted of 1x *Taq* DNA polymerase buffer (Roche), 200 μM of each dNTP, 500 nM of each primer, 0.02 unit/μl of *Taq* DNA polymerase (Roche). The thermocycle conditions were 2 min at 94^0C followed by 34 cycles of 10 sec at 94^0C, 30 sec at 60^0C, 90 sec at 72^0C, and a final incubation for 5 min at 72^0C.

B: Electrophoretic analysis of the PCR products from RLM-RACE using mycelial RNA. PCR products obtained with the 5'RACE outer primer and the primer sap4b were purified, and reamplified with the 5'RACE inner primer and the primer sap2h (lanes 1-3) or the 5'RACE inner primer and the primer sap3a (lane 4). Negative controls included a no template control (lane 1) and genomic DNA (lane 2). RT and PCR reaction conditions were the same as described in A.

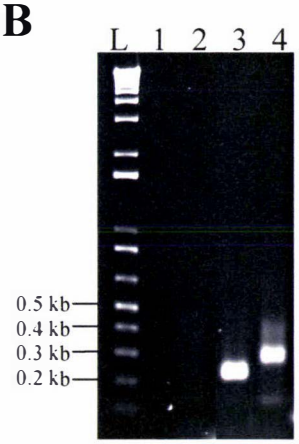
C: Sequencing was done using the primer sap3a. Sequence across the cDNA/adaptor oligonucleotide boundary for the PCR product shown in lane 1, panel A. The TSP is indicated by the vertical arrow. The sequence of the cDNA product (not shown in full) exactly matched the *SAP* promoter sequence downstream of the TSP. Sequencing of the PCR product from lane 4 gave the same result (data not shown).

D: Sequencing was done using the primer sap2h. Sequence across the cDNA/adaptor oligonucleotide boundary for the PCR product shown in lane 3, panel B. The TSP is indicated by the vertical arrow. The sequence of the cDNA product (not shown in full) exactly matched the *SAP* promoter sequence downstream of the TSP.

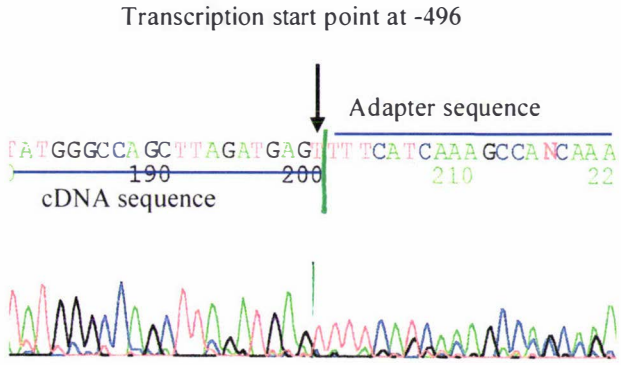
A


L 1 2 3 4 5 6
0.5 kb
0.4 kb
0.3 kb
0.2 kb

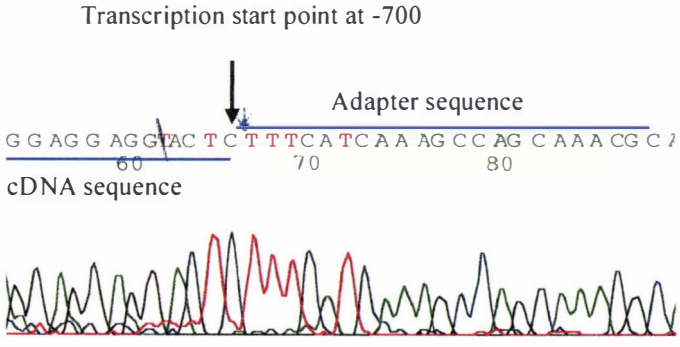
B


L 1 2 3 4
0.5 kb
0.4 kb
0.3 kb
0.2 kb

C


Transcription start point at -496
Adapter sequence
190
200
210
220
cDNA sequence

D


Transcription start point at -700
Adapter sequence
60
70
80
cDNA sequence

fluorescence was successfully used in several *Colletotrichum* species (Dumas *et al.*, 1999; Liu and Kolattukudy, 1999; Takano *et al.*, 2001), and in other fungi (Lorang *et al.*, 2001). GFP expression can be directly observed in individual cells or in a population of cells, both qualitatively and quantitatively. GFP has an advantage over other reporters (β-glucuronidase, β-galacturonidase, chloramphenicol acetyl-transferase, and firefly luciferase) that rely on cofactors or substrates for activity (Lorang *et al.*, 2001). Thus GFP was chosen as the reporter gene to use for the analysis of the *SAP* promoter.

There are several strategies available for the introduction of exogenous DNA into fungal cells (Mullins and Kang, 2001). PEG-mediated transformation of fungal protoplasts has been used for many years. More recently, *Agrobacterium*-mediated transformation, initially used for plant transformation, has been adapted for fungal transformation. Fungi that have been transformed in this way include *Colletotrichum gloeosporioides* (de Groot *et al.*, 1998). *Agrobacterium*-mediated transformation was considered to have some advantages in comparison with the conventional PEG mediated transformation. It can be used to transform fungal mycelium or conidia rather than protoplasts, whose preparation is laborious, and transformants obtained usually arise from a single copy integration event, which is desirable (Michielse *et al.*, 2005). Thus, a plasmid pFSG (Fig 3.5) in which the GFP coding sequence was placed under the control of the *SAP* promoter, was constructed. It was introduced into *Agrobacterium tumefaciens* and attempts made to transform *G. cingulata.* After a great deal of effort, no transformants were obtained and the strategy was abandoned. Instead, conventional PEG-mediated transformation of *G. cingulata* was performed using one or other of two constructs (plasmids pBHG and pBHS, Fig 3.5) in which the GFP coding sequence was placed under the control of either the *A.nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter (pBHG) or the *SAP* promoter (pBHS).

3.2.1 GFP-reporter plasmid construction

Firstly, the 3.2 kb *Hin*dIII fragment from the plasmid pFAT-3gfp, containing the hygromycin resistance cassette, was cloned into the *Hin*dIII site of pBluescript giving the plasmid pBH5 (Fig 3.5). The 2.3 kb *Not*I fragment from pFAT-3gfp, which

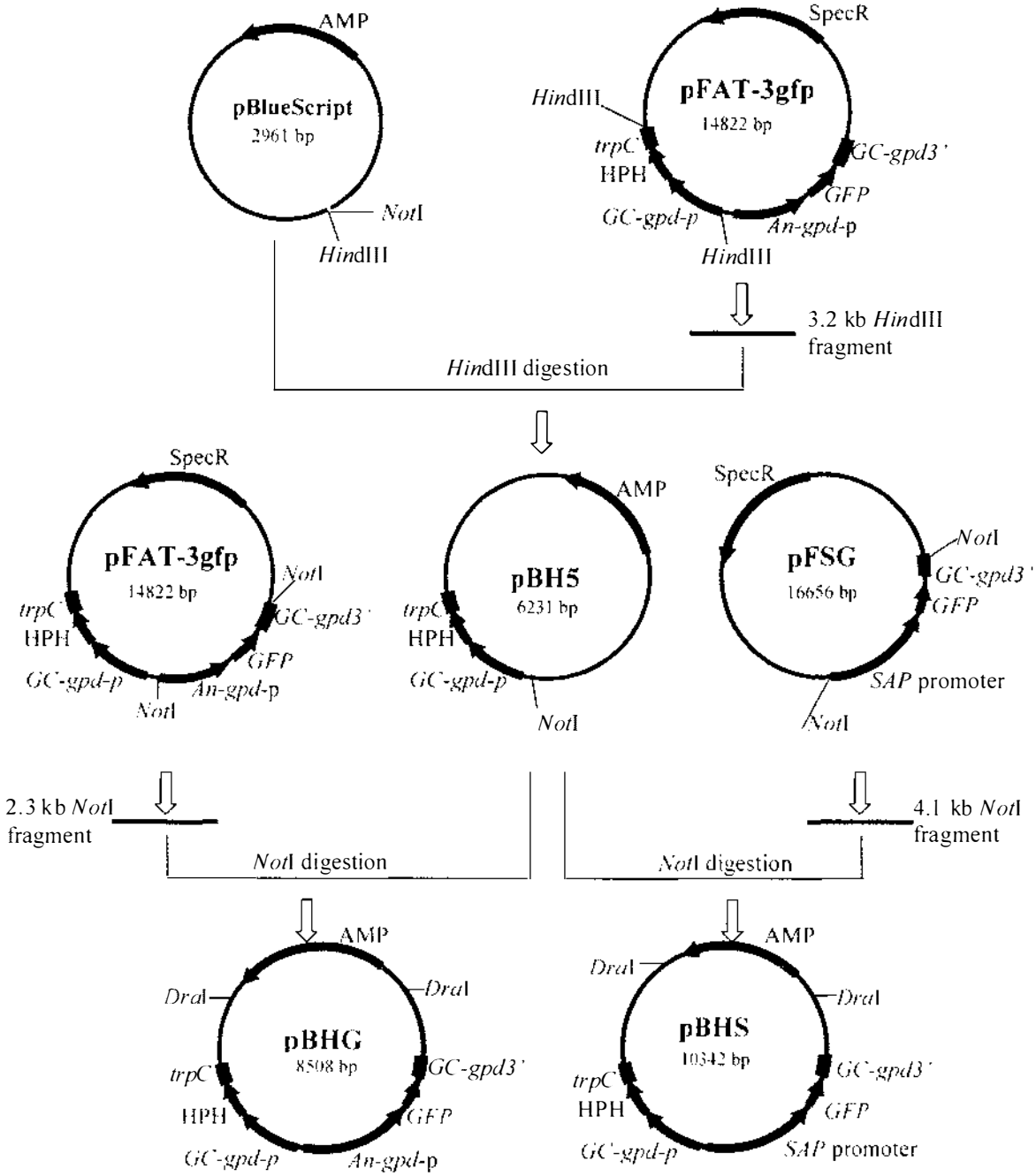


Figure 3.5 GFP reporter gene construction

The two reporter plasmids (pBHG and pBHS) were made as shown. Abbreviations used are as follows: *An-gpd-p*, *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter; *GC-gpd-p*, *G. cingulata* glyceraldehyde-3-phosphate dehydrogenase promoter; *trpC*, *Aspergillus nidulans trpC* terminator; *GC-gpd3'*, *G. cingulata gpd* terminator; HPH, hygromycin B resistant gene; GFP, GFP coding sequence; AMP, Ampicillin resistance gene; SpecR, spectinomycin resistance gene cassette.

contained the GFP coding sequence under the control of the *Aspergillus nidulans gpd* promoter, was then cloned into the *Not*I site of pBH5 resulting in plasmid pBHG (Fig 3.5). In a separate cloning reaction, the 4.1 kb *Not*I fragment from pFSG, which contained the GFP coding sequence under the control of the *G. cingulata SAP* gene promoter (3 kb), was also cloned into the *Not*I site of pBH5 resulting in plasmid pBHS (Fig 3.5).

A nested deletion series of the *SAP* gene promoter was also constructed by exonuclease III digestion of pBHS (Fig 3.6). Exonuclease III digests DNA with a recessed 3' terminus (Henikoff, 1984). The plasmid pBHS was therefore digested by *Kpn*I which produced a protruding 3' terminus (protective from exonuclease III digestion) and *Cla*I which produced a recessed 3' terminus (susceptible for exonuclease III digestion). The exonuclease III digestion, re-ligation and transformation were carried out as described in Section 2.5.3. Plasmids which contained 2.3 kb, 1.5 kb, 0.8 kb, 0.5 kb and 0.2 kb of the *SAP* promoter were selected by restriction digestion (data not shown).

3.2.2 Transformation of *G. cingulata*

Prior to transformation of *G. cingulata*, the plasmids were linearised by *Dra*I digestion to avoid single crossover homologous recombination between the fungal genome and either the *SAP* promoter or the *G. cingulata gpd* promoter region. This strategy was designed to promote random integration events. The type of integration that occurred, however, was not critical so long as the GFP coding region remained associated with the promoter present in the plasmid.

Transformation was carried out using protoplasts prepared from both mycelium and conidia as described in Section 2.10. Several protocols were tested and the final protocol used throughout this study was described in Section 2.10.3. The concentration of hygromycin B was found to be critical for successful selection of the transformants. The optimal concentration of hygromycin B was determined to be 200 μg/ml. A lower concentration (100 μg/ml) resulted in too many untransformed colonies growing, and a

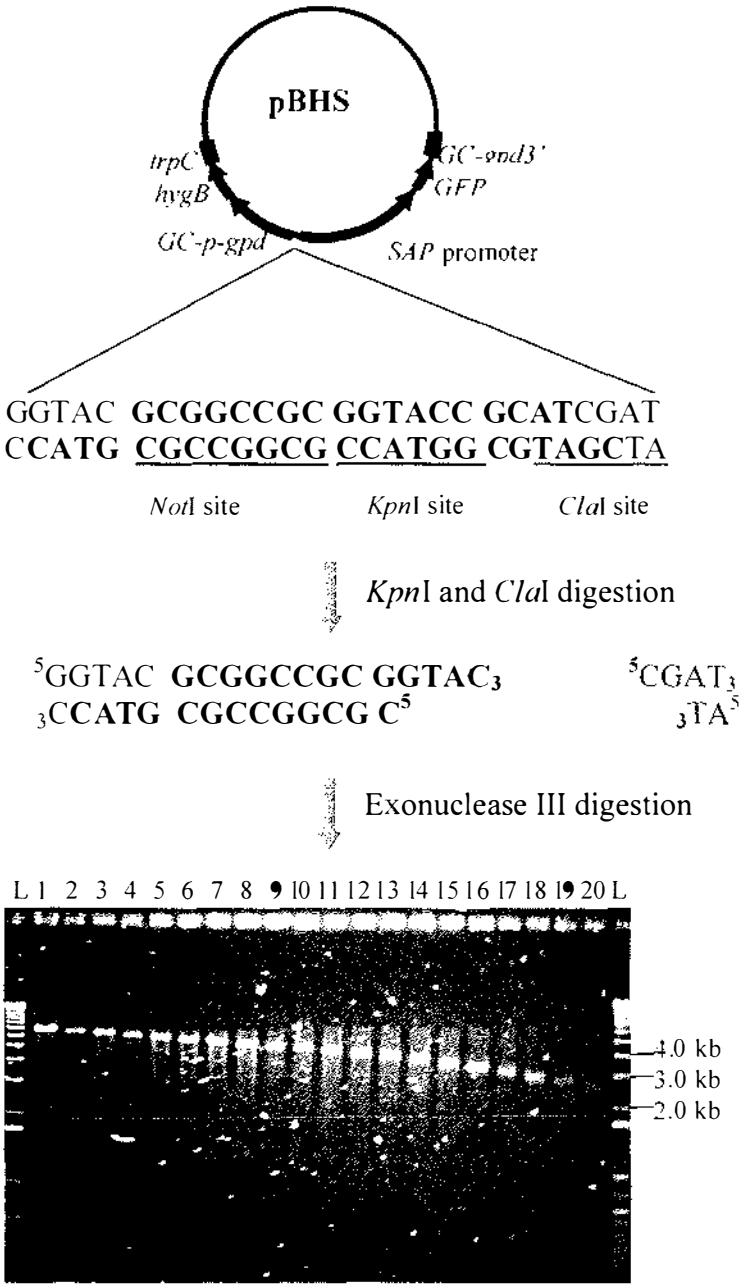


Figure 3.6 Generation of a *SAP* promoter deletion series for the *SAP* promoter-*GFP* reporter plasmid

The plasmid pBHS was digested with *Kpn*I and *Cla*I. Exonuclease III digestion followed by self ligation of the products generated a series of plasmids with a deletion at the 5' end of the *SAP* promoter. Exonuclease III digests were stopped at 30 second intervals from zero to 10 minutes (lane 1 to 20) and examined by agarose gel electrophoresis. Lanes L contained the 1 kb plus DNA ladder as the size marker. Selected samples were re-ligated and the plasmids used to transform *G. cingulata*.

higher concentration (300 μg/ml) resulted in no colonies growing through the agar overlay.

Transformants were obtained using the constructs described in Section 3.2.1. Single spore derived colonies were selected as described in Section 2.10.4.

3.2.3 Characterisation of transformants by PCR

Four independent *G. cingulata* transformants BHS1 to BHS4, derived from plasmid pBHS, were characterized by PCR using four pairs of primers as shown in Fig 3.7. The first pair of primers, T7 and L2079 would amplify a 1.4 kb product from the T7 primer binding site on the pBluescript backbone of the plasmid to a site within the *SAP* promoter. The second pair of primers, GFP1 and F2019 would amplify a 1.3 kb product from within the GFP coding region to a different site within the *SAP* promoter. The third pair of primers, sap1a and HygF would amplify a 4.8 kb product from within the *SAP* promoter to a site within the hygromycin resistance cassette. The fourth pair of primers, HygR and T3 would amplify a 1.6 kb product from within the hygromycin resistance cassette to the T3 primer binding site on the pBluescript backbone of the plasmid. DNA from all four transformants gave the expected PCR product with each of the four primer pairs, consistent with integration of the linearised plasmid in its entirety (Fig 3.7).

Eight independent *G. cingulata* transformants BHG1 to BHG8, obtained using plasmid pBHG, were characterized by PCR using a similar strategy to that described above. Two primer pairs were used in this analysis (Fig 3.8). The first pair of primers, T7 and GFP3132F would amplify a 1.0 kb product from the T7 binding site on the pBluescript backbone to a site within the GFP coding region. The second pair of primers, HygR and T3 would amplify a 1.6 kb product from a site within the hygromycin resistance cassette to the T3 primer binding site on the pBluescript backbone. As shown in Fig 3.8, these primer pairs amplified the predicted products.

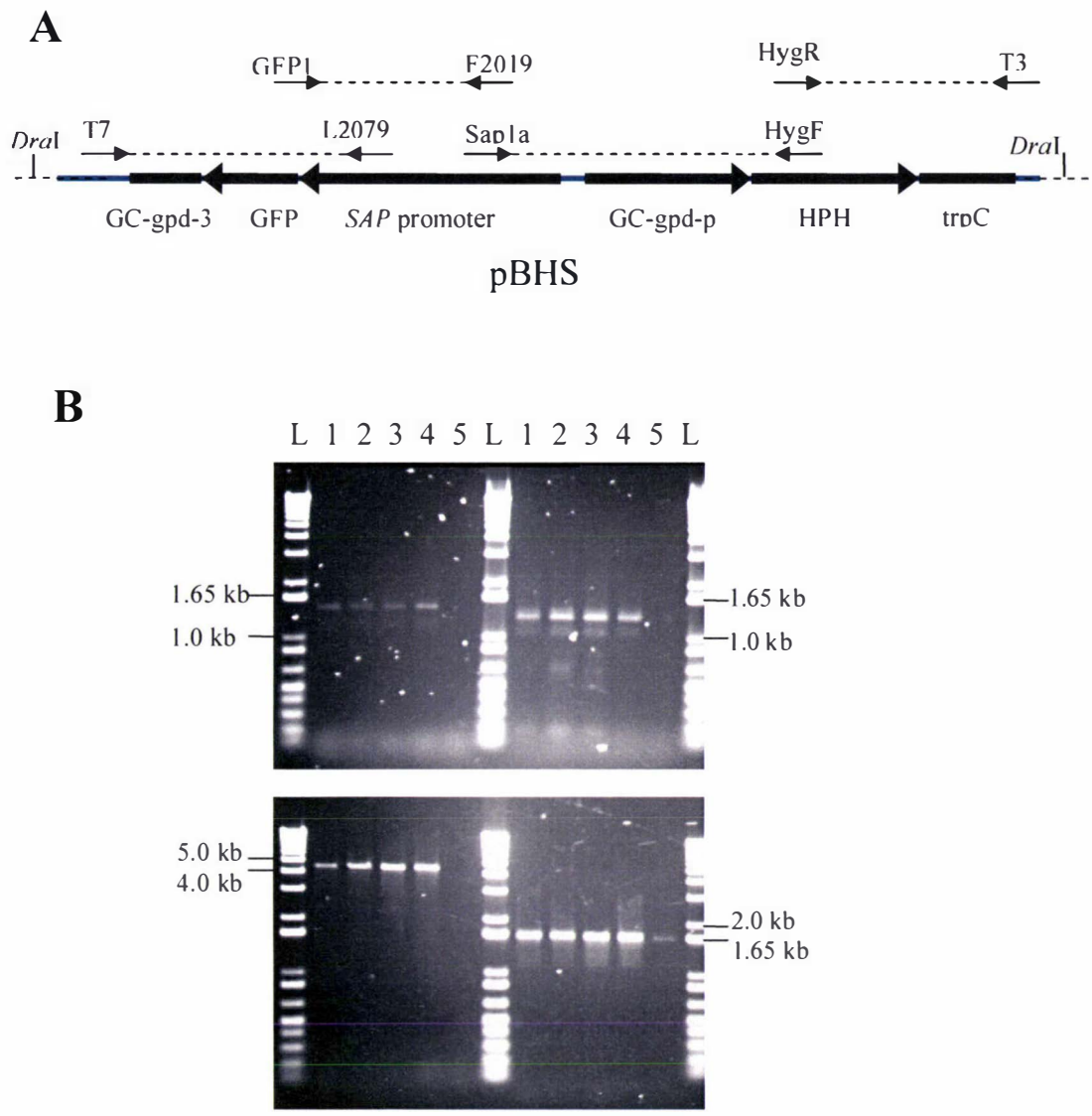


Figure 3.7 Characterisation of *G. cingulata* transformed with the plasmid pBHS

A: Position of the primers used in the PCR for characterisation of the transformants obtained with plasmid pBHS. The drawing is not to scale.

B: Electrophoretic analysis of the PCR products obtained using the primer pairs shown in panel A. The predicted products were observed for all four transformants. Upper left panel, primers T7 and L2079; upper right panel, primers GFP1 and F2019; lower left panel, primers sap1a and hygF; lower right panel, primers hygR and T3. Genomic DNA from BHS1 (lane 1), BHS2 (lane 2), BHS3 (lane 3), and BHS4 (lane 4) was used as the template in these PCR reactions. The negative control was a PCR reaction with no template (lane 5).

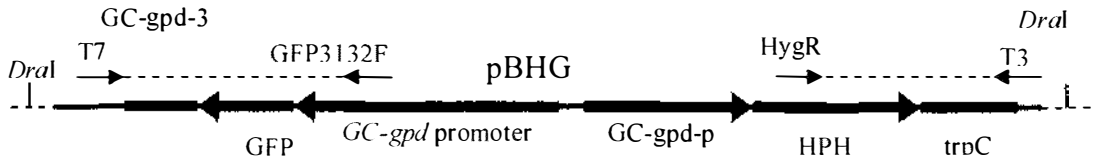
Figure 3.8 Characterisation of *G. cingulata* transformed with the plasmid pBHG

A: Position of the primers used in the PCR for characterisation of the transformants obtained with plasmid pBHG. The drawing is not to scale.

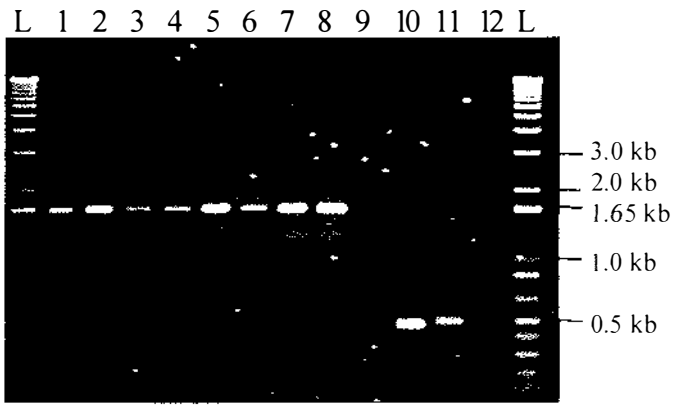
B: Agarose electrophoresis of the PCR products obtained using the primer pairs T7/GFP3132. Genomic DNA from transformants BHG1 to 8 (lane 1-8) as template (expected size: 1.65 kb). Wild type genomic DNA was used as the negative control (lane 9). The *G. cingulata Actin* (lane 10) *and sod* (lane 11) primers were used to amplify a fragment from wild type genomic DNA as the positive control for PCR reaction. Lane 12 was no-template control for the *sod* primers.

C: Agarose electrophoresis of the PCR products obtained using the primer pairs T3/HygR. Genomic DNA from transformants BHG1 to 8 (lane 1-8) as template (expected size: 1.0 kb). Wild type genomic DNA (lane 9) and a no-template reaction (lane 10) were used as negative controls.

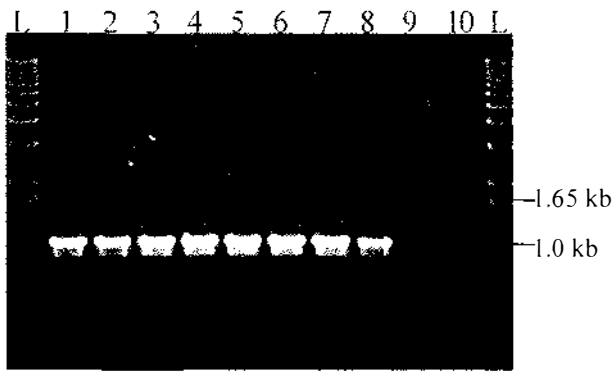
A


GC-gpd-3
T7
GFP3132F
pBHG
HygR
T3
DraI
DraI
GFP
GC-gpd promoter
GC-gpd-p
HPH
trpC

B


L 1 2 3 4 5 6 7 8 9 10 11 12 L
3.0 kb
2.0 kb
1.65 kb
1.0 kb
0.5 kb

C


L 1 2 3 4 5 6 7 8 9 10 L
1.65 kb
1.0 kb

The twelve transformants characterized above were examined for GFP fluorescence as described in the next section. Transformants obtained from the deletion series of the *SAP* promoter were not characterized by PCR analysis, because the fluorescence of these transformants was no different to that of the four transformants obtained with plasmid pBHS.

3.2.4 Fluorescence of the transformants

Appressoria of all strains (wild type, pBHG transformants and pBHS transformants, including the deletion series) were induced on wax-coated glass slides for 16 hours and examined with a fluorescence microscope. Images from the wild type, BHS1, BHS2, BHG1 and BHG2 transformants are shown in Fig 3.9. No noticeable difference in the intensity of fluorescence was observed in any of the transformants compared to the wild type. Mycelium of all strains was grown on PDA medium and on medium containing BSA as the sole nitrogen source, and fluorescence of the mycelium was examined at 2, 4, and 7 days. As in the case of appressoria, no noticeable difference in the intensity of fluorescence was observed for the transformants compared to the wild type (Fig 3.10).

3.2.5 Examination of *GFP* transcription in the transformants

Since no difference in fluorescence was observed between the wild type strain and the transformants, *GFP* expression was examined by RT-PCR. Appressorial and mycelial RNA was isolated from two pBHS derived transformants (BHS1 and BHS2), and two pBHG derived transformants (BHG1 and BHG2). cDNA was synthesized using random hexamer primers and then PCR, using the primer pair GFP1 and GFP3132, was performed to amplify a 207 bp fragment of the *GFP* transcript. The *GFP* transcript was not detected in RNA from either appressoria or mycelium of transformant BHS1. This was possibly due to poor RNA quality, or poor performance in the RT-PCR, because there were also no products observed in the positive control using the *G. cingulata sod* (superoxide dismutase) gene primers. The *GFP* gene was found to be expressed in transformants BHS2, BHG1 and BHG2 as shown in Fig 3.11. Although the amount of *GFP* transcript was not able to be quantitatively determined from the RT-PCR, there

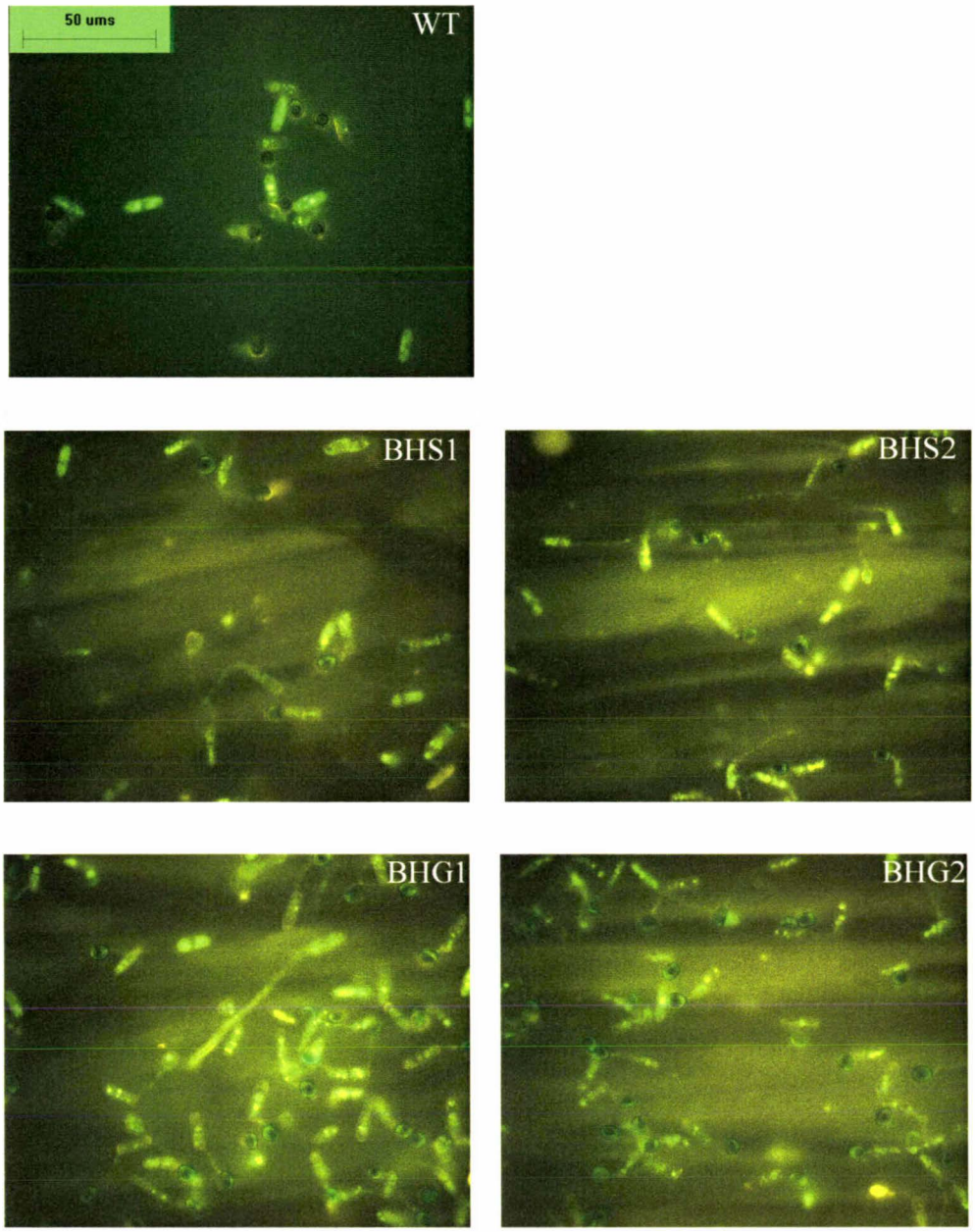


Figure 3.9 Fluorescence of appressoria

Appressoria of the wild type, two transformants obtained using the plasmid pBHG, and two transformants obtained using the plasmid pBHS were induced on wax coated glass slides for 16 hours and then examined using a fluorescence microscope with excitation wavelength at 485 nm, dichroic mirror at 510 nm and emission wavelength at 535 nm. The exposure time for each sample was the same in order to make comparison of different samples possible. No noticeable differences in the intensity of fluorescence were observed.

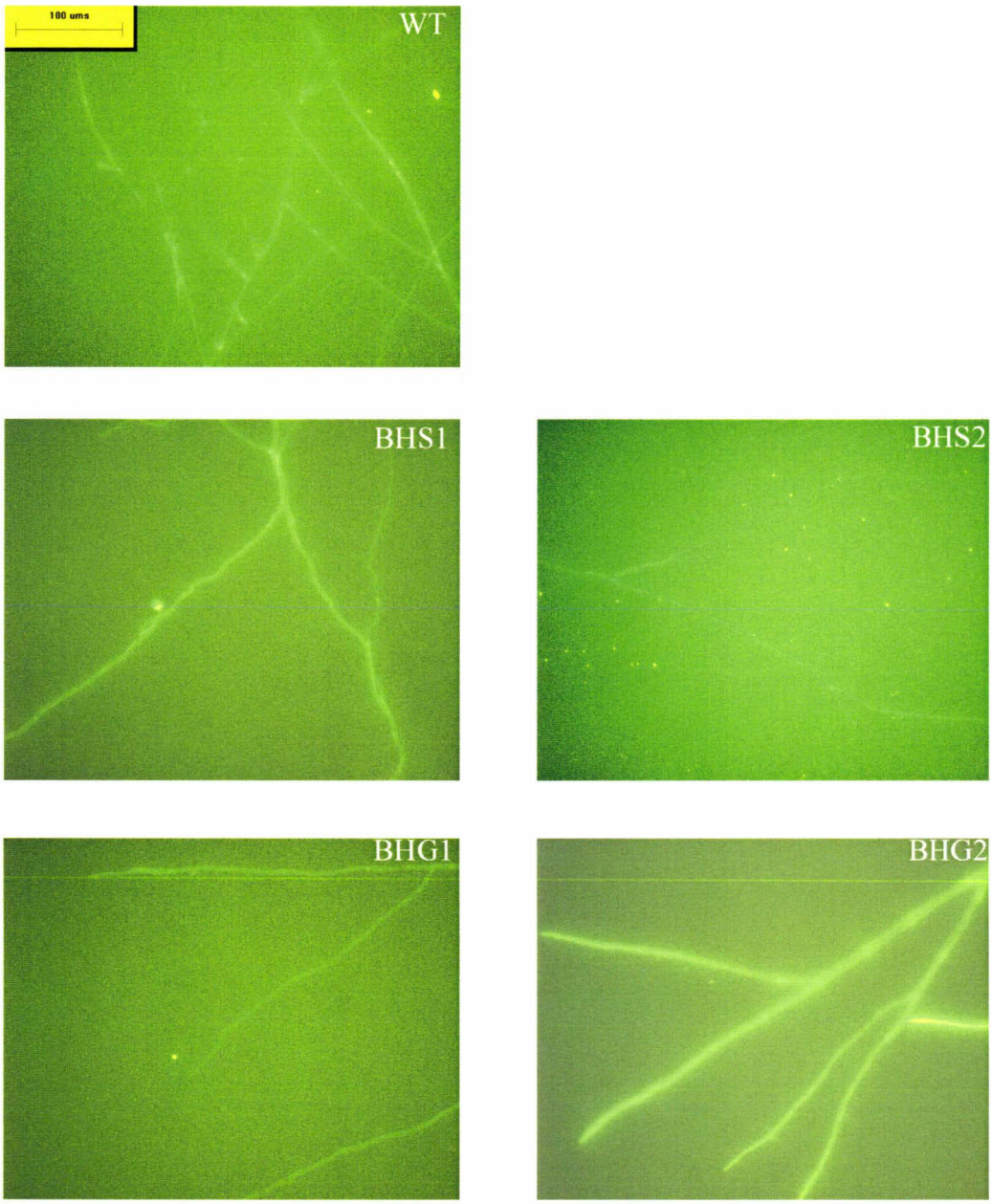


Figure 3.10 Fluorescence of mycelia

Mycelium of the wild type, two transformants obtained using the plasmid pBHG, and two transformants obtained using the plasmid pBHS were grown on the plates using BSA as solo nitrogen source. Plates at 2, 4, 7 days were examined with a fluorescence microscope with excitation wavelength at 485 nm, dichroic mirror at 510 nm and emission wavelength at 535 nm. Exposure time for each sample was set to be same in order to make comparison. No noticeable differences in the intensity of fluorescence were observed.

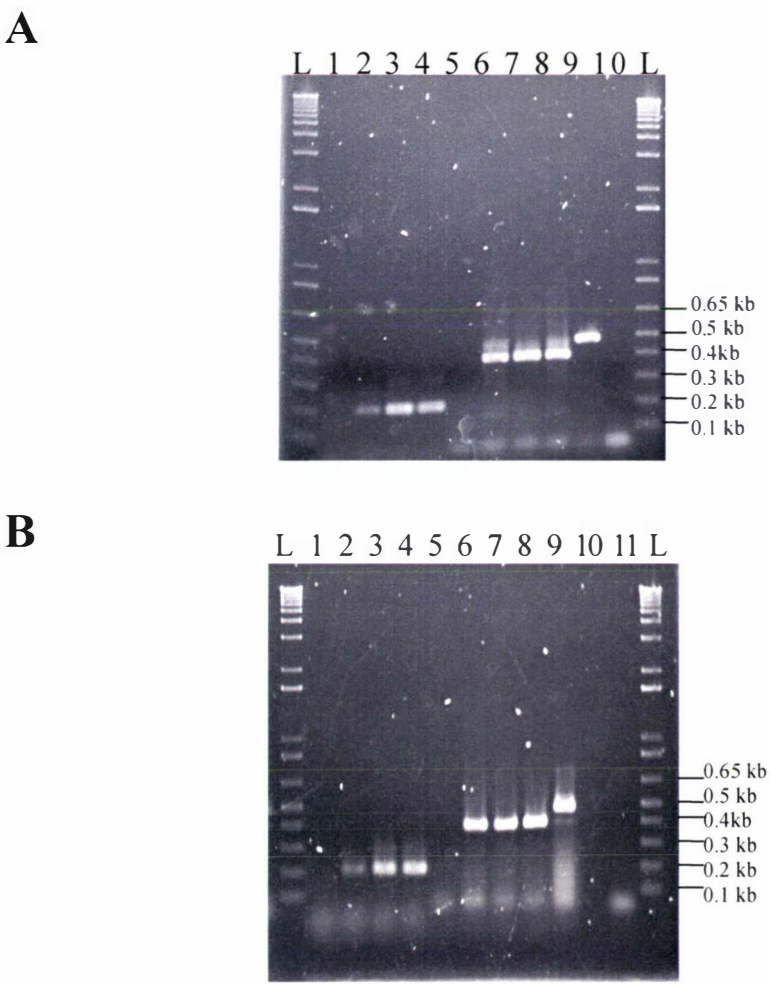


Figure 3.11 Detection of the *GFP* transcript in transformants by using RT-PCR

Transcription of the *GFP* gene in transformants was examined by RT-PCR. RNA was isolated from either appressoria or mycelia of four transformants and treated with DNaseI. cDNA was synthesized using random hexamer primers and the PCR was carried out using the *GFP* gene primer pair GFP1/GFP3132 (lanes 1-4), or the *sod* gene primer pair sodF/sodR (lanes 5-9) for both mycelial (panel A) and appressorial (panel B) cDNA. Products of the predicted size were obtained with RNA from transformant BHS2 (lanes 2, 6), BHG1 (lanes 3, 7) and BHG2 (lanes 4, 8), but not product for transformant BHS1 (lanes 1, 5). Amplification of genomic DNA using the primer pair sodF/sodR produced a product (lane 9) which was bigger than that from cDNA (lane 6-8) because an intron was present. The negative controls were a no template PCR reaction (lane 10) or genomic DNA as template (lane 11, panel B only) using the primer pair GFP1/ GFP3132.

seemed to be a reasonable level of expression by comparison with the intensity of the PCR product from the *sod* gene transcript. Expression of the *G. cingulata sod* gene was shown to be constitutive during vegetative growth and appressorium formation (Clark, 1998). That no differences in fluorescence intensity were observed between the wild type and these transformants may have resulted from the relatively low level of *GFP* transcripts, or failure to form functional GFP protein, or a relatively strong background fluorescence detected in the wild type.

3.3 Summary

This project began with the characterisation of the *SAP* promoter. The first experiment was to determine the transcription start point for the long transcript of the *SAP* promoter, which was thought to be expressed exclusively during the early stage of appressorium formation. RT-PCR revealed that this long transcript was also expressed in the later stages of appressorium formation, and during vegetative growth of mycelium. By using the RLM-RACE strategy, three transcription start points for the short, middle, and long transcripts were determined with RNA from vegetative mycelium. Two of these transcription start points were also shown to be used during appressoium formation. The evidence from RT-PCR suggested that the longer transcript was also produced by appressoria.

The second experiment involved the construction of *SAP*-GFP reporter plasmids and their integration into the *G. cingulata* genome. Although it was not possible to transform *G. cingulata* using *Agrobacterium*-mediated transformation, PEG-mediated transformation with a second series of constructs was successful. Even though a reasonable level of *GFP* expression was observed in the RT-PCR experiments, however, no differences in fluorescence intensity were distinguishable between the wild type and GFP reporter transformants. Therefore, no further attempts to study the *SAP* promoter were made.

Chapter four

Cloning and characterisation of the *StuA* gene

Since characterization of the *G. cingulata SAP* promoter had not provided sufficient information to continue with that strategy, the candidate gene approach was adopted as an alternative way to study the genes that are involved in appressorium formation and function. The APSES family regulates developmental transitions, although this transition differs in each fungal species. One member of this family has also been shown to be a downstream target of the cAMP signal transduction pathway in *Candida albicans* (Section 1.3.5.2). Appressorium development is a developmental transition involving morphological changes and it is controlled by the cAMP and MAPK signal transduction pathways. It was therefore considered likely a *G. cingulata* APSES transcription factor might be involved in appressorium formation and function. Six APSES genes from different fungi have been characterized and another two gene sequences are available. This information was used to isolate a member of this family from *G. cingulata* by PCR using degenerate primers. The gene isolated in this way was named *StuA*.

4.1 Cloning of a 5' fragment of the *G. cingulata StuA* gene

Cloning of the *G. cingulata StuA* gene began with the PCR amplification of a 5' fragment from the conserved APSES domain. Two degenerate forward primers (DF1 and DF2, Appendix 3) and two degenerate reverse primers (DR1 and DR2, Appendix 3) were designed for the PCR based on the alignment of the protein sequences of the APSES gene family (Fig 4.1). The predicted size of the PCR products from the different combinations of these primers was between 200 and 400 base pairs. In spite of the short (45 second) elongation time used for the reaction, multiple bands ranging in size up to 2 kb were observed following agarose gel electrophoresis of the PCR products. Four bands were recovered from the gel (Fig 4.2), re-amplified and sequenced. BLASTX analysis of one sequence (432 bp, named StuA432) identified an open reading frame that encoded a polypeptide sequence highly similar to proteins belonging to the APSES family (Fig 4.3). The other products were not derived from APSES domains. Alignment of the StuA432 nucleotide sequence with that of other genes in the APSES family also revealed the presence of two putative introns in this sequence (Fig 4.4). In order to

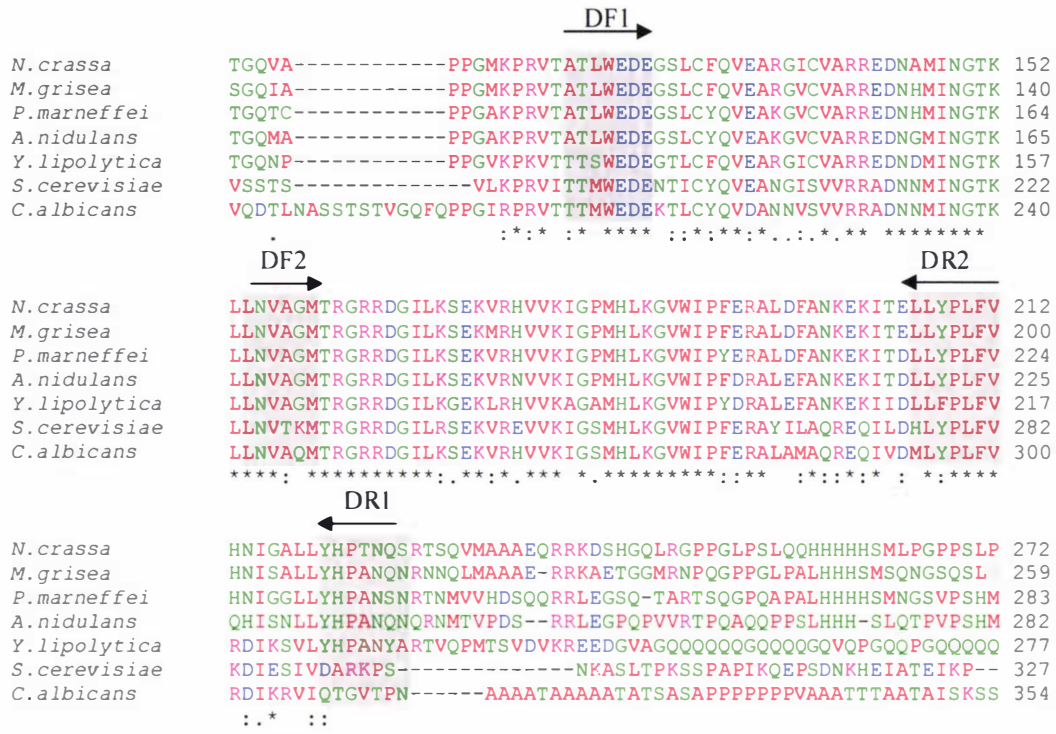


Figure 4.1 Design of degenerate primers for cloning the *G. cingulata StuA* gene

The polypeptide sequences from all known fungal APSES domain were aligned using the program ClustalW (http://www.ebi.ac.uk/clustalw/). The proteins used were the *S. cerevisiae PHD1* gene product (GenBank accession number U05241, Gimeno and Fink, 1994), the *C. albicans EFG1* gene product (GenBank accession number Z32687, Stoldt *et al.*, 1997), the *Yarrowia lipolytica MGF1* gene product (GenBank accession number AJ007730), the *Penicillium marneffei StuA* gene product (GenBank accession number AF436076, Borneman *et al.*, 2002), the *Aspergillus nidulans StuA* gene product (GenBank accession number M83569, Miller *et al.*, 1992), the *Neurospora crassa Asm-1* gene product (GenBank accession number U51117, Aramayo and Metzenberg, 1996) and the *Magnaporthe grisea* MG00692 hypothetical protein (http://www.broad.mit.edu/annotation/fungi/magnaporthe/).

A "*" indicates that the residues in that column are identical in all sequences in the aligment. A ":" indicates that conserved substitutions are found at that position in the alignment and A "." indicates that semi-conserved substitutions are observed.

The sequences selected for designing degenerate primers are shaded.

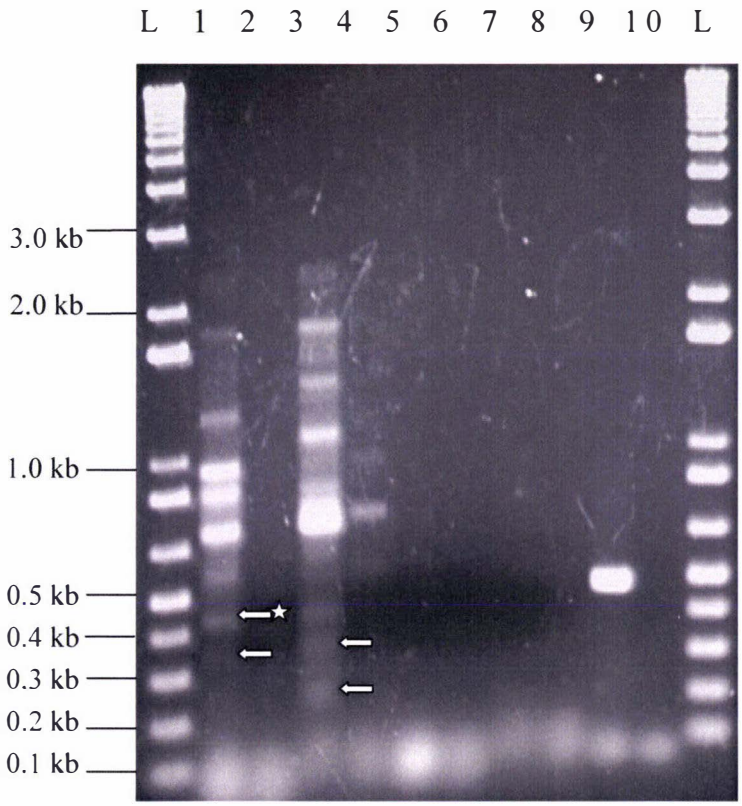


Figure 4.2 PCR products from amplification of *G. cingulata* DNA using degenerate primers designed to the APSES domain

The PCR contained either genomic DNA (10 ng) as template (lanes 1 to 4 and lane 9) or no template (lanes 5 to 8 and lane 10). The PCR products were analyzed by agarose gel electrophoresis. Degenerate primer pairs were used in the following combinations: lanes 1 and 5, DR2 and DF1; lanes 2 and 6, DR1 and DF2; lanes 3 and 7, DR2 and DF1; lanes 4 and 8, DR1 and DF2. A pair of primers from the *G. cingulata sod* gene was used as a control for the PCR (lane 9, genomic DNA as template; and lane 10, no template). Lanes L contained the 1 kb plus DNA ladder (Invitrogen) as size standard. Bands indicated by arrows were recovered from the gel, re-amplified and sequenced. The arrow with a star indicates the band from which the StuA432 fragment was obtained.

Figure 4.3 Alignment of the deduced StuA432 protein sequence with fungal APSES gene products

The deduced StuA432 protein sequence was aligned with APSES gene products using the program ClustalW. Only that part of the APSES proteins that aligned with the predicted *G. cingulata* protein sequence is shown. Symbols below the alignment have the same significance as in Fig 4.1. The APSES proteins used in this alignment are also the same as listed in Fig 4.1.

Figure 4.4 Nucleotide sequence and conceptual translation of the StuA432 fragment

Positions of the two primers used to amplify this fragment are underlined. The location of two putative introns is shaded. The orientation and location of two primers (iv_up primer and iv_down primer) designed for subsequent PCR, designed to obtain additional sequence, are underlined. The location of selected restriction sites is also shown. Arrows indicate the orientation of the primers.

obtain additional sequence of the *G. cingulata* gene, this 432 bp sequence was used to design primers (Fig 4.4) for two different PCR strategies.

The successful approach was based on the single specific primer PCR strategy in which the template is restriction-digested genomic DNA that has been ligated to a plasmid but not cloned (Shyamala and Ames, 1989). Genomic DNA was digested with the restriction enzymes *Hin*dIII, *Pst*I, and *Sac*I. The digests were ligated into pBlueScript II KS that had been cut with the same restriction enzyme. The ligation mixture was used as template for PCR containing one primer designed to anneal to the StuA432 sequence (Fig 4.4) and one of two vector-specific primers, either the T7 or T3 primer. Both vector-specific primers were used because the inserted restriction fragment could be ligated in either orientation with respect to the vector sequence.

In the PCR reaction using template prepared from the *Sac*I digest, the major product from primer pair iv-up/T7 was slightly smaller than that from the iv-up/T3 (Fig 4.5), suggesting that, although these two products arose from different templates, those templates contained a common insert which had been ligated to pBlueScript II KS in opposite orientations. The size difference of these two products resulted from the different distances between the *Sac*I site and the T7 or T3 primer binding sites in these two templates (Fig 4.6). These two PCR products were recovered from the gel, re-amplified and sequenced. Overlapping sequences were obtained that suggested the PCR product arose from a 1.7 kb *Sac*I fragment that, as expected, contained part of the StuA432 fragment. Alignment of the conceptual translation of the 1.7 kb *Sac*I fragment with the APSES transcription factors from *P. marneffei* (StuA), *A. nidulans* (StuA), *N. crassa* (Asm-1) and *M. grisea* (MG00692 hypothetical protein) revealed that this fragment also contained a possible translation start site. PCR using the other templates did not show a similar pattern (Fig 4.5) and was not taken further. At the same time, inverse PCR was attempted using self ligated restriction digests of genomic DNA as template. The restriction enzymes used were *Bam*HI, *Eco*RI, *Sal*I, and *Xho*I. No products were obtained and the inverse PCR strategy was not pursued further.

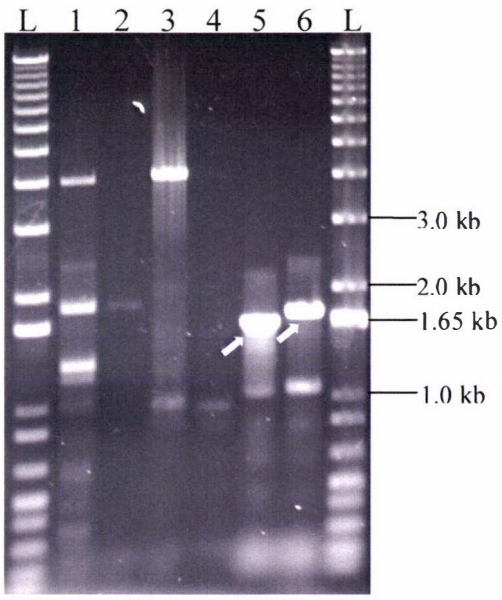
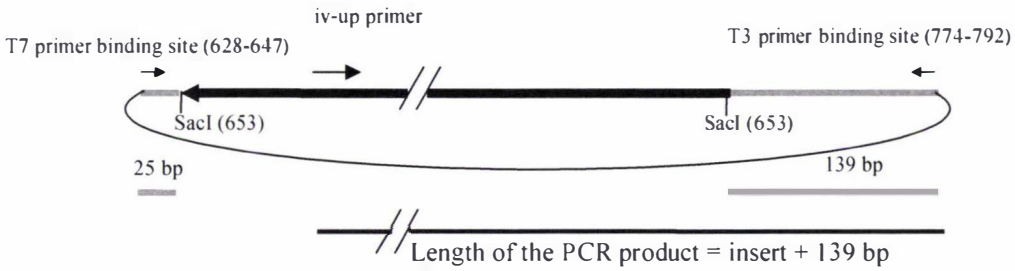


Figure 4.5 Analysis of PCR products obtained using the single specific primer PCR strategy

Following restriction digestion of genomic DNA and ligation of the products into the vector pBlueScript, the ligation reaction mixture was used as template in a PCR reaction with the following primer pairs: T7/iv_down (lane 1 and lane 3), T3/iv_down (lane 2 and lane 4), T7/iv_up (lane 5), and T3/iv_up (lane 6). Restriction enzymes used to digest the genomic DNA were: *Pst*I (lane 1 and lane 2), *Hin*dIII (lane 3 and lane 4), and *Sac*I (lane 5 and lane 6). PCR conditions were 1 cycle of 1 min at 94^0C, 34 cycles of 10 sec at 94^0C, 30 sec at 60^0C and 3 min at 72^0C, and a final incubation for 5 min at 72^0C. The PCR products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining. The 1 kb plus DNA ladder (lanes L) was used as size standard. The two PCR products referred in the text are indicated by arrows.

A



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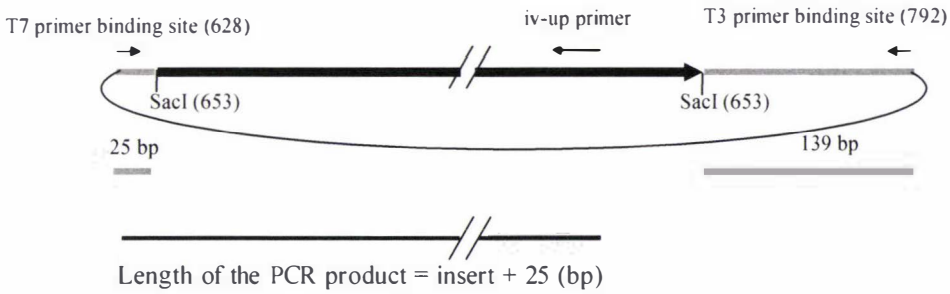


Figure 4.6 Strategy for identification of a specific PCR product based on the single specific primer PCR method

Ligation of *Sac*I digested genomic DNA into pBlueScript would result in two different templates (panels A and B). If the ligation mixture was used as template in separate PCR reactions using either the primer pair T3/iv_up (panel A) or the primer pair T7/iv_up (panel B), then the size of the two PCR products would differ because of the different distances between the T3 or T7 primer binding sites and the *Sac*I site in the vector. The two products, that differ in length by 114 bp, might be distinguished by agarose gel electrophoresis. The position of the *Sac*I site and the T3/T7 primer binding sites are shown (pBlueScript KS numbering).

Using the sequence information obtained from these two PCR products (StuA432 and the 1.7 kb *Sac*I fragment), two primers (1_24F and 580_up, both containing *Bgl*II restriction sites at their 5' ends to facilitate cloning) were designed to amplify a 595 base pair fragment (named the StuA595 fragment) from the ORF starting from the possible translation start codon. The single PCR product obtained with these primers was cloned into the vector pGEM-T Easy to generate a plasmid designated pGT595.

The 595 bp *Bgl*II fragment was used as the probe in a Southern blot analysis of *G. cingulata* genomic DNA (Fig 4.7). Only a single hybridizing band was observed for the *Bam*HI, *Eco*RI, *Sac*I, *Sal*I and *Xho*I digests. As expected, two hybridizing bands were observed in the *Pst*I and *Hin*dIII digests because these cutting sites were present within the region that hybridized to the StuA595 fragment. Based on the relative length of sequence to which the probe would hybridize, the different intensity of the hybridizing bands suggested that the 12 kb *Hin*dIII band and 2.0 kb *Pst*I band contained the 5' end of the probe whereas 5 kb *Hin*dIII band and 1.5 kb *Pst*I band contained the 3' end of the probe. Taken together, these results showed that there is a single copy of the *StuA* gene in the *G. cingulata* genome. The size of the hybridizing band in the *Sal*I and *Xho*I digests was 5.3 kb and 4.1 kb, respectively. It was considered that there was a good chance that these two fragments, either individually or together, would contain the entire gene because the estimated size of the *StuA* gene was 2 kb. Therefore, two subgenomic libraries, using either size-fractionated *Sal*I or *Xho*I digests were constructed, using the vector pBlueScript II KS, in order to clone the entire gene.

These libraries were screened by PCR using the same primer pair (1_24F and 580_up) that had been used to amplify the StuA595 fragment for the Southern analysis. One clone, pXho41, containing a 4114 base pair *Xho*I fragment, was identified by PCR screening. The *Xho*I insert was mapped (Fig 4.8) and sequenced. It contained 2.9 kb of the promoter and 1.2 kb of the *StuA* coding region. This was still only part of the open reading frame. From the Southern blot results shown in Fig 4.7 and the positions of the *Sal*I and *Hin*dIII sites in the pXho41 insert, the location of adjacent downstream *Sal*I and *Hin*dIII sites was deduced (Fig 4.8). Since the 5 kb *Hin*dIII fragment would provide more downstream sequence data than the 5.3 kb *Sal*I fragment, a subgenomic library

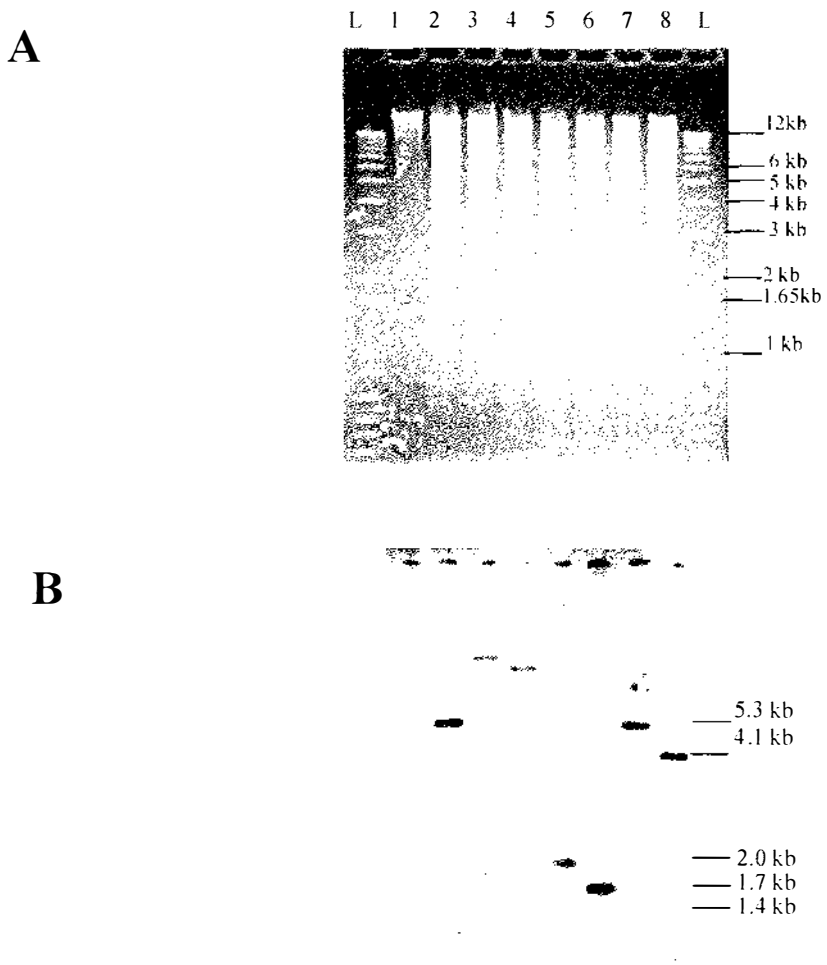


Figure 4.7 Southern blot analysis of *G. cingulata* genomic DNA

Panel A: Agarose gel electrophoresis of *G. cingulata* genomic DNA. About 10 μg of digested DNA was loaded in each lane. Lane 1, uncut DNA; lane 2, *Bam*HI digest; lane 3, *Eco*RI digest; lane 4, *Hin*dIII digest; lane 5, *Pst*I digest; lane 6, *Sac*I digest; lane 7, *Sal*I digest; lane 8, *Xho*I digest.

Panel B: Autoradiograph of the Southern blot of the gel shown in panel A probed with digoxigenin-11-dUTP labeled StuA595 fragment. Stringent wash (0.2 x SSC) was applied after hybridisation. The estimated size for each hybridizing band was as follows: *Bam*HI digest, 5.5 kb; *Eco*RI digest, 14 kb; *Hin*dIII digest, upper band was 12 kb and lower band was 5 kb; *Pst*I digest, upper band was 2.0 kb and lower band was 1.4 kb; *Sac*I digest, 1.7 kb; *Sal*I digest, 5.3 kb; *Xho*I digest, 4.1 kb.

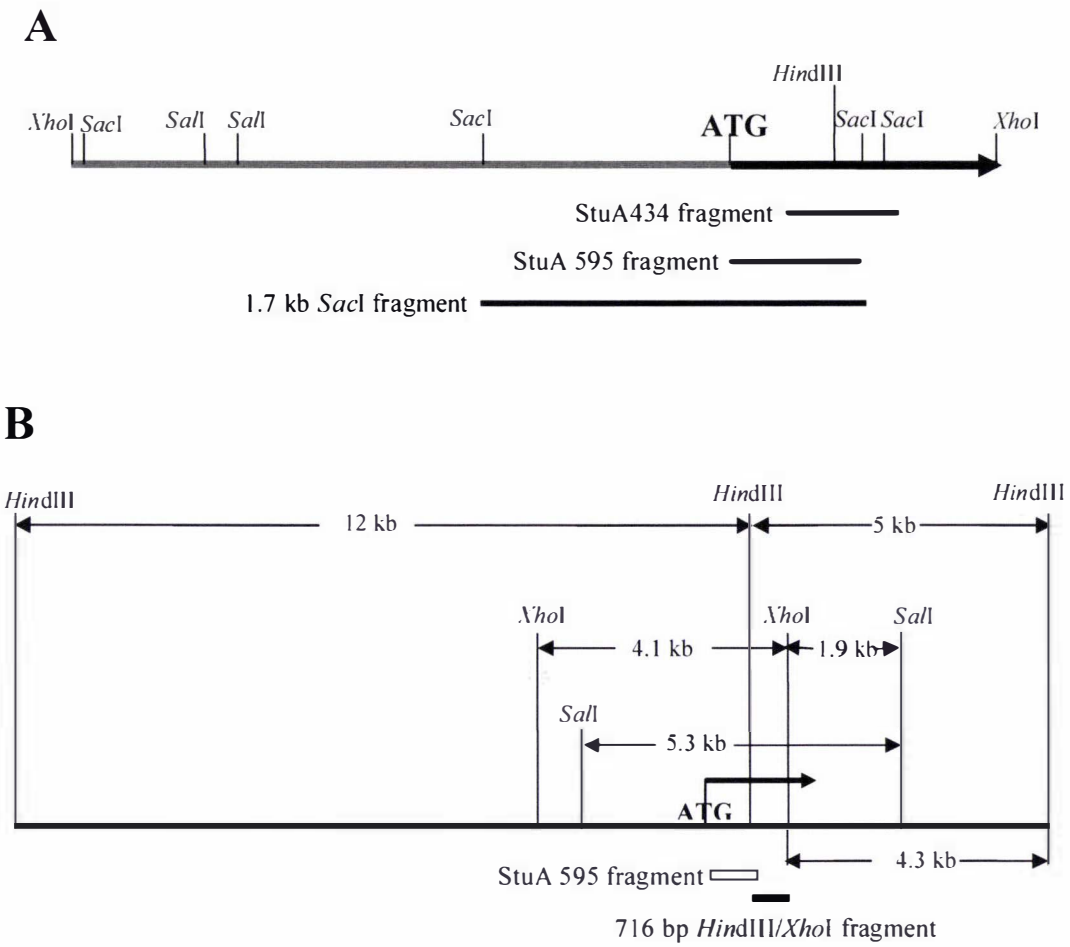


Figure 4.8 Maps of the 4114 bp *Xho*I fragment and the surrounding sequence

Panel A: Restriction sites and features revealed by sequencing of the 4114 bp *Xho*I fragment in pXho41 are shown. The *StuA* ORF is shown as a black arrow. The recognition sites of the restriction enzymes *Sal*I, *Hin*dIII, *Sac*I and a possible translation start site (ATG) are indicated. Positions of the previous identified fragments (StuA434, and the 1.7 kb *Sac*I fragment) and the probe (StuA595) are also shown.

Panel B: Positions of the *Sal*I and *Hin*dIII fragments, that hybridised to the StuA595 probe (open bar) in relation to the 4114 bp *Xho*I fragment as deduced from the position of the *Sal*I and *Hin*dIII sites in the *Xho*I fragment and the hybridisation pattern (Fig 4.7). The position of a 716 bp *Hin*dIII/*Xho*I fragment used as a probe in plasmid rescue experiments (Section 4.3.1) is also shown (black bar).

using size-fractionated *Hin*dIII-digested genomic DNA was constructed and screened by PCR using a pair of primers (iv_down/T7xho112) that were designed to amplify a 476 bp region of the known ORF downstream of the *Hin*dIII site present in the pXho41 insert. Several hundred colonies were screened but no positive clone was identified. This strategy was therefore abandoned. The remaining sequence of the *StuA* gene ORF and downstream flanking sequence was eventually obtained by another means and this will be described in Section 4.3. In the meantime, it was thought that the sequence obtained thus far was sufficient to investigate the function of the *StuA* gene by disruption of the gene and phenotypic analysis of the mutants.

4.2 Disruption of the *G. cingulata StuA* gene

To study the function of the *G. cingulata StuA* gene, targeted gene disruption was carried out. Bowen *et al.* (1995) successfully employed this strategy in their study of the *G. cingulata* pectin lyase gene. Furthermore, they compared the use of a deletion and a disruption vector. Transformation using a disruption vector was more efficient than with a deletion vector. With the deletion vector only 5 transformants were obtained from 7 transformation experiments, whereas with the disruption vector 19 transformants were obtained from 3 transformation experiments. Two of the three disruption mutants were single copy integration events, whereas both deletion mutants also contained additional copies of the deletion vector that had integrated ectopically (Bowen *et al.*, 1995). This disruption strategy has also been used successfully with the *G. cingulata* secreted aspartic peptidase (Plummer *et al.*, 2004). Another advantage of using a disruption vector was the ease of vector construction because only a small portion of the gene of interest was required. This meant that the sequence information obtained thus far for the *StuA* gene was enough to permit the use of such a strategy. Disruption vectors carrying fragments of *G. cingulata* as small as 505 bp have given efficient homologous recombination (Rikkerink *et al.*, 1994). Fragments of *G. cingulata* DNA over 600 bp are believed to be too large because they can result in unstable integration of the vector. Therefore a disruption vector containing a 595 bp fragment of the *G. cingulata StuA* gene was constructed.

The 595 bp *Bgl*II fragment from pGT595 was subcloned into the *Bgl*II site of the plasmid pAN7-1 (Punt *et al.*, 1987) to construct the disruption vector pAN595 (Fig 4.9). This plasmid has a hygromycin resistance gene under the control of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter from *A. nidulans*, for use as a selectable marker in transformation. The orientation of the insert in the disruption vector was established by restriction digestion and confirmed by sequencing (data not shown). In *G. cingulata*, homologous recombination between the *StuA* gene and the *StuA* gene fragment in the disruption vector pAN595 would result in insertion of the entire vector into the genome by a single crossover event. As a result, the *StuA* gene becomes disrupted by the hygromycin cassette.

G. cingulata protoplasts were prepared and transformed with the circular disruption vector pAN595 as described in Section 2.10.3. Three transformation experiments were performed and a total of forty transformants (designated SC1 to SC40) were obtained. In order to screen for *G. cingulata StuA* disruption mutants, single spore isolates of these hygromycin resistant colonies were obtained (Section 2.10.4) and subcultured on PDA plates containing 200 μg/ml hygromycin. Mycelium from these cultures was collected, freeze-dried and used to prepare genomic DNA for PCR and Southern blot analysis.

PCR analysis of transformants SC1 to SC9 is shown in Fig 4.10. Primers for PCR amplification were designed to identify homologous recombination events and disruption of the *G. cingulata StuA* gene. Primer 420F would bind upstream and 1752R downstream of the 595 bp fragment of the *StuA* gene present in the disruption vector. This primer pair would amplify a 1.3 kb fragment from wild type genomic DNA but an 8 kb fragment from the disruption mutants. However, under the PCR conditions used (elongation time was 90 seconds), no product would be expected from the disruption mutants. Primers pAN-forward and pAN662R anneal to the pAN7-1 backbone (Fig 4.10). The primer pair 420F/pAN-forward would amplify a 1.2 kb product and the pair 1752R/pAN662R would amplify a 1.3 kb product, if the disruption vector had integrated into the *StuA* locus as expected. For all nine transformants the expected products were observed (Fig 4.10).

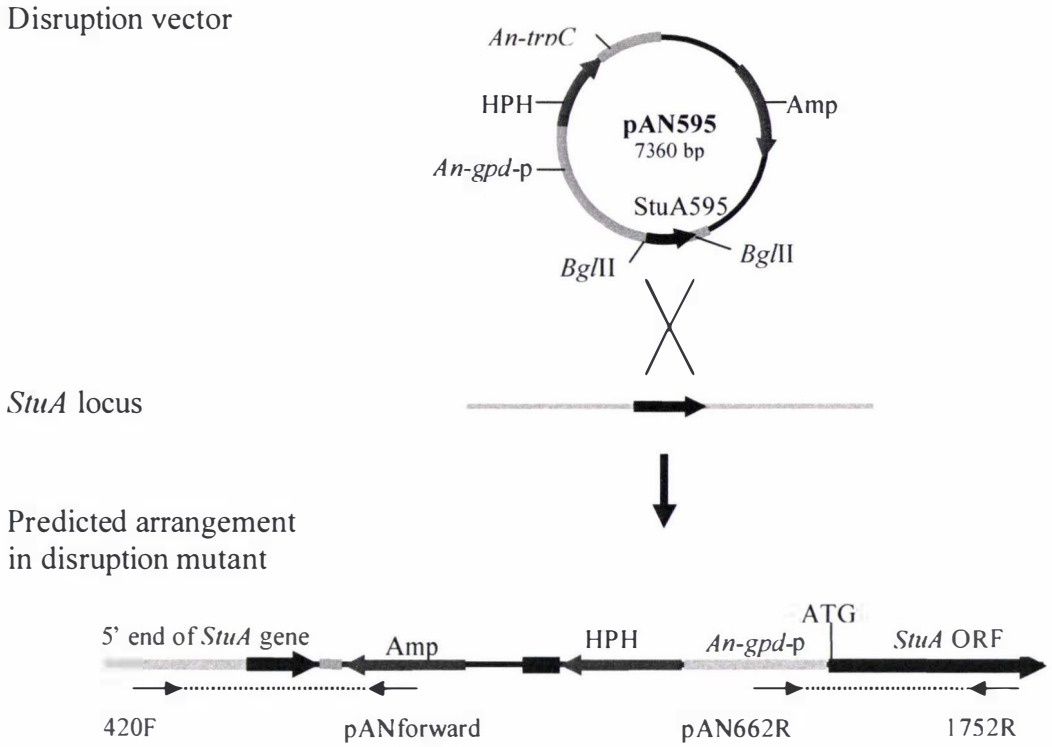


Figure 4.9 Strategy for disruption of the *G. cingulata StuA* gene

The 595 bp *Bgl*II fragment was excised from pGT595 and cloned into the *Bgl*II site of plasmid pAN7-1 to construct the disruption vector pAN595. This plasmid contains a hygromycin resistance gene (HPH) under the control of the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter (*An-gpd-p*) and the *A. nidulans trpC'* terminator (*An-trpC'*).

After the *G. cingulata* protoplasts were transformed with the disruption vector pAN595, homologous recombination by a single crossover event would occur between the *StuA* gene in the *G. cingulata* genome and pAN595, resulting in insertion of the entire vector into the genome and disruption of the *StuA* open reading frame. The predicted structure of the disrupted *StuA* gene and the primers used in PCR to detect this integration event are shown.

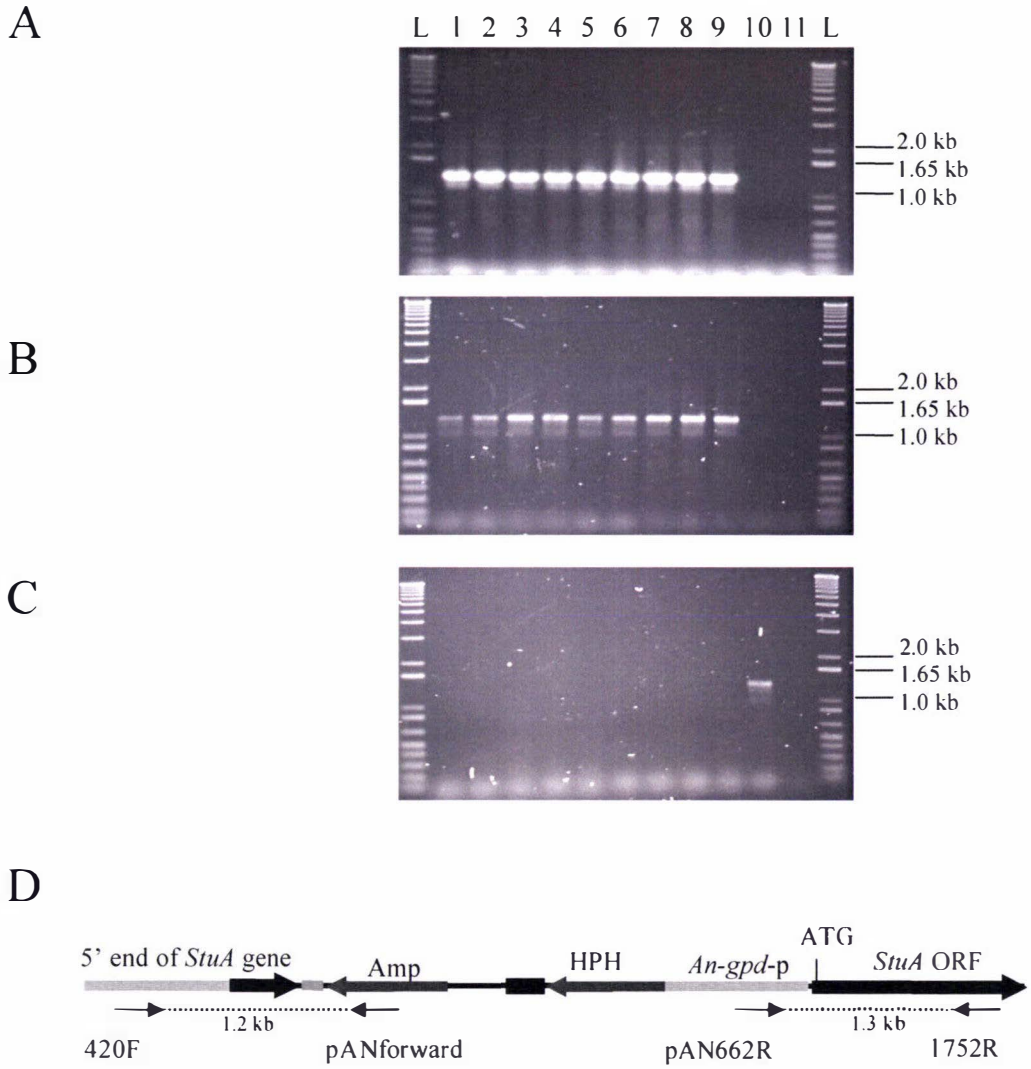


Figure 4.10 PCR analysis of the *G. cingulata StuA* disruption mutants

PCR products amplified with three pairs of primers using genomic DNA from SC1 (lane 1), SC2 (lane 2), SC3 (lane 3), SC4 (lane 4), SC5 (lane 5), SC6 (lane 6), SC7 (lane 7), SC8 (lane 8), SC9 (lane 9). Wild type genomic DNA was used as a positive control (lane 10). Lane 11 was the PCR reaction with no template as a negative control. Panel A: PCR using primers 420F and pAN-forward. Panel B: PCR using primers pAN662R and 1752R. Panel C: PCR using primers 420F and 1752R. With this pair of primers and a 90 second elongation time in the PCR reaction, product would only be expected for the DNA from the wild type. D: Map of the disrupted *StuA* gene.

Southern blot analysis of transformants SC1 to SC9 to confirm the disruption of the *StuA* gene was carried out using the 595 bp *Bgl*II fragment from pGT595 as probe. As expected from the Southern blot analysis of the wild type DNA (Fig 4.7), the probe hybridized to a 4.1 kb *Xho*I fragment of DNA from the wild type (Fig 4.11). This band was absent in all nine transformants. The probe hybridized to two *Xho*I fragments (9.3 kb and 1.75 kb) in all nine transformants, consistent with the integration of the disruption vector by a single crossover event at the *StuA* locus. An unexpected 7.1 kb hybridizing band was present in eight of the nine transformants (SC5 was the exception). The size of this band is slightly smaller than the size of the disruption vector itself (7.4 kb) which contains two *Xho*I restriction sites. The *Xho*I digestion of two tandem copies of the disruption vector integrated in the *StuA* locus would generate an additional 7.1 kb fragment, which would hybridize to the probe, and a 0.3 kb fragment which would not hybridize to the probe. The 7.1 kb band may therefore arise from tandem integration of the disruption vector (Fig 4.12). An additional Southern blot analysis was conducted in order to determine whether or not tandem integration of the disruption vector pAN595 had occurred in these transformants. The restriction enzyme *BstX*I was chosen for digestion of genomic DNA from the disruption mutants SC5, SC6 and the wild type strain. This enzyme was chosen because there was no *BstX*I restriction site anywhere in the disruption vector pAN595. As shown in Fig 4.13, the probe StuA595 hybridized to an 8 kb *BstX*I fragment in wild type DNA, a 15 kb *BstX*I fragment of DNA from disruption mutant SC5 and a 23 kb *BstX*I fragment of DNA from disruption mutant SC6. This indicated that the disruption mutant SC5 had just one copy of the disruption vector pAN595 integrated at the *StuA* locus (8 kb wild type fragment plus 7.4 kb of pAN595 =15.4 kb), whereas the disruption mutant SC6 had two tandem copies of the disruption vector pAN595 integrated at the *StuA* locus (8 kb wt fragment plus 2 x 7.4 kb of pAN595 =22.8 kb).

At this stage, it was observed that the appearance of the mycelium in the first batch of transformants SC1 to SC9 was quite different to that of the wild type strain. When growing on PDA plates in the dark, wild type colonies were white at the beginning (1-4 days) and gradually turned to black (4-7 days) with fluffy aerial hyphae. When growing under a light/dark cycle, wild type colonies were white at the beginning (1-4 days),

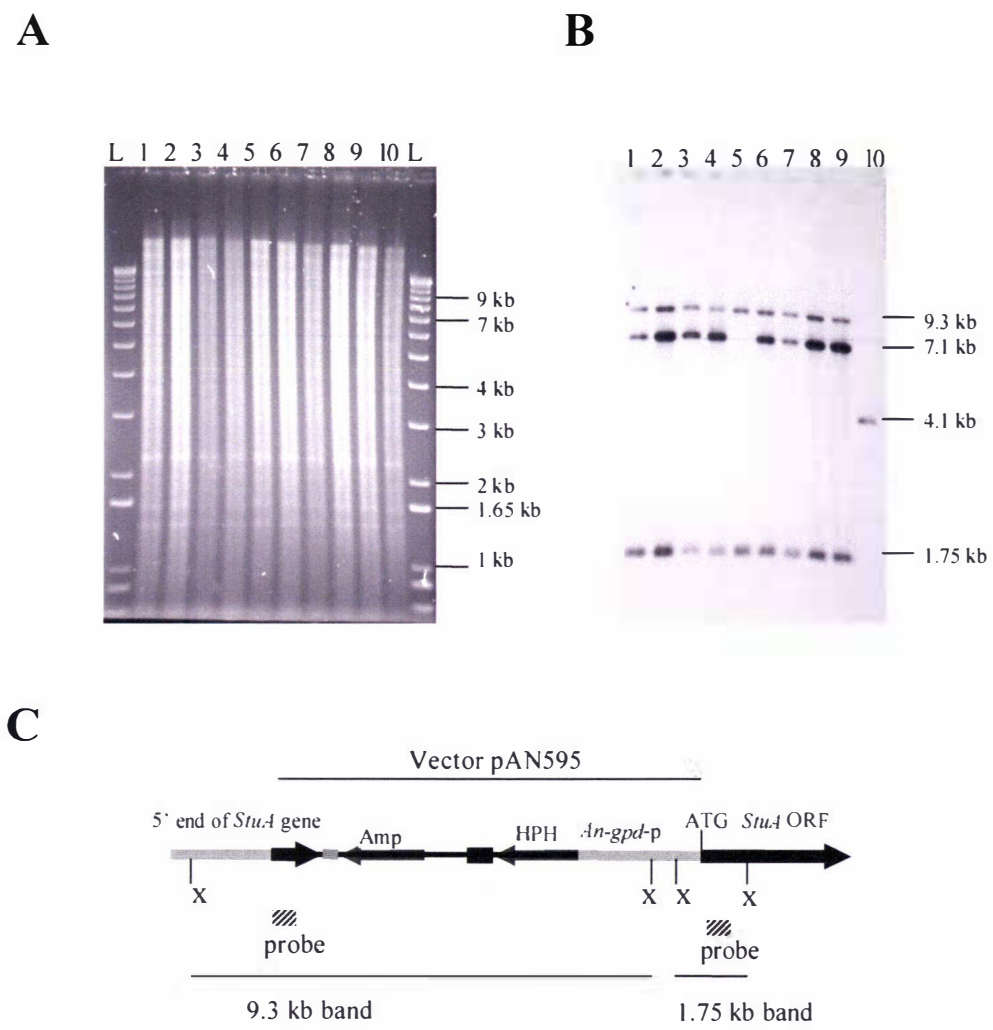


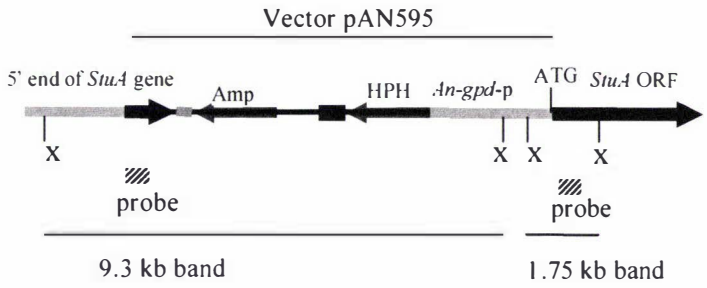
Figure 4.11 Southern blot analysis of *G. cingulata StuA* disruption mutants

Panel A: Agarose gel electrophoresis of *Xho*I digested genomic DNA from the disruption mutants SC1 (lane 1), SC2 (lane 2), SC3 (lane 3), SC4 (lane 4), SC5 (lane 5), SC6 (lane 6), SC7 (lane 7), SC8 (lane 8), SC9 (lane 9). Wild type genomic DNA was used as a positive control (lane 10).

Panel B: Autoradiograph of the Southern blot of the gel shown in panel A probed with Digoxigenin-11-dUTP labeled probe StuA595.

Panel C: Predicted hybridisation pattern for disruptants. X, *Xho*I.

A



B

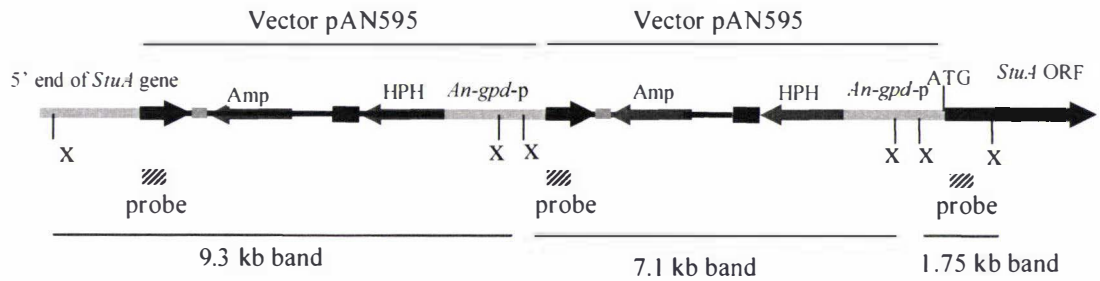


Figure 4.12 Predicted hybridisation patterns for different disruption mutants

Panel A: Predicted hybridisation pattern for a single copy integration event. The *Xho*I digested genomic DNA of the disruption mutant arising from the integration of a single copy of the disruption vector pAN595 would have a 9.3 kb fragment and a 1.75 kb fragment that hybridised to the probe StuA595.

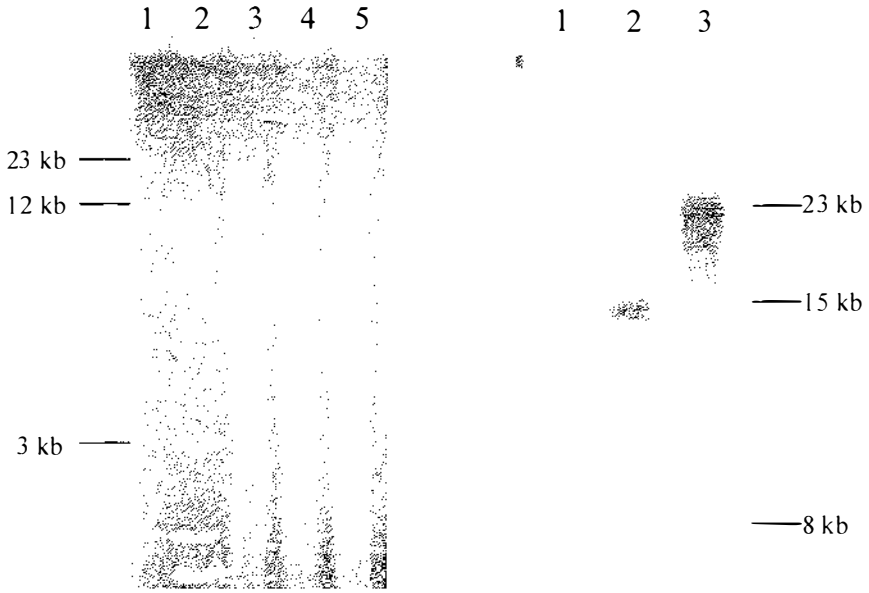
Panel B: Expected hybridisation pattern for tandem integration of two copies of the vector. The *Xho*I digested genomic DNA of a disruption mutant arising from the integration of two copies of the disruption vector pAN595 would have a 9.3 kb fragment, a 1.75 kb fragment and an additional 7.1 kb fragment that hybridised to the probe StuA595.

Figure 4.13 Southern blot analysis of disruption mutants SC5 and SC6

Panel A: Southern blot analysis of DNA from the wild type and the disruption mutants SC5 and SC6. Left panel: Genomic DNA from the wild type (lane 3), the disruption mutants SC5 (lane 4) and SC6 (lane 5) was digested using *BstX*I and separated on an agarose gel. Lane 1 is *Hind*III digested λ DNA and lane 2 is the 1 kb plus DNA ladder as size markers. Right panel: DNA from the gel in the left panel was blotted and hybridised to the DIG labelled StuA595 probe. Lane 1, wild type; lane 2, SC5; lane 3, SC6.

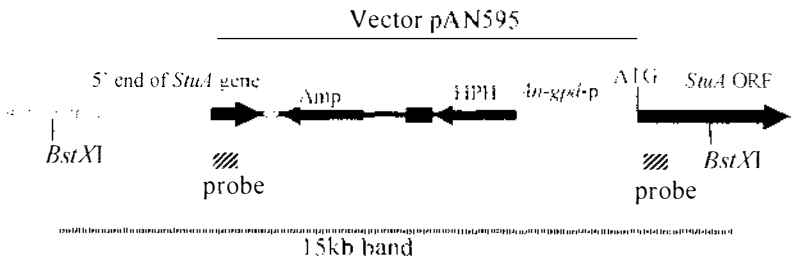
Panel B: Maps of the disrupted StuA gene arising from either integration of a single copy of the disruption vector (top) or two tandemly integrated copies of the disruption vector (bottom). Regions which would hybridise to the probe StuA595, the location of the BstXI restriction sites and sizes of hybridising fragments are shown.

A

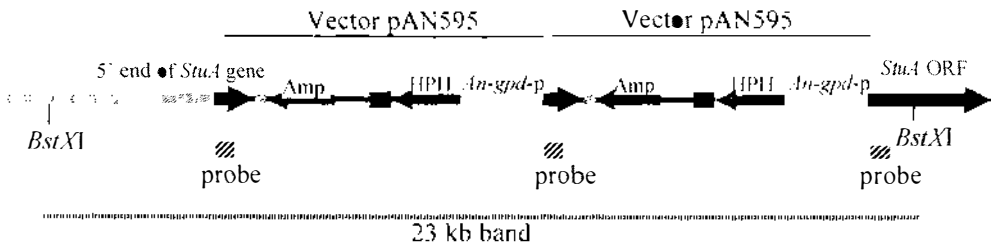


B

Single copy integration



Tandem integration



gradually turned to pink (4-7 days) and then black (>7 days). These colonies produced many conidia. For all nine transformants, aerial hyphae were absent or stunted and colonies were flat under either growth condition. The colonies remained a white colour when growing in the dark, or a pink colour when growing under the light/dark cycle, but never turned black under either condition. In addition, it was noticed that the mycelium of transformants was "wettable", and conidia production was greatly decreased compared to the wild type. These phenotypic changes may result from the disruption of the *StuA* gene.

To obtain additional transformants with just a single copy of the disruption vector integrated at the *StuA* locus, a second batch of transformants was screened. These transformants (SC10 to SC19) were analysed by PCR (data not shown) and Southern blot analysis (Fig 4.14). Transformants SC10, SC11, and SC13 displayed a hybridization pattern consistent with multi-copy integration of the disruption vector. They grew as flat, white mycelial colonies. Transformant SC19 contained multiple copies of the disruption vector integrated at the *StuA* locus. In addition, an ectopic integration event had occurred as shown by the presence of a 4.5 kb band that hybridized to the probe. The colony of transformant SC19 was also flat and white. In transformants SC12 and SC14 to SC17, a 4.1 kb band that hybridized to the probe was observed as in the wild type strain but there were additional bands that hybridized to the probe. These were of different sizes in each of the transformants. This suggested that these transformants arose from ectopic integration of the disruption vector without affecting the *StuA* gene. The colonies of these transformants had a similar appearance to that of the wild type. These results further confirmed that it was disruption of the *StuA* gene that was responsible for the visual changes in the mycelial colonies. DNA from transformant SC18 was not properly digested and the bands that hybridized to the probe were hard to interpret. However, the appearance of the colony was similar to that of the transformants that clearly arose from an ectopic integration event alone (SC14-SC17) and it was not analysed further.

From the third transformation experiment, only seven transformants (SC23, SC31-35 and SC40) that showed flat white mycelial colonies were screened by Southern blot

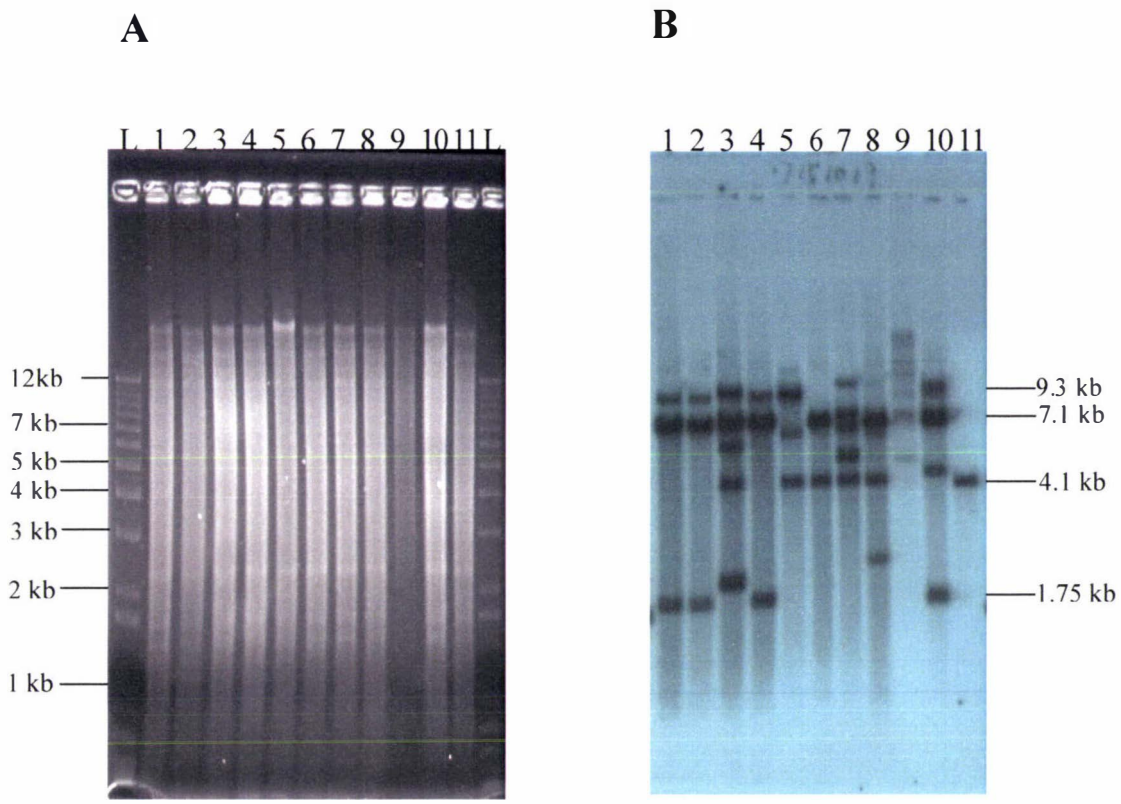


Figure 4.14 Southern blot analysis of the disruption mutants SC10 to SC19

Panel A: Agarose gel of the *Xho*I digested genomic DNA of disruption mutants SC10 (lane 1), SC11 (lane 2), SC12 (lane 3), SC13 (lane 4), SC14 (lane 5), SC15 (lane 6), SC16 (lane 7), SC17 (lane 8), SC18 (lane 9), SC19 (lane 10), and the wild type (lane 11). Lanes L were 1 kb plus DNA ladder (Invitrogen).

Panel B: Autoradiograph of a Southern blot of the gel shown in panel A probed with the Digoxigenin-11-dUTP labelled probe StuA595.

analysis (data not shown). The transformants SC31 and SC40 were free of ectopic integration events and showed a disrupted *StuA* locus. Therefore they were chosen for a second Southern blot analysis to determine the number of copies of the disruption vector that had integrated at the *StuA* locus (Fig 4.15). Transformant SC40 displayed a hybridization pattern that was consistent with the integration of a single copy of the disruption vector integrated at the *StuA* locus, whereas transformant SC31 displayed a hybridization pattern that was consistent with the integration of two copies of the disruption vector.

The three transformation experiments are summarized in Table 4.1. Of a total of 39 transformants analysed, 20 transformants displayed a flat white mycelial colony morphology on PDA plates (20/39), 17 of these transformants were disruption mutants as confirmed by Southern blot analysis (17/20), but only 2 of the disruption mutants arose from integration of a single copy of the disruption vector (2/17). Six of the disruption mutants that arose from tandem integration of the disruption vector also contained ectopic integration events. It appeared that the recombination efficiency was high and this was possibly the reason for the high ratio of multi-copy integration.

Table 4.1 Summary of the three transformation experiments

Experiment	Number of transformants analysed	Flat white mycelial colony	Black mycelial colony
1	9	9	0
2	10	4	6
3	20	7	13
Total	39	20	19

Transformants SC40 and SC5 were used for further analysis of the *StuA* gene. Transformants SC14 and SC17 were included as controls because the disruption vector had integrated ectopically and the *StuA* locus remained intact in these transformants. The appearance of the colonies of these transformants growing on PDA plates is compared in Fig 4.16. Radial growth rates for the wild type, the ectopic transformants (SC14 and SC17), and the single copy transformants (SC5 and SC40) were compared

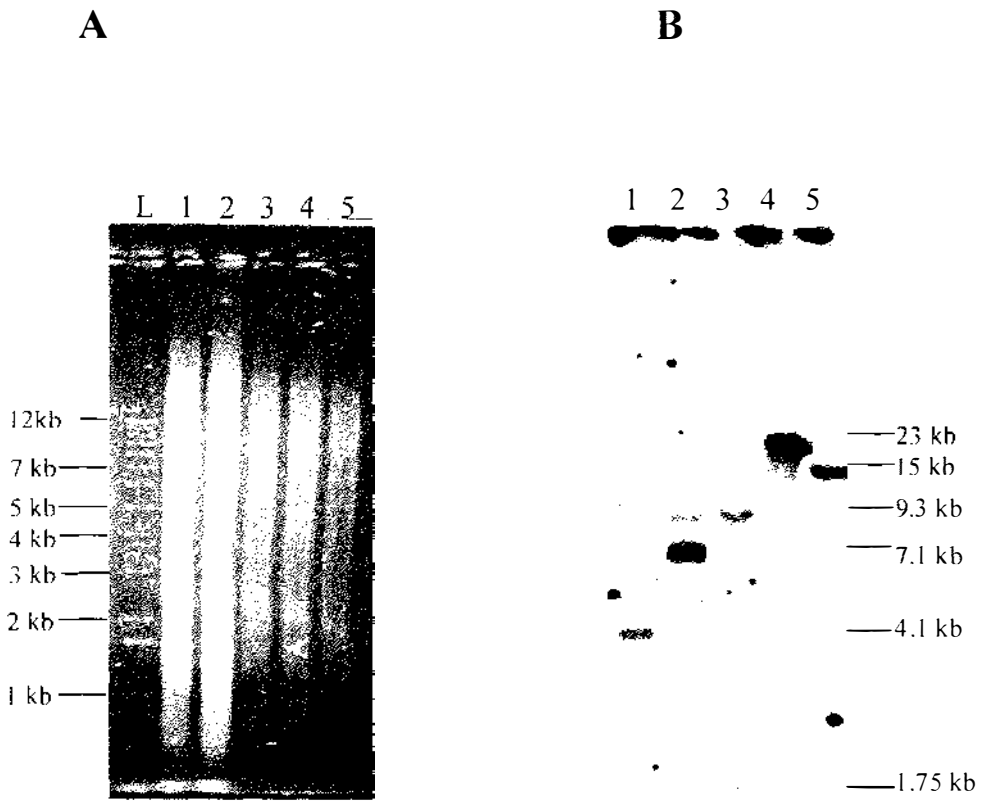


Figure 4.15 Southern blot analysis of the disruption mutants SC31 and SC40

Panel A: Agarose gel electrophoresis of the *Xho*I digested genomic DNA of the wild type (lane 1), disruption mutants SC31 (lane 2), SC40 (lane 3), and the *BstX*I digested genomic DNA of SC31 (lane 4), SC40 (lane 5). 10 μg of digested DNA was loaded in each lane. Lane L was the 1 kb plus DNA ladder (Invitrogen).

Panel B: Autoradiograph of a Southern blot of the gel shown in panel A probed with the Digoxigenin-11-dUTP labelled probe StuA595. For maps of the disrupted *StuA* gene see Fig 4.13.

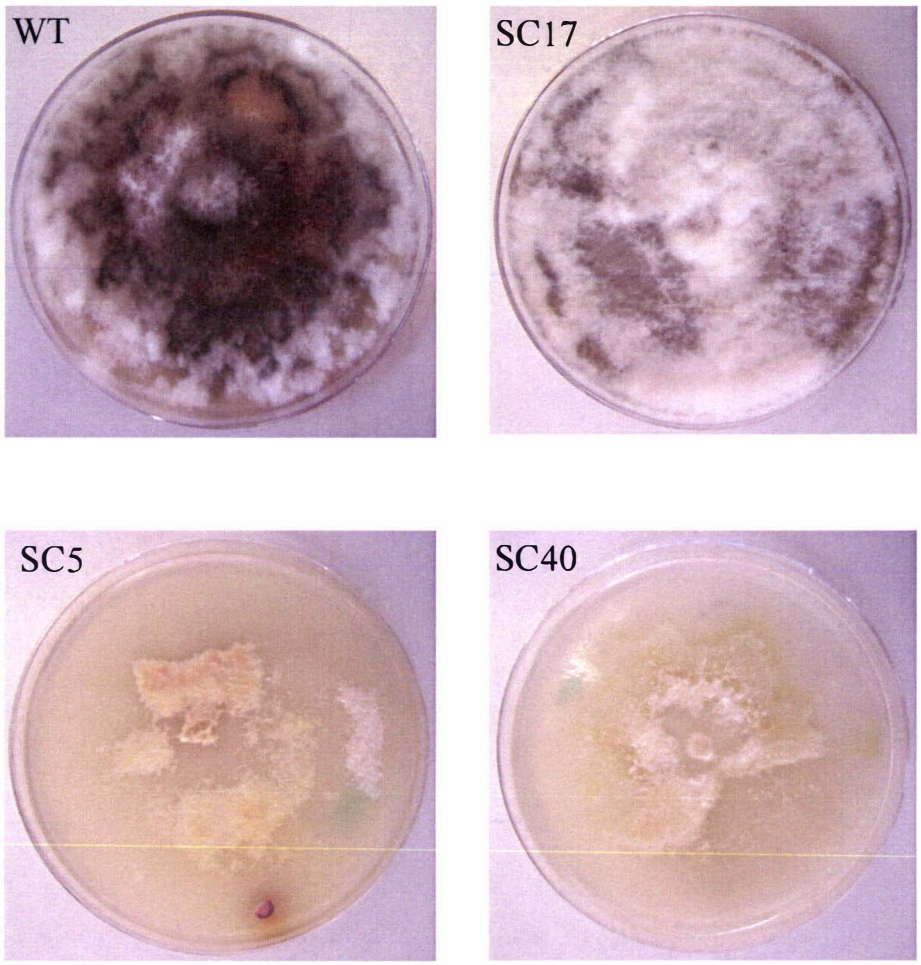


Figure 4.16 Colony morphology of the disruption mutants

Mycelium of *G. cingulata* wild type, the ectopic transformant SC17 and the disruption mutants (SC5 and SC40) was grown on PDA medium for 7 days at 25^0C in the dark. The ectopic transformant SC17 showed thick, fluffy black mycelium, similar in appearance to the wild type. The disruption mutants SC5 and SC40 showed flat white mycelium.

on PDA plates as described in Section 2.11.1. The growth rate (Table 4.2) of these strains was similar, indicating the disruption of the *StuA* gene did not affect vegetative growth of *G. cingulata*. It was noticed that wild type like sectors (Fig 4.17, panel A) appeared on most PDA plate cultures of the disruption mutants SC5 (5 out of 6) and SC40 (5 out of 6). This suggested that the mutants were reverting to the wild type in these cultures and this may interfere with further characterization of these transformants. To determine if these sectors had reverted to the wild type with a restored *StuA* gene, the mycelium from these sectors was collected and DNA extracted. The DNA was analyzed in a PCR reaction using primer pairs that were used in the screening of the transformants. As shown in Fig 4.17 (panel B), the DNA from these sectors was a mixture of transformant DNA and wild type DNA. These disruption mutants were not stable, possibly due to the instability of the single crossover integration of the disruption vector. Therefore no further characterization of these mutants was carried out. Instead the deletion strategy was revisited (Section 4.5).

Table 4.2 Growth rate of the *G. cingulata* wild type, disruption mutants and ectopic transformants

Strains	Growth rate at 22^{0}C (mm/hr)	Growth rate at 25^{0}C (mm/hr)
Wild type	0.72 ± 0.22	0.81 ± 0.14
SC14	0.72 ± 0.26	0.94 ± 0.25
SC17	0.74 ± 0.24	0.94 ± 0.23
SC5	0.62 ± 0.23	0.70 ± 0.24
SC40	0.62 ± 0.20	0.79 ± 0.30

Growth rate was determined by measuring the diameter of the mycelial colony growing on PDA plates inoculated with a 5 mm plug of mycelium. All strains were at 25^{0}C in the dark or at 22^{0}C under a light/dark cycle. SC14 and SC17 were the transformants derived from ectopic integration of the disruption vector. SC5 and SC40 were the disruption mutants derived from integration of a single copy of the disruption vector. No significant difference (Student's t-test, $P > 0.1$, $n = 6$ plates) in growth rate was observed between the wild type and SC14 or SC17. Similarly for the wild type and SC5 or SC40.

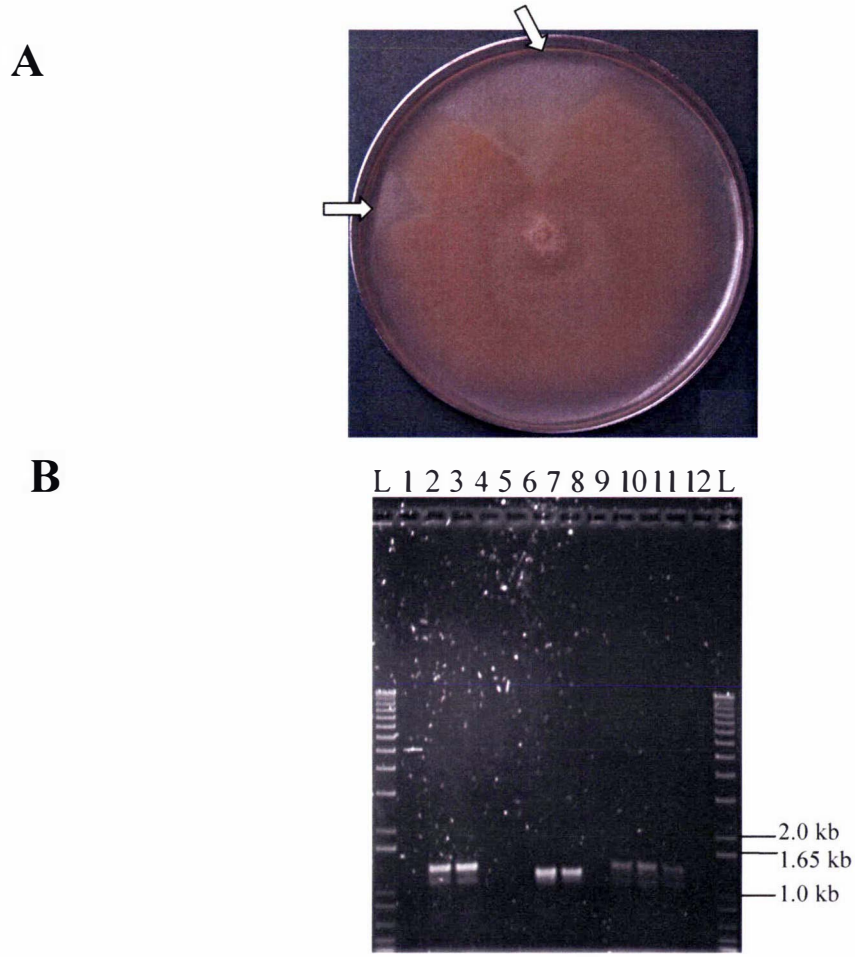


Figure 4.17 Reversion of the disruption mutant and PCR analysis of DNA from the colony

Panel A: Mycelium of the disruption mutant SC5 growing on a PDA plate. Two sectors are indicated by arrows.

Panel B: PCR products amplified with three pairs of primers using genomic DNA from wild type (lanes 1, 5 and 9), disruption mutants SC5 (lanes 2, 6 and 10), SC40 (lanes 3, 7 and 11). A PCR reaction with no template was used as a negative control. Primers used for lane 1-4 were 420F and pAN-forward, which would amplify a 1.3 kb product from SC5 and SC40 but no product from wild type. Primers used for lane 5-8 were pAN662R and 1752R, which would amplify a 1.2 kb product from SC5 and SC40 but no product from wild type. Primers used for lane 9-12 were 420F and 1752R, which would amplify a 1.4 kb product from wild type, whereas no product was obtained with DNA from a pure colony of the disruption mutant (Fig 4.10). The presence of products in lanes 10 and 11 suggested that reversion had occurred in both disruption mutants.

4.3 Cloning of the remainder of the *StuA* gene and sequence analysis of the gene

Although the possibility of obtaining useful gene deletion mutants was reportedly very small (Bowen, 1995), the deletion strategy seemed to be the only approach left. A deletion construct requires placing two fragments of DNA flanking the target gene on either side of a selectable marker. A fragment from the promoter region of *StuA* could be obtained from the plasmid pXho41, which contained an *Xho*I fragment covering 2.6 kb of the *StuA* promoter region plus part of the ORF. But DNA from downstream of the *StuA* ORF was also needed. Since no useful plasmids had been obtained from the subgenomic library screen, plasmid rescue using disruption mutants SC5 and SC40 provided an alternative way to obtain the required DNA.

4.3.1 Cloning of a 3' fragment of the *StuA* gene by plasmid rescue

The enzyme used for plasmid rescue should not digest the pAN7-1 backbone, nor cut the known sequence 3` to the insertion site. Therefore *Eco*RV was chosen to digest genomic DNA from the disruption mutants SC5 and SC40. The digested DNA was self-ligated and used to transform *E. coli* (Section 2.10.5). Only circularized *Eco*RV fragments containing pAN595 would replicate in *E. coli* because only these molecules would contain an origin of replication and the antibiotic resistance gene. No clones were obtained with DNA from the disruption mutant SC5. Three clones were obtained using DNA from the disruption mutant SC40. The low efficiency of transformation might have resulted from the low efficiency of blunt end ligation and the high ratio of non-replicable DNA to replicable DNA in the transformation reactions. *Eco*RV digestion of these three clones showed that one (pERV2) was about 7 kb in size whereas the other two were about 13 kb in size. The 7 kb plasmid was smaller than the disruption vector itself and was therefore not analysed further. Both of the two larger plasmids (pERV1 and pERV3) were examined by Southern blot analysis using a 716 bp *Hin*dIII/*Xho*I fragment from pXho41 as probe (named probe HX, location of the probe with respect to the 4.1 kb *Xho*I fragment in pXho41 is shown in Fig 4.8).

The Southern blot hybridization pattern for pERV1 was not consistent with the known restriction map and was therefore not analysed further. Southern blot analysis of pERV3 is shown in Fig 4.18. Using the information from the Southern analysis of wild type genomic DNA (Fig 4.7), the sequences of the plasmid pXho41 (Fig 4.8) and pAN595 (Fig 4.10, the disruption vector), a physical map of the *Hin*dIII, *Sal*I and *Xho*I restriction sites on pERV3 was generated (Fig 4.19). Subsequently the 4 kb *Hin*dIII/*Eco*RV fragment of pERV3 was subcloned into pBlueScript II KS (the resulting plasmid was designated pHE40) for further analysis. This fragment was chosen because it contained the longest 3' region downstream of the *StuA* ORF and its 5' end overlapped with the 3' end of pXho41 by 716 bp. Restriction fragments of plasmid pHE40 were subcloned into pBlueScript II KS and sequenced on both strands. The sequence of the 716 bp overlapping region was identical to the sequence previously obtained from the plasmid pXho41.

Plasmids pXho41 and pHE40 were used to assemble an insert (Fig 4.20) that covered the entire *StuA* locus for use in preparing a probe for Southern analysis of deletion mutants and in the construct of a complementation vector (Sections 4.4 and 4.5). Both plasmids have a *Not*I site in the pBlueScript II KS multiple cloning sites and another site within the 716 bp region of overlap. These two plasmids were therefore digested with *Not*I. The 4.1 kb fragment from pHE40 and the 6.3 kb fragment from pXho41 were purified by gel electrophoresis and ligated to generate a pBlueScript based plasmid pXHE which contains a 7432 bp insert. This insert comprised 2.7 kb of the 5' flanking region, the 2.1 kb *StuA* open reading frame and 2.5 kb of the 3' flanking region. To further confirm that the insert in plasmid pXHE correctly represented the *StuA* locus in the *G. cingulata* genome, a series of PCR reactions were performed. Two primers that would anneal upstream of the *StuA* ORF in pXHE were used as forward primers, and one primer that would anneal downstream of the *StuA* ORF in pXHE were used as reverse primer to amplify part of the insert in pXHE and the *StuA* ORF from the *G. cingulata* genomic DNA. PCR products from two different combinations of these primers were analysed by gel electrophoresis. For each primer pair, both templates gave products of the same size (Fig 4.21). The product obtained from the *G. cingulata*

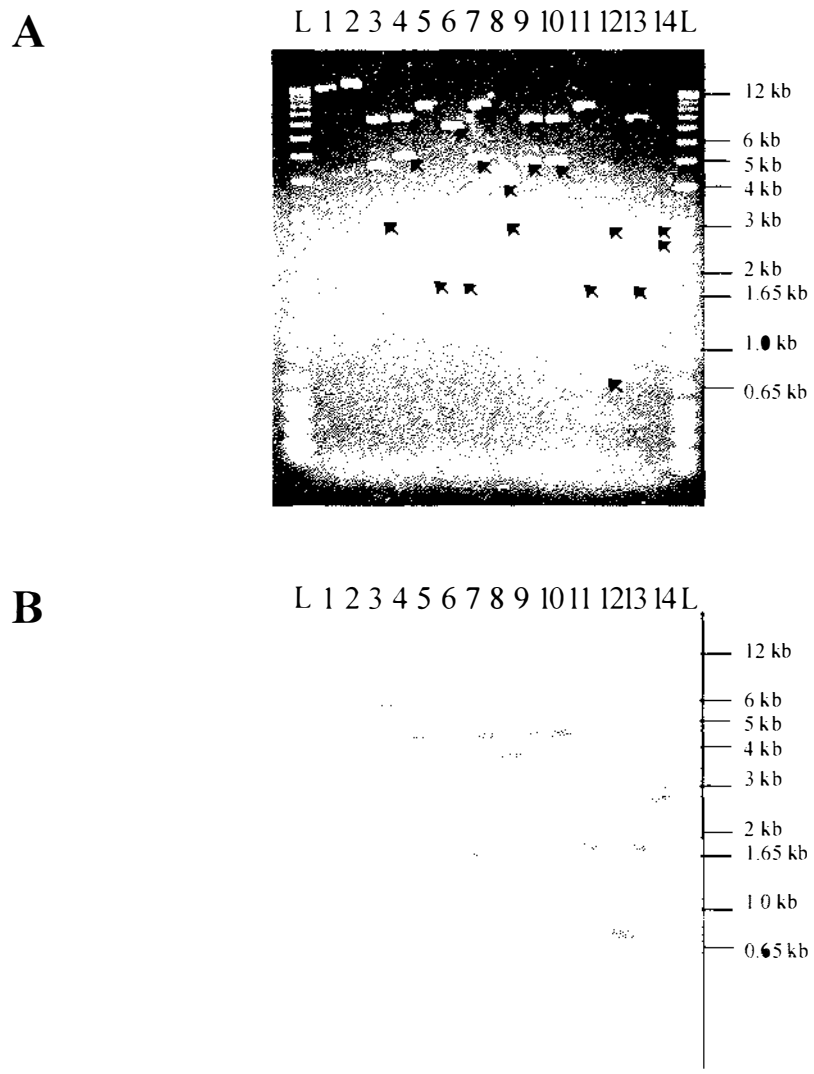
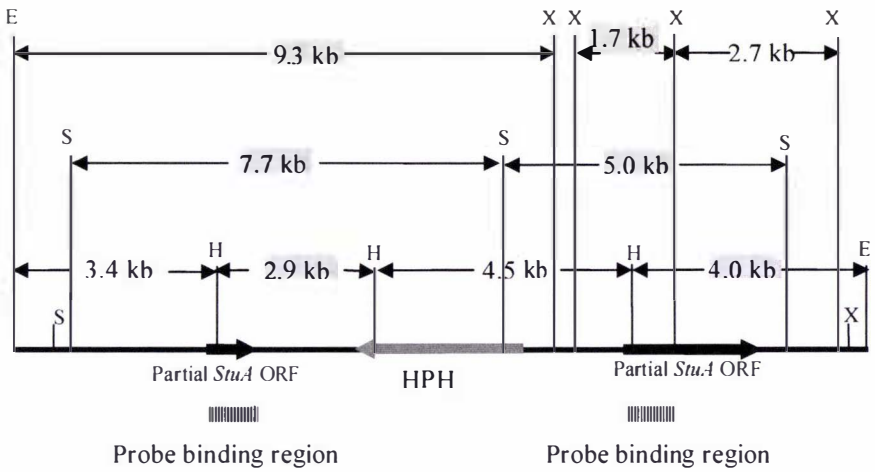


Figure 4.18 Southern blot analysis of pERV3

Panel A: Agarose gel electrophoresis of various restriction enzyme digests of plasmid pERV3. Lane 1, uncut plasmid; lane 2, *Eco*RV digest; lane 3, *Hin*dIII digest; lane 4, *Sal*I digest; lane 5, *Xho*I digest; lane 6 *Sac*I digest; lane 7, *Bam*HI digest; lane 8, *Eco*RV/*Hin*dIII digest; lane 9, *Eco*RV/*Sal*I digest; lane 10, *Sal*I digest; lane 11, *Eco*RV/*Xho*I digest; lane 12, *Hin*dIII/*Xho*I digest; lane 13, *Xho*I/*Sal*I digest; lane 14, *Sal*I/*Hin*dIII digest. Bands that hybridised to the probe (panel B) are indicated by arrows.

Panel B: Autoradiograph of the Southern blot of the gel shown in panel A probed with the digoxigenin-11-dUTP labeled 716 bp *Hin*dIII/*Xho*I fragment.



pERV3

Figure 4.19 Physical map of the plasmid pERV3

The restriction map has been drawn for the plasmid that had been linearised with *Eco*RV. The sizes of those fragments that hybridised to the 714 bp probe HX are shaded. The locations at which the 714 bp probe HX should bind are shown below the map.

E, *Eco*RV; H, *Hin*dIII; S, *Sal*I; X, *Xho*I.

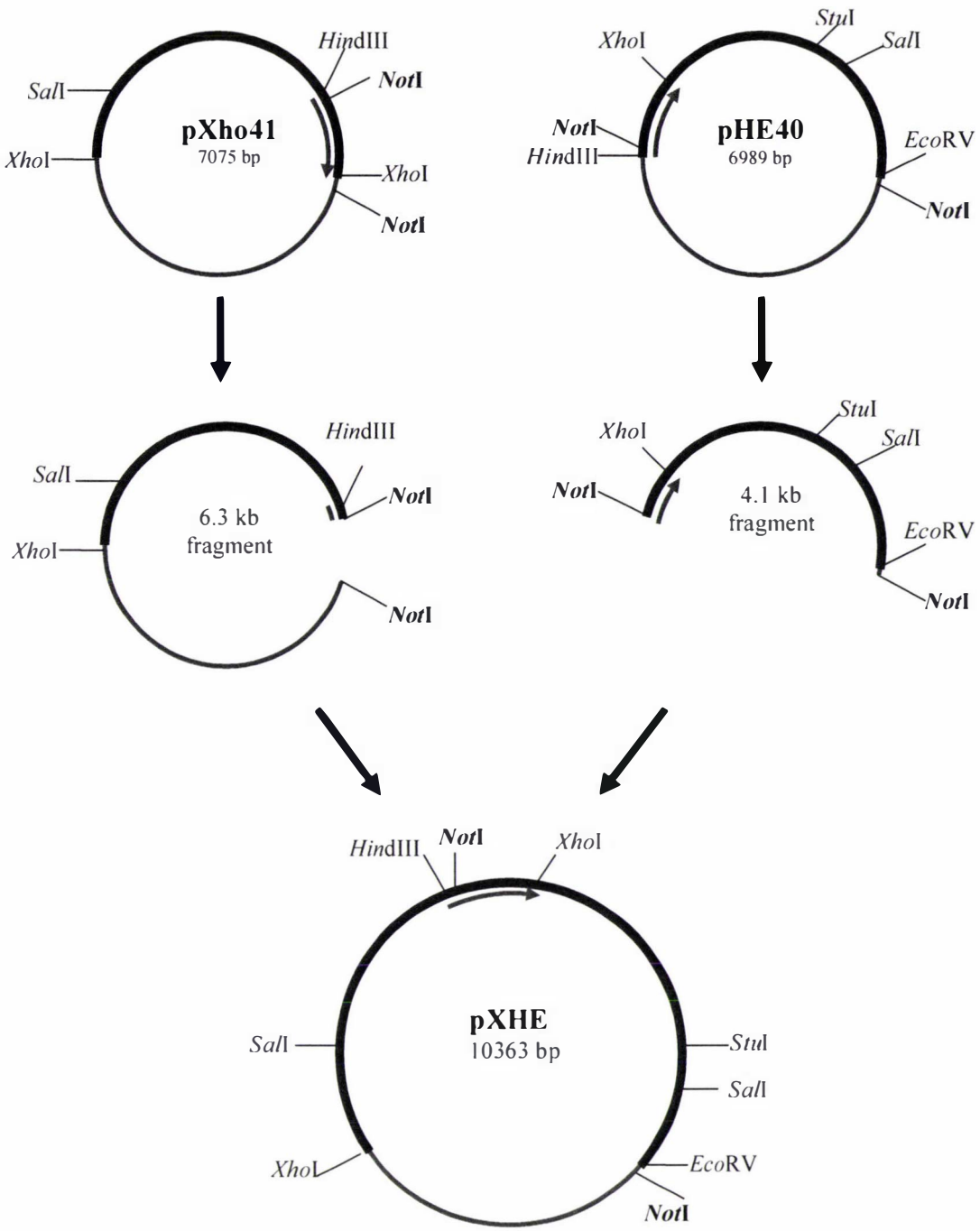
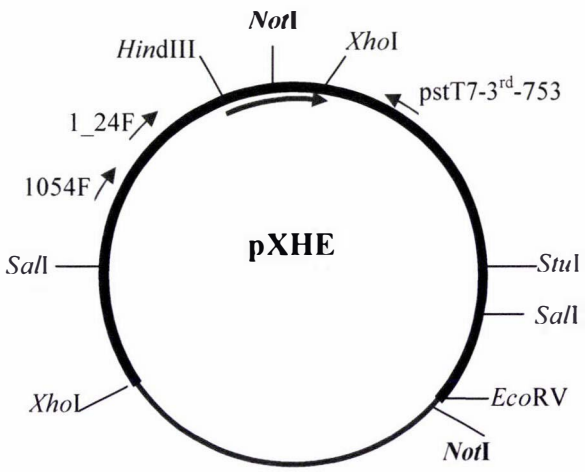


Figure 4.20 Assembly of the plasmid pXHE

Plasmid pXHE was assembled from the plasmids pXhoI and pHE40 both of which were digested with *Not*I. The 4.1 kb fragment from pHE40 and the 6.3 kb fragment from the pXho41 were ligated to generate plasmid pXHE.

A



B

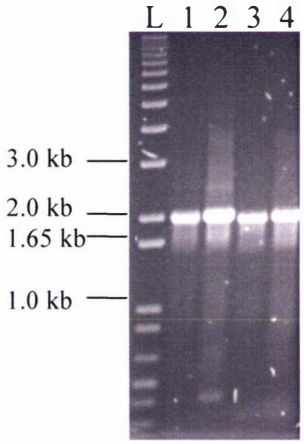


Figure 4.21 Confirmation of the structure of the insert in the plasmid pXHE

Panel A: Positions of five primers are shown with respect to the plasmid pXHE. PCR products would span the junction between the regions of the insert derived from plasmids pXho41 and pHE40.

Panel B: Comparison of the PCR products from the plasmid pXHE and genomic DNA. *G. cingulata* genomic DNA (lanes 1 and 3) and the plasmid pXHE (lanes 2 and 4) were used as templates. Primers 1054F and pstT7-3rd-753 were used to get the product in lane 1 and lane 2. Primers 1-24F and pstT7-3rd-753 were used to get the product in lane 3 and lane 4.

genomic DNA with the primer pair 1-24F/pstT7-3rd-753 was sequenced. This product spanned the *Not*I site in the insert in plasmid pXHE and the sequence exactly matched the sequence of the plasmid in this region. This confirmed that the insert in plasmid pXHE was indeed a genuine copy of the *G. cingulata* genome.

4.3.2 Analysis of the sequence of the *StuA* gene and its flanking regions

The nucleotide and deduced protein sequence for *StuA* is shown in Fig 4.22. The presumptive translation start site and the open reading frame for the *StuA* gene was identified based on a comparison of the deduced protein sequence with the other protein sequences in the APSES family (see Fig 4.1 for a list of these sequences) by a BLASTX search. The presumptive translation start site was recognized 243 bp upstream of the start site previously proposed for the StuA432 sequence (Fig 4.3, 4.4). However the sequence surrounding this start codon GGTAC**ATG**AG is not an exact match to the Kozak consensus sequence (GCCRCC**ATG**GC) (Kozak, 1981). The *StuA* open reading frame is disrupted at its 5' end by two introns, which are 53 bp and 57 bp in size, respectively. The length and position of these two introns are similar to those in the *M. grisea* hypothetical gene MG00692. The conserved sequences of intron/exon boundaries are present. The existence of these two introns was confirmed by a comparison of this genomic sequence with the mRNA sequence obtained in the RACE experiment performed later (Section 4.3.3). An intron internal consensus sequence PyGCTAACN was found in the second intron but not in the first one (Fig 4.22).

4.3.3 Analysis of the *StuA* mRNA

RACE (rapid amplification of cDNA ends, Section 2.9.5) was carried out to determine the 5' end and 3'ends of the *StuA* mRNA as outlined in Fig 4.23. This is a method to amplify DNA sequences from a messenger RNA (mRNA) template between a defined internal site and unknown sequences at either the 5' or the 3' ends of the mRNA. Total RNA from mycelium was treated with DNase I and checked for any contaminating DNA by RT-PCR using the size difference between the PCR products from cDNA and the corresponding genomic DNA containing an intron. A different strategy from that


XhoI
EcoRV
SacI
BrlA binding site
BrlA binding site
SalI
SmaI
SalI
BrlA binding site
BrlA binding site

*Pst*I

1381 CCTGCAGCACCCTGGCTTTACCTTGCCTACCTTGACCTTAGCTGCACCGGTCGGGGGCGT

1441 GGATGTATGATCTTGAGGACCGTAGTCTGTAGATGGGGTGGAGGGACTTCGACCTGCCTC

1501 CTCTTCTTTTCTCTCACCACCGAAAACAAAACCTTCCCACGCCCGGCTTCTTTCTTTTCC

1561 TTCTACCTCTCACGACTCCACCAACCACCTCCAATACTCCCTTAGGACAGCCCAAGAGGC

1621 GAGGACGGTAAGTCTGAACCCGAACTTCTTCTATGCTCCCAATGCCTCTTCACCAGCCCT

AbaA binding site

1681 CCTACCCTTTCCCATAAGCGCCATCTCAGAACGAAAAGTCCTTGGTTTGGTTTCCATTCC

1741 TGCATCTCTGCTGCTGCACCCTCCACCCCCATCTTACACGCCAACTCTAGCTGATGTACC

*Sac*I

1801 TGCATTAGAAGAGTTGGGTGAGCTCGACGAAGAGAGAAGAAGAAAAACAAGAAACCAACG

1861 ACCTCGCTCGAAGTCCACGATTTGTTGTCTGGTCTCTGCGAAAGTGTTTGTGTGTTCTGC

1921 TTGTTCGGTACCTCTGCTTCGATAAGACCTTTCATACAAACGGCCTCCACCGCCTGTTAC

AbaA binding site

1981 TCAGCTCCCCATTTCACAAGAAAAAAGGGCCTCGCTTCCTACCGTCACCGTCCT

2041 CGGCGCCTTCGTGCAACCCGCCAAGCAAGTAAAGAACCAAGAGGTCAACCCAATCTCTTG

2101 TATTTACTCTGCCCTTCGACGACCGACGTCTGAAGACTACTCTCTATTCGACCTTACCGA

2161 TACGAAATAGCCATTGCAACAGTTCCCACGGCTTGACACAATCAGATGCTAACGTTACTG

2221 AAGCTCTGATTTCTTTTTTAACGACTGCTTACGACGCCCCACGACCGTCCTGATCTGTTT

AreA binding site

2281 TCACTCAAGAAAAGTTCTCGTACCACGGTTCCATCTTGCCTGGTAGCACCCTCA

Transcription start site

2341 CCGCTCGAACAACCTGACGTTTTTTTTTTGCGCCGTCAAACCTCGCCAACTGATTCCAAC

cAMP response motifs

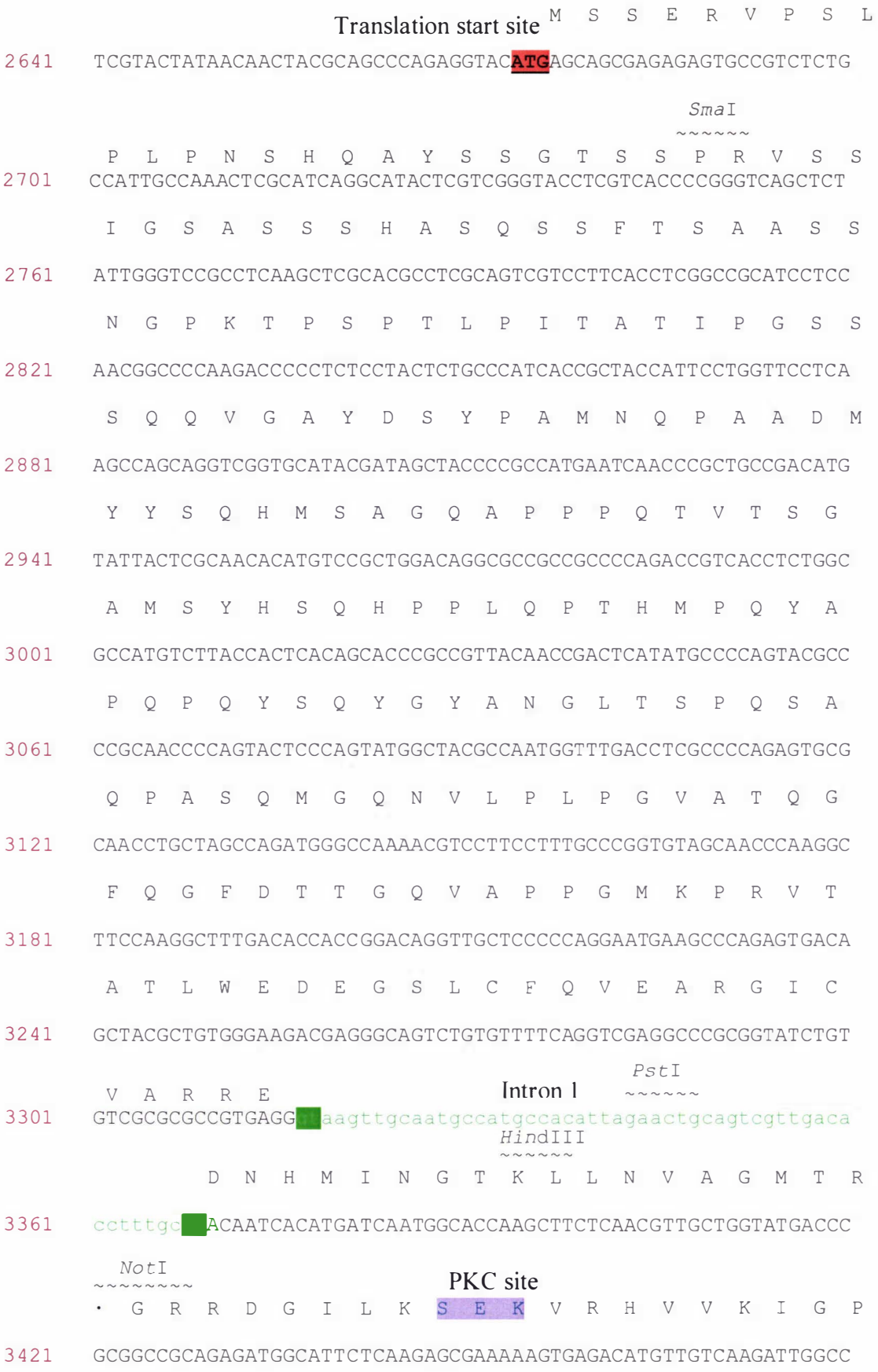
2401 TGCGCTATCGACTGCGGCGATTGGACAGGTCAATTTGACTGAACAACGCTCGACGCCTGG

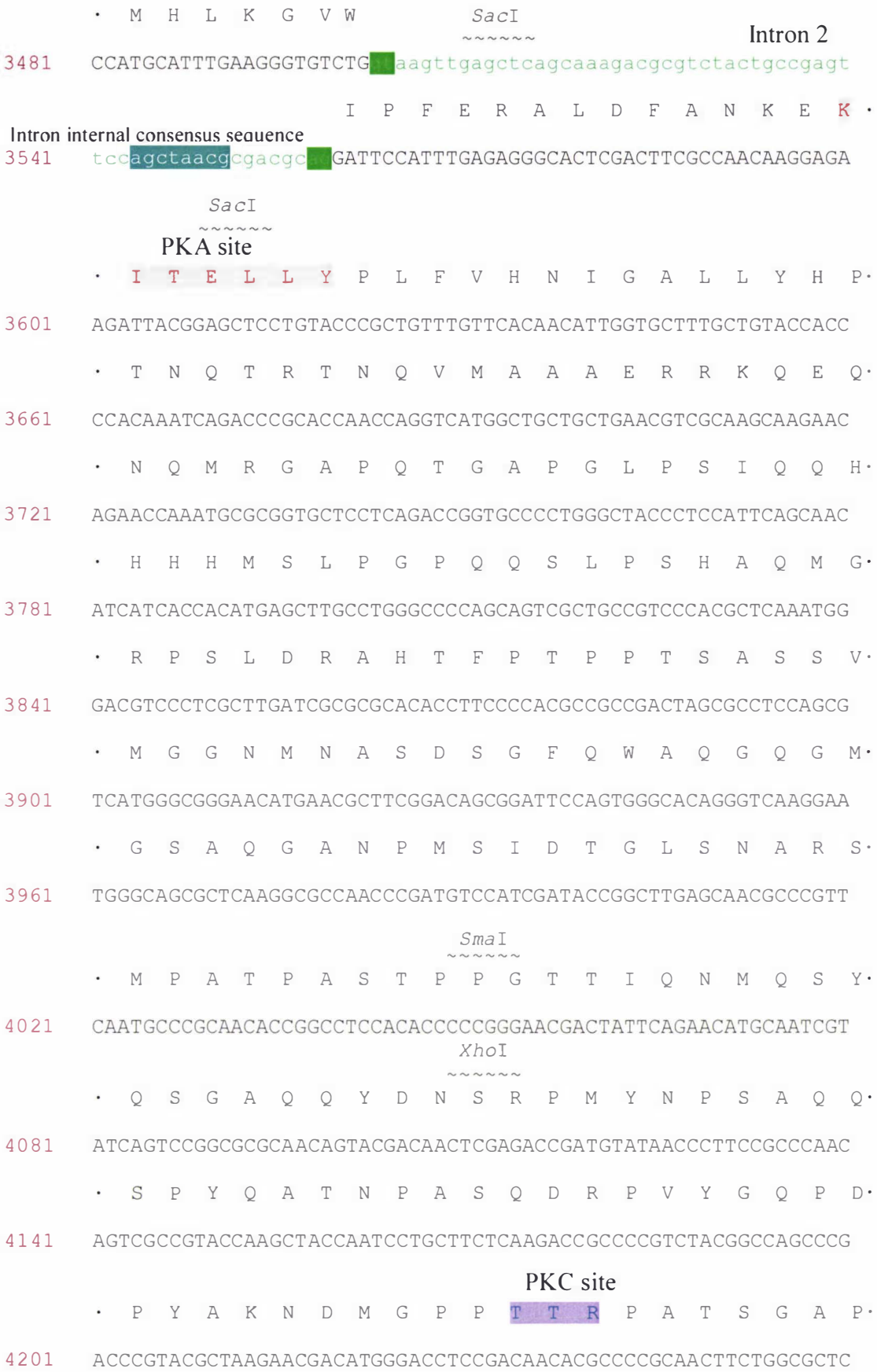
2461 AAAGACAATTGCTGCTCTTTCCACGATCGTGTGTTTTTGCAGCTGCCGCAGTCCAGAGGC

2521 TCGCTTCGCGACTCGGACTCCACTGACTGTCTGGCGTCAGGTACGAACCTTCGGGGCAAC

*Sma*I

2581 ACCGTGAAGCTGGATCTACCGTCGATTTCTCAAGTGCAAACCCGGGGACCGACCGACGTG


Translation start site
M S S E R V P S L
2641 TCGTACTATAACAACTACGCAGCCCAGAGGTACATGAGCAGCGAGAGAGTGCCGTCTCTG
SmaI
P L P N S H Q A Y S S G T S S P R V S S
2701 CCATTGCCAAACTCGCATCAGGCATACTCGTCGGGTACCTCGTCACCCCGGGTCAGCTCT
I G S A S S S H A S Q S S F T S A A S S
2761 ATTGGGTCCGCCTCAAGCTCGCACGCCTCGCAGTCGTCCTTCACCTCGGCCGCATCCTCC
N G P K T P S P T L P I T A T I P G S S
2821 AACGGCCCCAAGACCCCCTCTCCTACTCTGCCCATCACCGCTACCATTCCTGGTTCCTCA
S Q Q V G A Y D S Y P A M N Q P A A D M
2881 AGCCAGCAGGTCGGTGCATACGATAGCTACCCCGCCATGAATCAACCCGCTGCCGACATG
Y Y S Q H M S A G Q A P P P Q T V T S G
2941 TATTACTCGCAACACATGTCCGCTGGACAGGCGCCGCCGCCCCAGACCGTCACCTCTGGC
A M S Y H S Q H P P L Q P T H M P Q Y A
3001 GCCATGTCTTACCACTCACAGCACCCGCCGTTACAACCGACTCATATGCCCCAGTACGCC
P Q P Q Y S Q Y G Y A N G L T S P Q S A
3061 CCGCAACCCCAGTACTCCCAGTATGGCTACGCCAATGGTTTGACCTCGCCCCAGAGTGCG
Q P A S Q M G Q N V L P L P G V A T Q G
3121 CAACCTGCTAGCCAGATGGGCCAAAACGTCCTTCCTTTGCCCGGTGTAGCAACCCAAGGC
F Q G F D T T G Q V A P P G M K P R V T
3181 TTCCAAGGCTTTGACACCACCGGACAGGTTGCTCCCCCAGGAATGAAGCCCAGAGTGACA
A T L W E D E G S L C F Q V E A R G I C
3241 GCTACGCTGTGGGAAGACGAGGGCAGTCTGTGTTTTCAGGTCGAGGCCCGCGGTATCTGT
PstI
V A R R E
Intron 1
3301 GTCGCGCGCCGTGAGGtaagttgcaatgccatgccacattagaactgcagtcgttgaca
HindIII
D N H M I N G T K L L N V A G M T R
3361 cctttgcACAATCACATGATCAATGGCACCAAGCTTCTCAACGTTGCTGGTATGACCC
NotI
PKC site
· G R R D G I L K S E K V R H V V K I G P
3421 GCGGCCGCAGAGATGGCATTCTCAAGAGCGAAAAAGTGAGACATGTTGTCAAGATTGGCC


· M H L K G V W
SacI
Intron 2
3481 CCATGCATTTGAAGGGTGTCTG aagttgagctcagcaaagacgcgtctactgccgagt
I P F E R A L D F A N K E K ·
Intron internal consensus sequence
3541 tccagctaacg cgacgc GATTCCATTTGAGAGGGCACTCGACTTCGCCAACAAGGAGA
SacI
PKA site
· I T E L L Y P L F V H N I G A L L Y H P·
3601 AGATTACGGAGCTCCTGTACCCGCTGTTTGTTCACAACATTGGTGCTTTGCTGTACCACC
· T N Q T R T N Q V M A A A E R R K Q E Q·
3661 CCACAAATCAGACCCGCACCAACCAGGTCATGGCTGCTGCTGAACGTCGCAAGCAAGAAC
· N Q M R G A P Q T G A P G L P S I Q Q H·
3721 AGAACCAAATGCGCGGTGCTCCTCAGACCGGTGCCCCTGGGCTACCCTCCATTCAGCAAC
· H H H M S L P G P Q Q S L P S H A Q M G·
3781 ATCATCACCACATGAGCTTGCCTGGGCCCCAGCAGTCGCTGCCGTCCCACGCTCAAATGG
· R P S L D R A H T F P T P P T S A S S V·
3841 GACGTCCCTCGCTTGATCGCGCGCACACCTTCCCCACGCCGCCGACTAGCGCCTCCAGCG
· M G G N M N A S D S G F Q W A Q G Q G M·
3901 TCATGGGCGGGAACATGAACGCTTCGGACAGCGGATTCCAGTGGGCACAGGGTCAAGGAA
· G S A Q G A N P M S I D T G L S N A R S·
3961 TGGGCAGCGCTCAAGGCGCCAACCCGATGTCCATCGATACCGGCTTGAGCAACGCCCGTT
SmaI
· M P A T P A S T P P G T T I Q N M Q S Y·
4021 CAATGCCCGCAACACCGGCCTCCACACCCCCGGGAACGACTATTCAGAACATGCAATCGT
XhoI
· Q S G A Q Q Y D N S R P M Y N P S A Q Q·
4081 ATCAGTCCGGCGCGCAACAGTACGACAACTCGAGACCGATGTATAACCCTTCCGCCCAAC
· S P Y Q A T N P A S Q D R P V Y G Q P D·
4141 AGTCGCCGTACCAAGCTACCAATCCTGCTTCTCAAGACCGCCCCGTCTACGGCCAGCCCG
PKC site
· P Y A K N D M G P P T T R P A T S G A P·
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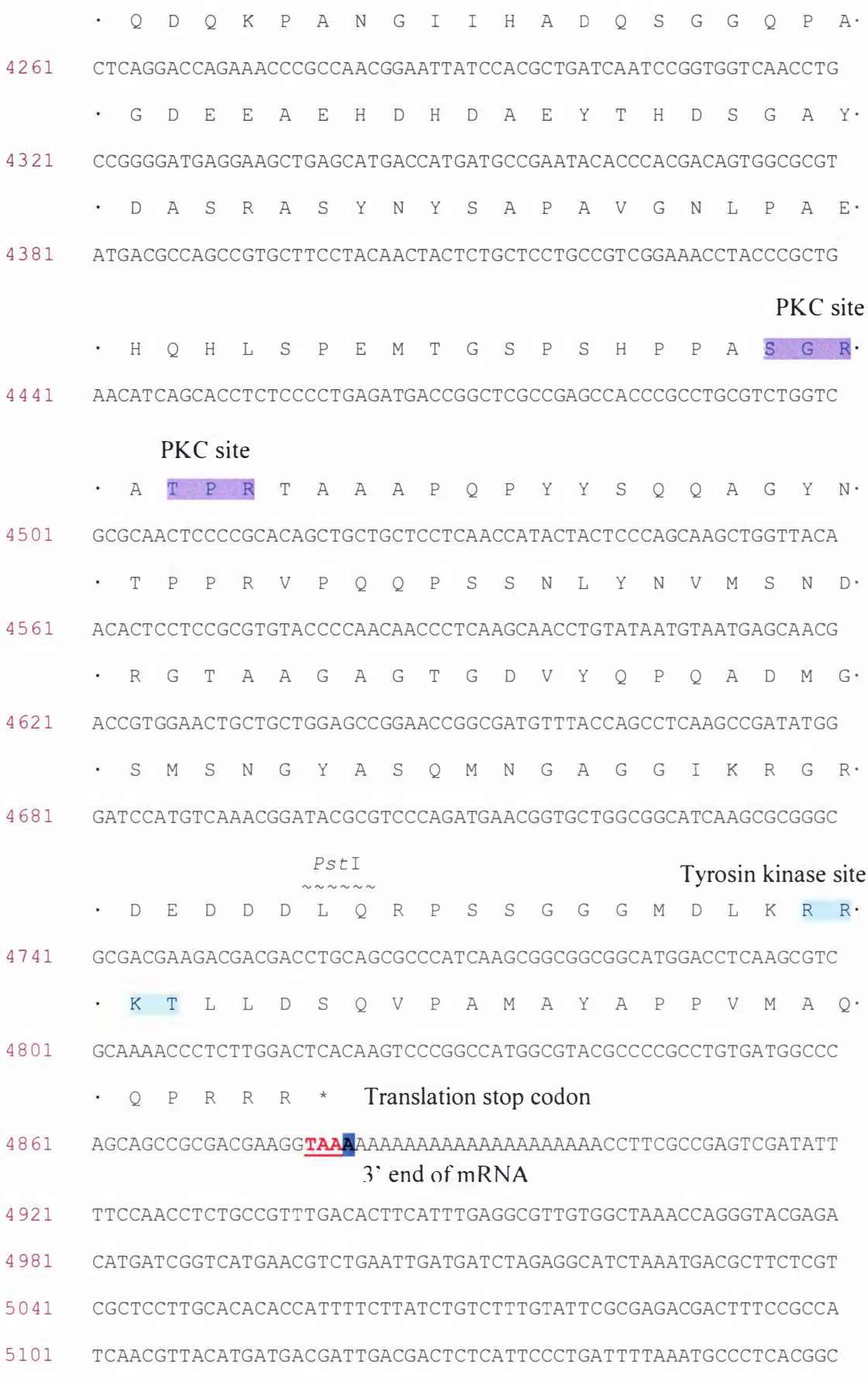
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· G D E E A E H D H D A E Y T H D S G A Y·
4321 CCGGGGATGAGGAAGCTGAGCATGACCATGATGCCGAATACACCCACGACAGTGGCGCGT
· D A S R A S Y N Y S A P A V G N L P A E·
4381 ATGACGCCAGCCGTGCTTCCTACAACTACTCTGCTCCTGCCGTCGGAAACCTACCCGCTG
PKC site
· H Q H L S P E M T G S P S H P P A S G R·
4441 AACATCAGCACCTCTCCCCTGAGATGACCGGCTCGCCGAGCCACCCGCCTGCGTCTGGTC
PKC site
· A T P R T A A A P Q P Y Y S Q Q A G Y N·
4501 GCGCAACTCCCCGCACAGCTGCTGCTCCTCAACCATACTACTCCCAGCAAGCTGGTTACA
· T P P R V P Q Q P S S N L Y N V M S N D·
4561 ACACTCCTCCGCGTGTACCCCAACAACCCTCAAGCAACCTGTATAATGTAATGAGCAACG
· R G T A A G A G T G D V Y Q P Q A D M G·
4621 ACCGTGGAACTGCTGCTGGAGCCGGAACCGGCGATGTTTACCAGCCTCAAGCCGATATGG
· S M S N G Y A S Q M N G A G G I K R G R·
4681 GATCCATGTCAAACGGATACGCGTCCCAGATGAACGGTGCTGGCGGCATCAAGCGCGGGC
PstI
Tyrosin kinase site
· D E D D D L Q R P S S G G G M D L K R R·
4741 GCGACGAAGACGACGACCTGCAGCGCCCATCAAGCGGCGGCGGCATGGACCTCAAGCGTC
· K T L L D S Q V P A M A Y A P P V M A Q·
4801 GCAAAACCCTCTTGGACTCACAAGTCCCGGCCATGGCGTACGCCCCGCCTGTGATGGCCC
· Q P R R R * Translation stop codon
4861 AGCAGCCGCGACGAAGGTAAAAAAAAAAAAAAAAAAAAAACCTTCGCCGAGTCGATATT
3' end of mRNA
4921 TTCCAACCTCTGCCGTTTGACACTTCATTTGAGGCGTTGTGGCTAAACCAGGGTACGAGA
4981 CATGATCGGTCATGAACGTCTGAATTGATGATCTAGAGGCATCTAAATGACGCTTCTCGT
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5101 TCAACGTTACATGATGACGATTGACGACTCTCATTCCCTGATTTTAAATGCCCTCACGGC

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5221  TTATTCCTTTTTTTGTTTTTCTCATTGATCCTCGGGGCAACATACACACGTAAGAGACGG
                                                  SacI
                                                  ~~~~~~
5281  AGATTTGGGACCCCATGGCGCTTCCCTTCGCCTGTTATGAGCTCGGGCGAAGTAGCCACA

5341  CTACTTTGAGGACTGATCACCTACATTACCTTGTTTCCGCTTTCCTTTTCTGTCTTGAAT

5401  ACAGGGGGGATCTGGCACCGAGCGTGTCAAGGGAAGGATGATGACTTTATGATGAATTTT

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5641  TCAGACCGACATGATGTATTTGCAACCTCGACATCAAACACTAGAATGCTTAATAGGTGA

5701  CTTTCTGCCGATTTTCAGCTCTCTGTTTTAAAACGCCTCGATGAGAATATTCACGAAGTA
                              StuI
                              ~~~~~~
5761  TCAAACGAAAACATATGGAGAAAGGCCTGTCAAATCCAAAACTAGGTGTCACTCGCGCAA

5821  CGACATCATATATGCTTAAACCATGCTTACTAGAAGTGTGCATTTTGTAGGTAGGTCCTC

5881  TTTGTCAGAAGAACGGGACTTTAAATCAATTCAAGTATGTGCAGTCAACGAATGAAGGCG

5941  TGGGTGTACTCCCGGTTAAACCCCGGATGTGTGTGCTTTCCGAGGCAAGTAAGCCGGAGC

6001  TGACGTGCCTTCCTTCTCACCTTGCTACCCTTGCTACTGCGGGACGCGATCAACTGCGTG
      SalI
      ~~~~~
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6121  TTACTTCCTGTGACGTCTTTTTCCATTTTGTTTTTTGGCACATCTTTCGCACTGGCAATA

6181  GGCTTTTCTCCCCTCTTCCTCTTGATGATCACGAGAGAATTGGCAACATAGCACGGCGCG

6241  GTCGGATTCAGCCATAAATCGCGATCGCCCACAAACTCGATCGACCAGCTCCAACGATGG

6301  CTACTCCGACTCAGATGAAGCATGCCGCTTCGCAACAAGGCCGGACACCTTCGCAGCTTG
      PstI
      ~~~~~~
6361  CTGCAGCGACACCACCGGTCTCGACACCCTTCTCAGCGTCCCAGACCCATGCAGCCTTCT

6421  CCCCTAGGGGTCCTCGGTCTTCGCCTCAGCAGTTTAAGAAATCGCCTGCGACCTCGACTA

6481  CTCTTATGGGACACCCTAATGGCGCTCTCAACTTCGACAGTCCGTCTGCCGCAGCAGCTA

6541  TGGGCGCCCTTGGAATAAGCGGCGGATTGGATATTGGCTTGGATCATGTTGGTGTCGGAG

6601  GATTCGGTGGTCTGGGAGGAATGGGCGAGGATGACAAGATCAAGCGCCTGGATTCGGTCA

6661  TTGATATTCTTGGGGTCAGTCAAACCTGTCAAATTGCTTCTGCGACGAAGACTGACTAGT
```

```
                                                        XhoI
                                                       ~~~~~~
6721   TTTTCTGGCAGAAAGCGAAAGGACGAGTGAGCGAAGCGGGCCTCGAGCGACTGACCCTCC
6781   GTACAGGTCTTACTAGCATCTGGGACGAGCACCGGTCGCCCGACGGCAGGAAGACCAAGA
6841   TGCTTGTCATAGCAGGACAGGCTCTGACGGTGGACATTGTCTTGAACAACAACGTTGTGG
6901   AGAACGTCTCGTTGACGTTCCCGGAGTCGGCCCCAATCGTGACGAGGCACAAAGACCAAG
                  XhoI
                 ~~~~~~
6961   CCGCCAAGATTCTACTCGAGGATCTCCAGCTTCGGCCGGACCAAAGTCCTCTGACCAAGA
7021   CCCTGGACAAATTCTCTGAGAATCTGGAGCGCCTCGCAAATCTTGACAAGCTCAGTGTCA
7081   TCCCCGGACTTGACTGCCAGGAGGCTCTGGCTGGCATCTTTGAGTGCTTGGAGCGTCTCT
                         PstI
                        ~~~~~~
7141   TCCGTTGGGAGCTGTCGAAGCTGCAGGAAGATGCCGCGATGGGCGGGAAGCCAGCGCGCT
7201   TTCTTCAAACCACAGTCATGTGCATGAAAAGCGGTCGCCCGATGATGCACGCCAGAGACA
7261   TGGTCGGCCTCAGTGTCGAGTATTGGACGGAAAGACGTCATGTCATACCCAAGACGGAGA
7321   CCGTCCGATACTGCGAGAAGCAAGAGAAGGTCTGGTCCATTCTCATAGGCTGCAAAGCGC
                                                      EcoRV
                                                     ~~~~~~
7381   TAGATGACGGAGAGTTATTCCCGCCCGTCCGCGTATCAGAGAGCTGGATATC
```

Figure 4.22 Sequence of the *StuA* gene and its flanking region

The 7,432 bp nucleotide sequence from the *StuA* locus contains a 2,204 bp open reading frame. The polypeptide sequence is shown above the nucleotide sequence. The 5' and 3' ends of the mRNA determined by RACE are indicated. Two introns, determined by RACE and BLASTX analysis are shown in lower case letters. The conserved sequences of intron/exon boundaries are indicated by shaded letters. A cAMP response element and putative transcription factor binding sites for the *A. nidulans* transcription factors BrlA, AbaA, and AreA are indicated by shaded letters. The putative phosphorylation sites on the polypeptide for PKC, PKA and tyrosine kinase are indicated by shaded letters. Selected restriction sites are shown above the nucleotide sequence.

The location of the StuA432 fragment (Fig 4.4) is from nucleotide position 3241 to 3673; the 1.7 kb *Sac*I fragment (Fig 4.6) is from 1825 to 3515; the StuA595 fragment (Fig 4.8) is from 2918 to 3513; the 716 bp *Hin*dIII/*Xho*I fragment is from 3394 to 4110; the insert in pXho41 is from 1 to 4110; the insert in pHE40 is at 3394-7432; the 2.0 kb *Sma*I fragment and the 2.6 kb *Xho*I fragment used for deletion vector construction cover the regions from 676 to 2624 and from 4110 to 6763, respectively; the Sal53 probe (Fig 4.27) is from 729 to 6061.

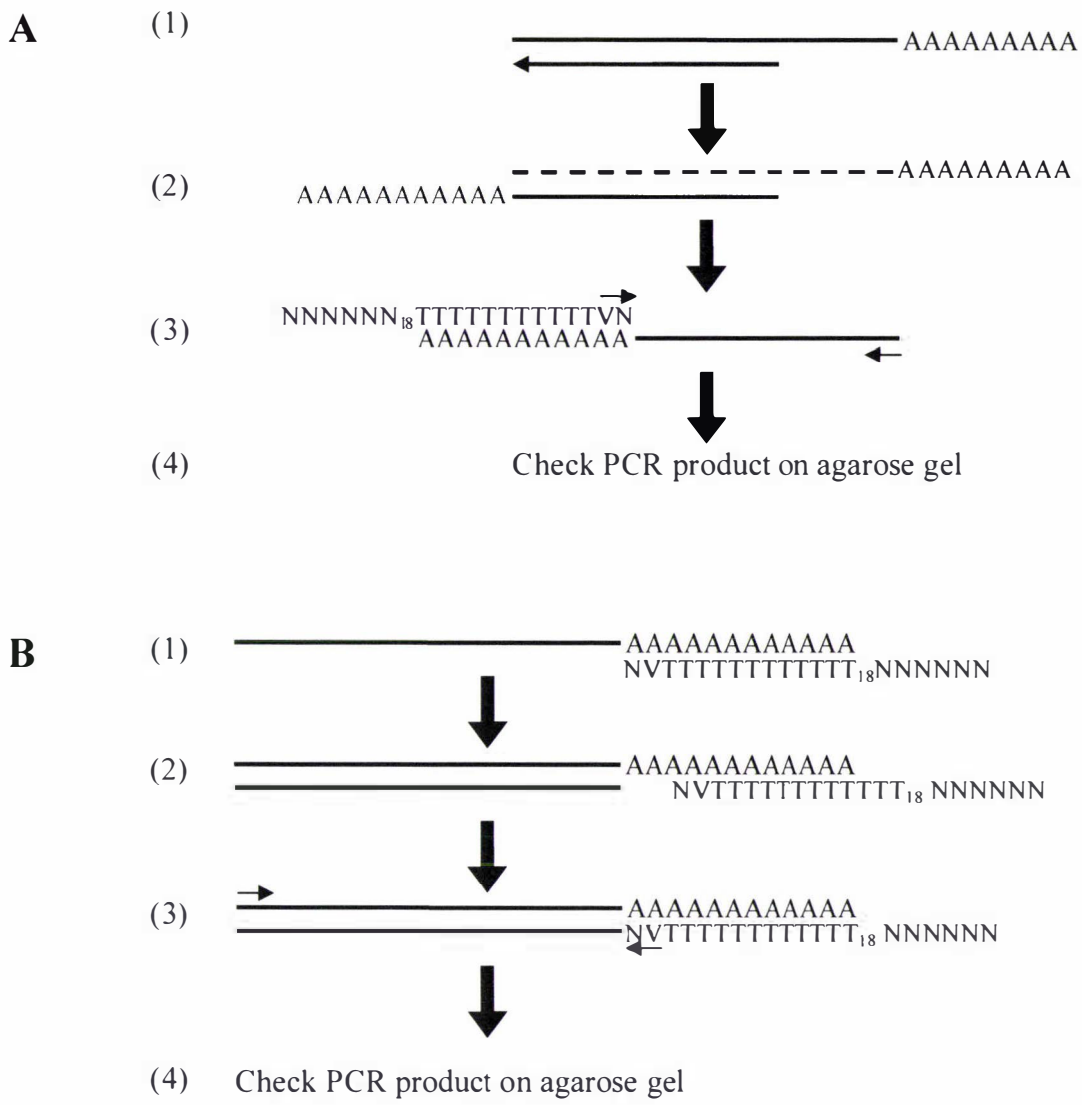


Figure 4.23 5'RACE and 3'RACE strategies

Panel A: 5'RACE. The first strand cDNA was synthesised using a reverse transcriptase with a gene specific primer 1752R (step 1). A poly(A)-tail was added to the cDNA using terminal transferase (step 2). PCR was performed using a nested gene specific primer iv_up, the Oligo dT-anchor primer and the A-tailed cDNA as template (step 3). The PCR product was examined by agarose gel electrophoresis and cloned (step 4).

Panel B: 3'RACE. The first strand cDNA was synthesised using MMLV reverse transcriptase with a polyT primer which has random sequence at the 5' end of the T residues (step 1 and 2). The cDNA is used as template in a PCR using a gene specific primer and another primer that anneals to the 5'end of the polyT primer (step 3). The PCR product was examined by agarose gel electrophoresis and cloned (step 4).

described in Section 3.1 was used in the 5'RACE experiment. The first strand cDNA was synthesized from total RNA using the primer 1752R (Appendix 3) and Expand reverse transcriptase (Roche). After purification of cDNA reactions, terminal transferase (Roche) was used to add a homopolymeric A-tail to the 3' end of the cDNAs. The poly(A)-tail is used due to the weaker A/T binding than G/C binding, therefore longer stretches of A residues are required before the oligo dT-anchor primer will bind to an internal site and truncate the amplification product. The A-tailed cDNA was amplified by PCR using a nested gene specific primer iv_up (Appendix 3) and the oligo dT-anchor primer. The oligo dT-anchor primer was a mixture of oligonucleotides carrying a non-T nucleotide (*i.e.* A, C or G) at their 3' end. By this means the oligo dT-anchor primer is forced to bind to the start of the poly(A)-tail. Thus, the actual length of the poly(A)-tail has no influence on the site of priming. The PCR products were analysed on an agarose gel. As shown in Figure 4.24, the PCR product from the 5'RACE reaction was a single sharp band, this indicated that the *G. cingulata StuA* gene may have only one major transcription start point. This band was recovered from the gel, cloned into the vector pGEM-T Easy (Promega) and sequenced. The absence of the 53 bp intron indicated that this sequence was from mRNA. The transcription start point was determined by identification of the nucleotide immediately downstream of the primer sequence. The results showed that the transcription start point of the *StuA* gene is a G residue 302 bp upstream of the presumptive translation start site.

In the 3'RACE experiment, first strand cDNAs were synthesised using Expand Reverse Transcriptase (Roche) and the oligo(dT)-anchor primer. The specific *StuA* cDNA was then amplified by PCR using a gene specific primer (1054F, Appendix 3) which annealed to the predicted 5' untranslated region, and a primer (3'RACE outer primer) that annealed to the complementary strand of the oligo(dT)-anchor primer. A pair of nested primers (1-24F/3'RACE inner primer, Appendix 3) was used in a second PCR. Three PCR products were detected by agarose gel electrophoresis (Fig 4.24). The strongest band was about ~2 kb, which was consistent with the expected size of the *StuA* ORF. This band was therefore cloned and sequenced. The sequencing revealed that this cDNA ended one nucleotide after the translation stop codon. The other two weaker bands (1.5 kb and 3.7 kb) were thought to be non-specific products because they were

less abundant. The 1.5 kb product was shorter than the *StuA* ORF and could not have resulted from an alternative splicing event. However, following northern blot analysis of the *StuA* gene (Section 4.6.4) it was realized that the 3.7 kb band might be a true product from the *StuA* mRNA. The 2 kb band may have resulted from the binding of the oligo-dT anchor primer to the run of adenosine nucleotides immediately after the stop codon. The same situation may also affect the 5'RACE result. There is run of a 10 thymine nucleotides immediately upstream of the 5' end of the RACE product. This would be converted to a run of adenosine nucleotides in the cDNA and the oligo-dT anchor primer used in the subsequent PCR reactions may have bound to this sequence rather than the true end of the cDNA. This would have result in a shorter product which would not indicate the true transcription start site. Using RLM-RACE may overcome this problem because the primer used in PCR would anneal to the adapter which contains random sequence rather than a run of adenosine nucleotides. Time constrictions prevented a re-examination of this question.

The primers used for these two RACE experiments were designed to make cDNA products that overlapped. Assembly of the two sequences provided at least a partial sequence for the *StuA* mRNA. The ORF from this mRNA sequence was consistent with the ORF predicted from the genomic sequences. This sequence is 2408 nucleotides in length containing a 302 bp 5' untranslated region and a 2094 bp open reading frame that ended at a TAA stop codon. The open reading frame encodes a 698 residue protein that has a predicted unmodified molecular mass of 77.8 kD. The deduced protein sequence was aligned with the protein sequences of other members of the APSES family using the Vector NTI program and the results are shown in Fig 4.25 and Table 4.3. The StuA protein displayed a high degree of identity and similarity with other APSES family protein sequences, especially in the N-terminal APSES domain. The sequences in the C-terminal part of the molecule were more divergent. The highest identity/similarity is between the StuA protein and the *N. crassa* ASM1 protein (52.6% identity/59.9% similarity, in overall protein sequence; 98.2% identity /98.2% similarity, in the 113 amino acid APSES domain). Using the ScanProsite program (http://www.gene-regulation.com/), a number of phosphorylation sites were found for Protein kinase C (241-243, 484-486, 571-573 and 575-577), Tyrosine kinase (273-279), and cAMP- and

Figure 4.24 Determination of 5' and 3' end of the *G. cingulata StuA* mRNA

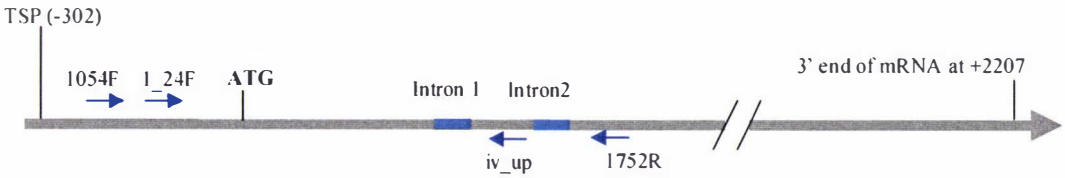
Panel A: The position and orientation of the primers used in the 5' and 3'RACE are shown relative to the ATG start codon, and two introns within the *StuA* gene. The transcription start point and the 3' end of the mRNA were determined and their locations are indicated. The sequence of the primers is given in Appendix 3. The figure is not to scale.

Panel B: Electrophoretic analysis of the PCR products from the 5'RACE (left panel) and 3'RACE (right panel) using mycelial RNA. In 5' RACE, cDNA was synthesized using the primer 1752R at 42^0C for 45 min and A-tailed with terminal transferase. PCR was performed using the primer iv_up and the oligo dT-anchor primer (lane 1), or the primer iv_up only (lane 2), or the oligo dT-anchor primer only (lane 3). The primer pair 1_24F/1752R was used to amplify genomic DNA (lane 4) or A-tailed cDNA (lane 5) as the positive controls. A no template negative control (lane 6) was included. In the 3'RACE, cDNA was synthesized using the oligo(dT)-anchor primer. PCR product was obtained using the primer pair 1054F/3'RACE outer primer followed by a second amplification using the primer pair 1_24F/3'RACE inner primer (lane 7), or the primer 1_24F only (lane 8), or the 3'RACE inner primer only (lane 9). The controls were the same as in the 5'RACE (lanes 10-12). PCR reactions consisted of 1x *Taq* DNA polymerase buffer (Roche), 200 µM of each dNTP, 500 nM of each primer, 0.02 unit/µl of *Taq* DNA polymerase (Roche). The thermocycle conditions were 2 min at 94^0C followed by 34 cycles of 10 sec at 94^0C, 30 sec at 60^0C, 120 sec at 72^0C, and a final incubation for 5 min at 72^0C.

Panel C: Sequence across the cDNA/adaptor oligonucleotide boundary for the PCR product shown in lane 1 (B, left panel). The TSP is indicated by the vertical arrow. The sequence of the cDNA product (not shown in full) exactly matched the *StuA* sequence downstream of the TSP.

Panel D: Sequence across the cDNA/adaptor oligonucleotide boundary for the major PCR product shown in lane 1 (B, right panel). The 3' end of the mRNA is indicated by the vertical arrow. The sequence of the cDNA product (not shown in full) exactly matched the *StuA* sequence upstream of the 3' end of the cDNA.

A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1_24F
ATG
Intron 1
Intron2
3' end of mRNA at +2207
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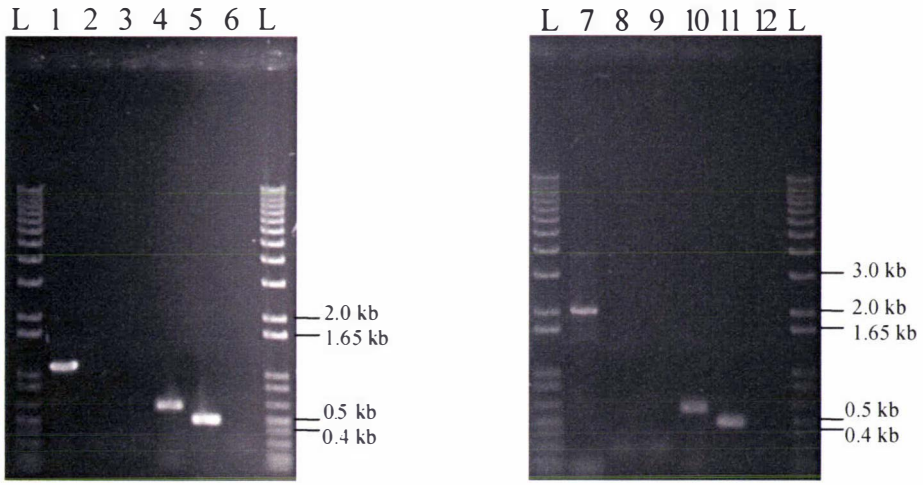
1752R

B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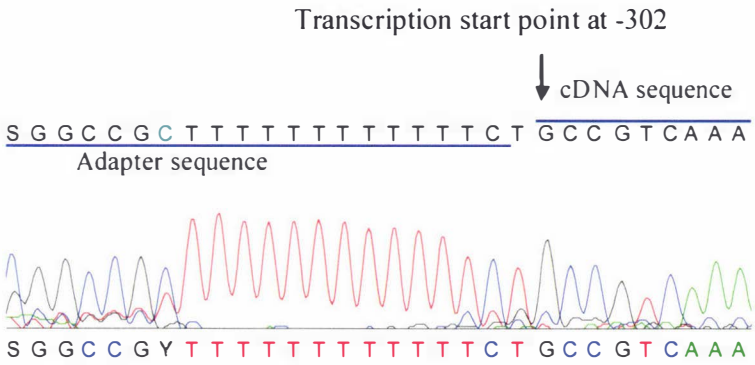
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Adapter sequence
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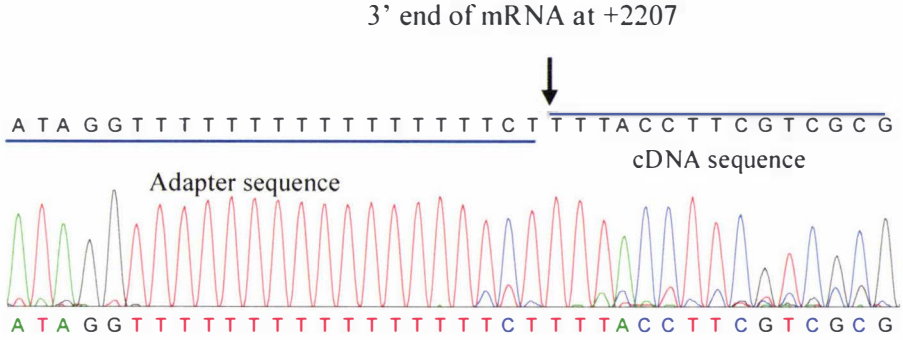
D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cDNA sequence
Adapter sequence
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P.marneffei
A.nidulans
M.grisea
G.cingulata
N.crassa
F.oxysporum
Y.lipolytica
S.cerevisiae
C.albicans


P.marneffei TARTSQGPQAPALHHHHSMNGSVPSHMPQASASTPQTNGRPELNRAHTFPTPPASASSLI 316
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M.grisea GMR-NPQ-GPPGLP--ALHHHSMSQNGSQ-SLS--GNIGRPSLDRAHTFPTPPTSASSAV 284
G.cingulata QMRGAPQTGAPGLPSIQQHHHHMSLPGPQQSLPSHAQMGRPSLDRAHTFPTPPTSASSVM 374
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Y.lipolytica ----------INTPSTAEYYQQGNPAYKAAPT 373
S.cerevisiae ----------
C.albicans ----------DQYLQQQQQQQQQMYGYQSNYYQGGAAN 461
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C.albicans SSYYPN----------YYQQQQPNYASSYPYQQQQQKQQQQQ 493
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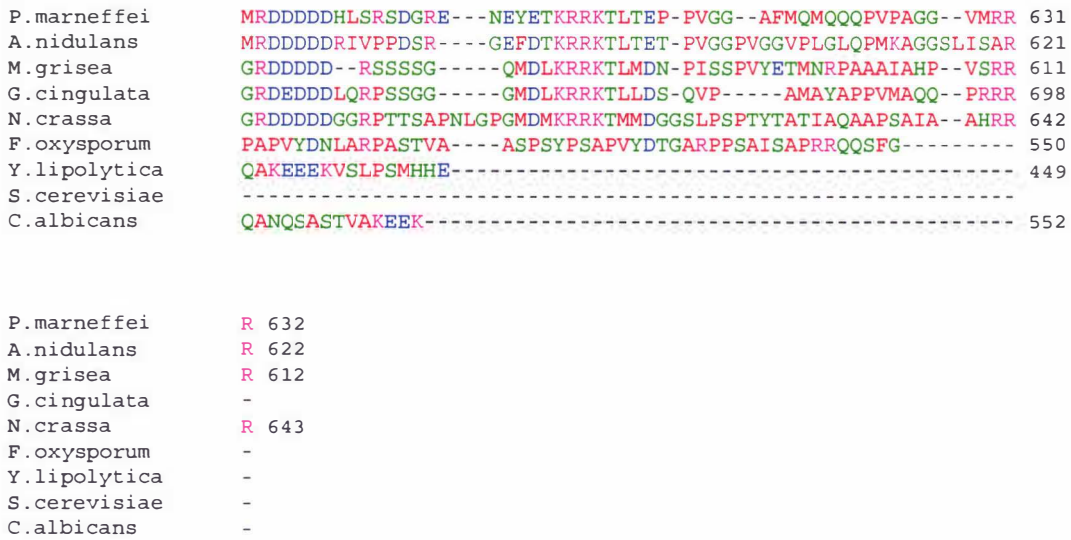


Figure 4.25 Alignment of the StuA protein sequence with other proteins from the APSES family

The deduced *StuA* protein sequence was aligned with other APSES gene products using ClustalW. Symbols below the alignment have the same meaning as described in Fig 4.1. The APSES proteins used in this alignment are also the same as listed in Fig 4.1, with the addition of the FoStuA protein (Ohara and Tsuge, 2004).

The presumptive N-terminal amino sequence is longer compared to those of the other filamentous fungal *StuA* gene products, because an in-frame ATG, upstream of the ATG adapted as the translation start codon in other studies, was used to be the translation start codon for the *G. cingulata StuA* gene. Whether this is genuine will need the confirmation from the StuA protein sequence which has not been reported yet.

Table 4.3 Comparison of the StuA protein with homologous proteins from the APSES family

Protein/gene name	Organism	Genbank accession No	Identity/similarity with GC-StuAp in overall sequence	Identity/similarity with GC- StuAp within the APSES domain	Reference
ASM-1	*Neurospora crassa*	U51117	52.6%/59.9% (632)*	98.2%/98.2% (113)*	(Aramayo and Metzenberg, 1996)
MG00692	*Magnaporthe grisea*		50.6%/58.8% (612)	95.6%/97.3%(113)	http://www.broad.mit.edu/ annotation/ fungi/magnaporthe/
StuA	*Penicillium marneffei*	AF436076	39.6%/47.5% (633)	92.0%/97.3%(113)	(Borneman *et al.*, 2002)
StuA	*Emericella nidulans*	M83569	40.4%/50.8% (622)	86.7%/92.0%(113)	(Miller *et al.*, 1992)
FoSTUA	*Fusarium oxysporum*	AB180746	30.6%/38.0% (550)	95.6%/98.1%(113)	(Ohara *et al.*, 2004)
Hypothetical protein	*Yarrowia lipolytica*	AJ007730	24.2%/30.1% (449)	79.6%/89.4%(113)	(Dujon *et al.*, 2004)
EFG1	*Candida albicans*	Z32687	19.6%/28.4 (552)	67.3%/78.8%(113)	(Stoldt *et al.*, 1997)
PHD1	*Saccharomyces cerevisiae*	U05241	14.1%/20.9% (366)	64.6%/75.2%(113)	(Gimeno and Fink, 1994)

Homologs of the StuA protein sequence were aligned with the Vector NTI program. In the 4th and 5th column, the first value is the percentage of identity between the *G. cingulata* StuAp and the protein from fungus shown in 2nd column. The second value is the percentage of similarity between these proteins.

* indicates the numbers of amino acid residues used in the comparison.

cGMP-dependent protein kinase (672-675). However, the *in vivo* role of these sites remains to be determined.

The promoter sequences of the APSES gene family are divergent. The *StuA* promoter sequence also showed low identity to the promoters of other genes in this family. The transcription factor binding sites in the *StuA* promoter region were identified manually or using the TRANSFAC program (http://www.gene-regulation.com/). No TATA box was identified. No typical cAMP response element (TGACGTCA) was found in the promoter region, but two CGTCA motifs were identified at -320 and -326 on opposite strands. These elements could be configured to function cooperatively in response to cAMP stimulation (Fink *et al.*, 1988; Montminy, 1997), suggesting that the expression of this gene may be regulated by cAMP levels or cAMP response element binding proteins. There are two potential binding sites (CATTCC, at -663 and -939) for the *A. nidulans* transcription factor AbaAp (Andrianopoulos and Timberlake, 1994), which functionally cooperates with the *StuA* gene to regulate the differentiation of the asexual reproductive structure (Miller *et al.*, 1992). There are four potential binding sites (YCCCTYT/G, at -1317, -1802, -2177 and -2270) for the *A. nidulans* transcription factor BrlAp, which is known to regulate expression of the *A. nidulans StuA* gene (Wu and Miller, 1997). There is one potential binding sites (TATCGT, at -364) for the *A. nidulans* transcription factor AreA (Kudla *et al.*, 1990; Peters and Caddick, 1994), which is the major positive-acting nitrogen regulator and a sequence-specific DNA-binding protein. This suggested that the expression of the *StuA* may be regulated by the homologs of these proteins in *G. cingulata*. However, the exact role of these elements remains to be determined by further investigation.

4.4 Deletion of the *G. cingulata StuA* gene

To generate a mutant in which the *StuA* gene had been deleted, a gene deletion vector was constructed in three steps (Fig 4.26). In the first step, a 2.0 kb *Sma*I fragment from the *StuA* promoter region (corresponding to nucleotides 673 to 2626 in Fig 4.22) was subcloned from plasmid pXho41 into the *Sma*I site of pBlueScript II KS. A plasmid with the desired orientation was selected by restriction enzyme mapping. This plasmid was designated pSma20. Then, a 2.6 kb *Xho*I fragment containing part of the *StuA* gene

and sequence downstream of the gene (corresponding to nucleotides 4109 to 6767 in Fig 4.22) was cut from plasmid pHE40 and inserted into the *Xho*I site of pSMA20. The orientation of the insert was again checked by restriction enzyme mapping. The selected plasmid was designated pSX. Finally, a 3.2 kb *Hin*dIII fragment that contained a hygromycin resistance cassette was transferred from the plasmid pFAT3 (Fig 3.5) into the *Hin*dIII site of plasmid pSX. This final construct was designated plasmid pSXH. The orientation of the *Hin*dIII fragment in pSXH was determined by restriction digestion.

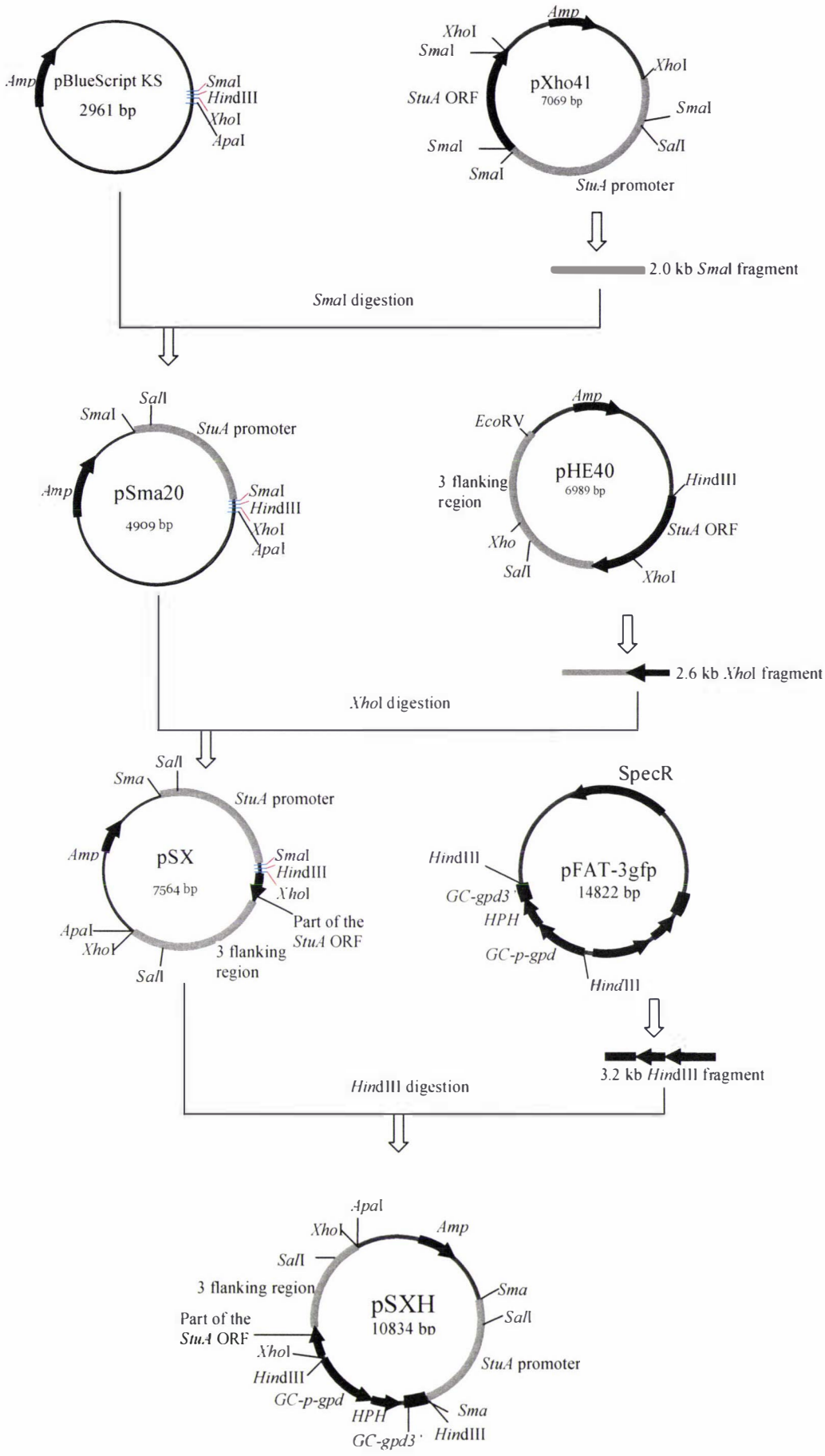
In the wild type *G. cingulata* genome, the region between the 2.0 kb *Sma*I and 2.6 kb *Xho*I fragments contained most (1373 bp) of the *StuA* ORF. After transformation of *G. cingulata* with plasmid pSXH, this region would be replaced by the hygromycin resistance cassette if a double crossover event occurred between the *StuA* locus and pSXH.

G. cingulata protoplasts were prepared, using spores collected from a 7 day old PDA plate, by digestion with Glucanex. Protoplasts (4×10^8) were transformed with 20 μg of pSXH linearised by *Apa*I (which cut the pBlueScript II KS backbone once). The transformation mixture was plated on regeneration plates. Three days after applying the hygromycin overlay, 280 colonies ranging from 3 to 9 mm in diameter had grown through the overlay. Sixty colonies (designated DC1 to DC60) were selected randomly and transferred to PDA plates containing hygromycin (200μg/ml). The appearance of the colonies for all 60 transformants was flat and white, resembling that of the disruption mutants SC5 and SC40. This suggested that all of them were deletion mutants. Single spore isolates from 20 (DC41 to DC60) of them were selected and subcultured on PDA plates. Mycelium from 7 day old plates was collected and freeze-dried to extract genomic DNA for subsequent analysis.

PCR analysis was performed on these 20 transformants. Three pairs of primers were used (Fig 4.27). One set of primers (xho43 and hyg1, Appendix 3) was designed to amplify a 3.4 kb fragment across the *Sma*I fragment used to target the deletion cassette

Figure 4.26 Construction of a *StuA* gene deletion vector

Construction of the *StuA* gene deletion vector was as shown. Abbreviations used are as follows: *GC'-gpd-p*, *G. cingulata* glyceraldehyde-3-phosphate dehydrogenase promoter; *GC'-gpd3'*, *G. cingulata gpd* terminator; HPH, hygromycin B resistant gene; Amp, Ampicillin resistant gene; SpecR, spectinomycin resistance gene cassette. The unique *Apa*I site which was used to linearise the deletion vector is shown.


pBlueScript KS
2961 bp
Amp
SmaI
HindIII
XhoI
ApaI
pXho41
7069 bp
Amp
XhoI
SmaI
StuA ORF
SmaI
SmaI
XhoI
SmaI
SalI
StuA promoter
2.0 kb SmaI fragment
SmaI digestion
pSma20
4909 bp
SalI
SmaI
StuA promoter
Amp
SmaI
HindIII
XhoI
ApaI
pHE40
6989 bp
Amp
EcoRV
3 flanking region
HindIII
StuA ORF
Xho
SalI
XhoI
2.6 kb XhoI fragment
XhoI digestion
pSX
7564 bp
SalI
Sma
StuA promoter
Amp
SmaI
HindIII
XhoI
Part of the StuA ORF
ApaI
XhoI
SalI
3 flanking region
pFAT-3gfp
14822 bp
SpecR
HindIII
GC-gpd3
HPH
GC-p-gpd
HindIII
3.2 kb HindIII fragment
HindIII digestion
pSXH
10834 bp
ApaI
XhoI
Amp
SalI
3 flanking region
Sma
SalI
Part of the StuA ORF
XhoI
HindIII
StuA promoter
GC-p-gpd
HPH
Sma
GC-gpd3
HindIII

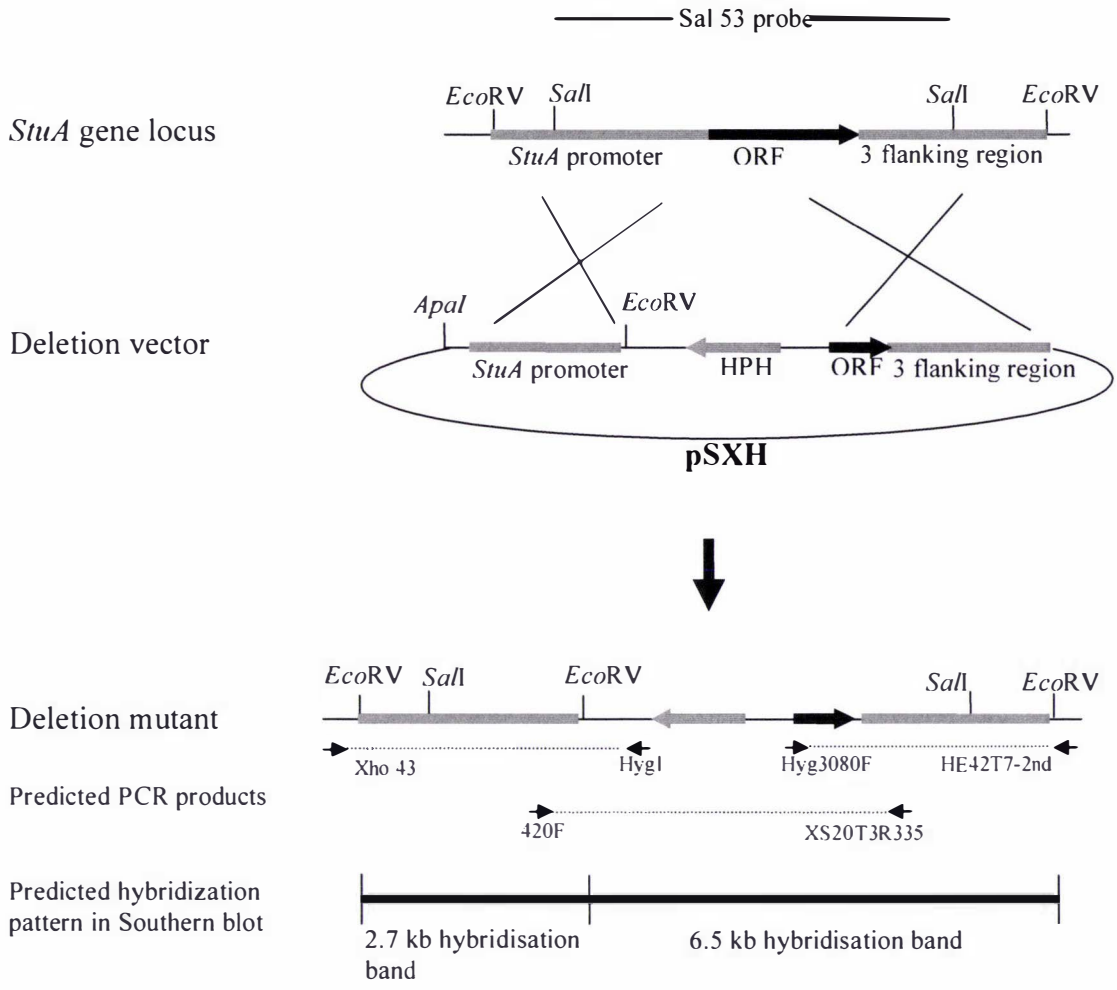


Figure 4.27 Strategy used for deletion of the *StuA* gene and predicted results

The deletion vector pSHX contained the hygromycin resistant cassette (HPH) flanked by 5' and 3' regions of the *StuA* gene. After transformation of *G. cingulata* protoplasts, homologous recombination would occur between the deletion vector pSXH and the *G. cingulata* genome by a double crossover event. The resulting deletion mutant can be screened by PCR using three pairs of primers, which would detect recombination in 5' or 3' region of the *StuA* gene and the existence of the hygromycin resistant cassette. Southern blot analysis of *Eco*RV digested genomic DNA from the deletion mutant would display a 2.7 kb and a 6.5 kb band that hybridised to a 5.3 kb *Sal*I fragment of the *StuA* locus (the Sal53 probe).

to the *StuA* locus. A second set of primers (hyg3080F and HE42T7-2nd, Appendix 3) was designed to amplify a 3.0 kb fragment across the *Xho*I fragment used to target the deletion cassette to the *StuA* locus. The third set of primers (420F and XS20T3R335, Appendix 3) was designed to amplify a 4.0 kb fragment across either the hygromycin resistance cassette in the deletion mutants or a 2.2 kb fragment from wild type DNA. All twenty transformants showed the expected pattern in the three PCR reactions (Fig 4.28).

Southern blot analysis of all 20 transformants was performed to confirm the results of the PCR analysis. Plasmid pXHE (Fig 4.20) was digested with *Sal*I and the 5.3 kb *Sal*I fragment containing the *StuA* gene was used as probe (designated probe Sal53). This fragment contained the *StuA* ORF plus 1.95 kb of 5' flanking and 1.17 kb of 3' flanking sequence. Genomic DNA was digested with *Eco*RV. This would generate a 7.4 kb fragment of genomic DNA containing the *StuA* gene from wild type DNA. EcoRV digestion of genomic DNA from the deletion mutant would generate two fragments, that would hybridize to probe Sal53 (a 2.7 kb fragment and a 6.5 kb fragment), because a single *Eco*RV site is present in the hygromycin resistance cassette. The presence of these two hybridizing fragments would confirm the correct integration of the hygromycin resistance cassette at both ends of the *StuA* locus. As shown in Fig 4.29, all twenty transformants showed the expected hybridization pattern, indicating that all of them were deletion mutants. An extra 3.0 kb band was present in transformant DC41. A single crossover event occurring at either the 5' or 3' end of a circular deletion vector would not generate such a hybridization pattern, so this represented an additional random insertion of the deletion vector somewhere else in the genome.

The phenotype of the deletion mutants will be described in Section 4.6. The next section describes the construction of *StuA* complementation mutant. This complementation mutant was required in order to confirm that the *StuA* gene itself and not some other undetected events were responsible for the phenotypic changes observed in these deletion mutants.

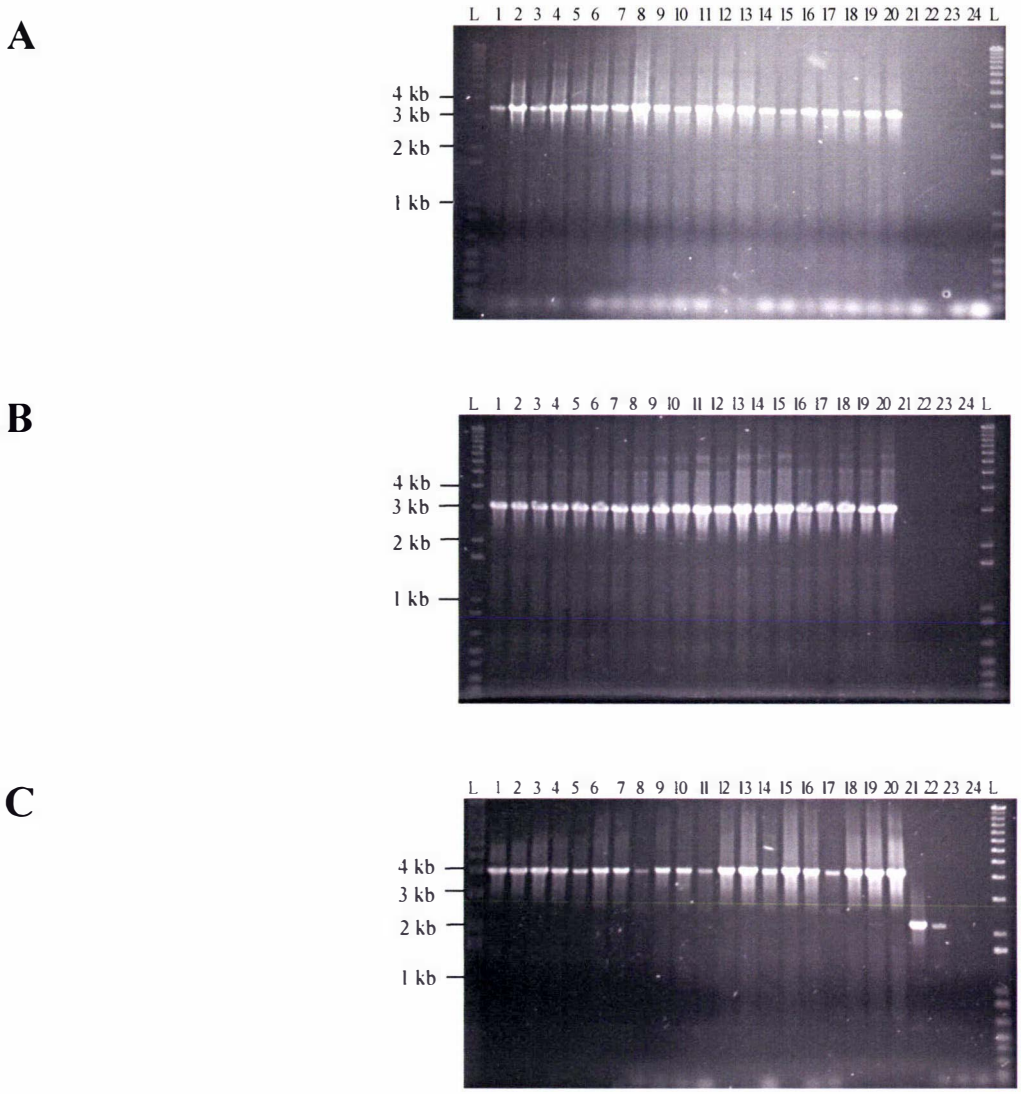


Figure 4.28 PCR analysis of the *G. cingulata StuA* deletion mutants

PCR products amplified with three pairs of primers using genomic DNA from the deletion mutants DC41 to DC 60 (lanes 1 to 20). Wild type genomic DNA was used as positive control (lanes 21 and 22). Lanes 23 and 24 were the no template negative controls. Panel A: PCR using primer Xho43 and Hyg1; panel B: PCR using primer Hyg3080F and HE42T7-2nd; and panel C: PCR using primer 420F and XS20T3 R335.

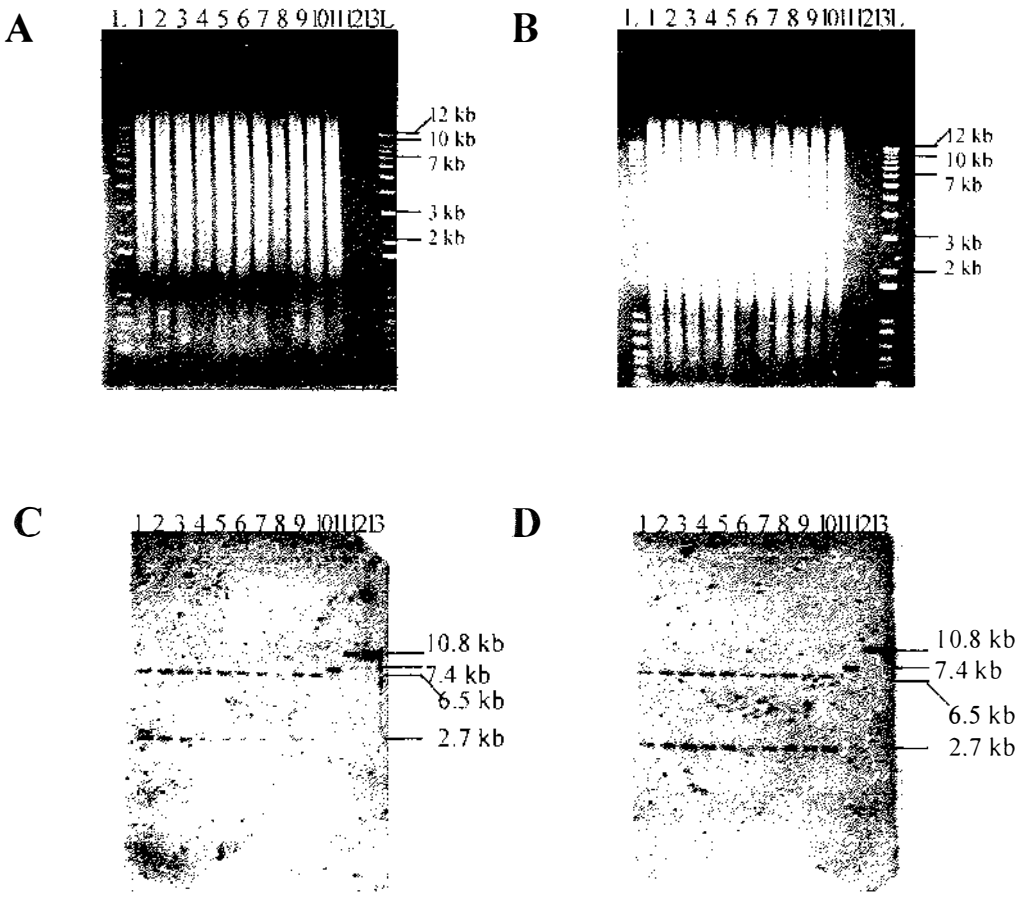


Figure 4.29 Southern blot analysis of the deletion mutants

Panel A: Agarose gel electrophoresis of *Eco*RV digested genomic DNA of the deletion mutants DC41-DC50 (lanes 1-10), wild type (lane 11), 20 ng and 100 ng of plasmid pSHX DNA (lanes 12 and 13) which was linearised with the restriction enzyme *Apa*I. Lane L was the 1 kb plus DNA ladder (Invitrogen).

Panel B: Lanes 1-10 were *Eco*RV digested genomic DNA of the deletion mutants DC51-DC60. Other lanes were the same as in panel A.

Panel C: Autoradiograph of the Southern blot of the gel shown in panel A probed with Digoxigenin-11-dUTP labelled probe Sal53.

Panel D: Autoradiograph of the Southern blot of the gel shown in panel B probed with Digoxigenin-11-dUTP labelled probe Sal53.

4.5 Restoration of the *G. cingulata StuA* gene function

Construction of a complementation vector required a plasmid which contained the entire *StuA* gene and a second selectable marker. Phleomycin was chosen as the second selectable marker because it strongly inhibited the growth of both the wild type (Debbie Hudson, unpublished) and deletion mutant. The *StuA* gene was therefore introduced into the cloning vector pBC-phleo (Silar, 1995). The pBC-phleo plasmid contained a phleomycin resistance cassette in a pBC SK backbone (Strategene). Two different constructs were made. One was designed for integration into the genome by random insertion or a single crossover event. The other was designed for targeted integration by double crossover events involving the *StuA* sequences flanking the StuA/phleomycin resistance cassette in the restoration vector and the *StuA* sequences flanking the hygromycin resistance cassette in the genome of the deletion mutant. In the later case it was not complementation but rather replacement of the deleted gene with a new intact gene.

4.5.1 Single crossover strategy for complementation of the *StuA* deletion

The complementation vector was constructed by introducing the 5.3 kb *Sal*I fragment which contained the *StuA* ORF plus 1.95 kb of the 5' flanking and 1.17 kb of the 3' flanking sequence, from plasmid pXHE (Fig 4.21) into pBC-phleo. The 5.3 kb *Sal*I fragment could not be cloned directly into the *Sal*I site in the pBC-phleo multiple cloning site because a second *Sal*I site is present in the phleomycin resistance gene cassette. Therefore the 5.3 kb *Sal*I fragment was excised from plasmid pXHE, gel purified, end-filled with Klenow, A-tailed with *Taq* polymerase and cloned into the plasmid pGEM-T Easy. The 5.3 kb *Sal*I fragment was excised from pGEM-T Easy using *Eco*RI and cloned into the *Eco*RI site in the multiple cloning site of pBC-phleo. The orientation of the *Sal*I fragment was determined by restriction enzyme mapping (data not shown) and a clone containing the insert orientated as shown in Fig 4.30 was designated pBPS53 and used for subsequent work.

Due to the low level of conidia production by the deletion mutant on PDA plates (described in detail in Section 4.6), it was difficult to obtain sufficient spores to make protoplasts. A method for induction of conidia production, based on that used with *Penicillium* (Roncal and Ugalde, 2003), was therefore adapted for *G. cingulata* (Section 2.10.1). The deletion mutant DC43 was grown in PDB liquid medium (at 25^0C for 3-5 days) and the mycelium was collected. This wet mycelium was homogenized and transferred to the conidia induction medium (containing 12.5% glucose) and incubated at 25^0C for a further 3 days or until the culture turned slightly pink. The yield of conidia from 800 ml of the induction medium was sufficient to make protoplasts for transformation.

Protoplasts of the deletion mutant DC43 were transformed with the complementation vector pBPS53. The aim of this transformation was to introduce a single copy of pBPS53 into the genome while retaining the hygromycin resistance cassette. Although this vector had been designed for integration into the genome by a random insertion event, the frequency of random integration of circular compared to linear DNA was not known, so a mixture of circular plasmid and pBPS53 linearized with *Xba*I was used in the transformation. Integration might either be random or occur by single crossover between the promoter element and the vector. The overlay used on the regeneration plates contained both phleomycin and hygromycin. Without hygromycin selection pressure, a double crossover event could occur between the complementation vector (pBPS53) and the host genome in which the hygromycin resistance gene cassette was replaced by the wild type *StuA* ORF, because the complementation vector contained sequence flanking both sides of the hygromycin resistance cassette. At the same time, the phleomycin resistance cassette in pBPS53 would then be lost and the resulting transformants would not be able to grow on phleomycin plates. Alternatively, this excised cassette could possibly be randomly inserted somewhere in the genome, the resulting transformant would grow in phleomycin containing medium and be selected. This was not desirable because such as a transformant could not be distinguished from a wild type *G. cingulata* strain with a phleomycin resistance cassette randomly inserted in the genome. With hygromycin selection pressure, the complementation vector could be randomly inserted somewhere in the genome or a targeted insertion into the *StuA* locus

could occur by a single crossover event, these could be distinguished by PCR and Southern blot analysis.

Five days after overlaying the PDA containing phleomycin (final concentration 8 μg/ml) and hygromycin (final concentration 200 μg/ml), 102 transformant colonies had grown through the PDA overlay and twenty three colonies were transferred to fresh plates for further growth. Four transformants (R532-2, R532-9, R532-10 and R532-11) that looked like the wild type (black colony and aerial hyphae) and three transformants (R532-4, R532-6 and R532-8) that looked like the deletion mutant (white colony and flat mycelium) were chosen for Southern blot analysis.

*Eco*RV was chosen to digest the genomic DNA of the complementation transformants because it was used in Southern blot analysis of the deletion mutants. If only one copy of the complementation vector had integrated into the *StuA* locus of the deletion mutant by a single crossover event, then the Southern blot hybridization pattern using the 5.3 kb *Sal*I fragment as probe (probe Sal53) for the complementation mutants can be predicted (Fig 4.30). The 6.5 kb hybridizing band (compare Fig 4.30 to Fig 4.27) would still be present but since there are two *Eco*RV sites in the complementation vector (pBPS53), the 2.7 kb hybridizing band in the deletion mutant (refer to Fig 4.27) would be replaced by 6.0 and 7.1 kb hybridizing bands in the complementation mutants (Fig 4.30).

If the complementation vector was randomly integrated anywhere else in the genome of the deletion mutant, the 6.5 kb and the 2.7 kb hybridizing bands in the deletion mutant would be retained in the resulting transformant, and additional hybridizing band(s) will be observed but the number and size of these additional bands can not be predicted.

In the Southern blot analysis shown in Fig 4.31, the four transformants (R532-2, R532-9, R532-10 and R532-11), that looked like wild type, showed the predicted hybridization pattern. Homologous recombination by a single crossover event had occurred between the promoter region of the *StuA* gene and the complementation vector. This seemed to result in the restoration of *StuA* function because their mycelium was fluffy and the colony black like that of the wild type.

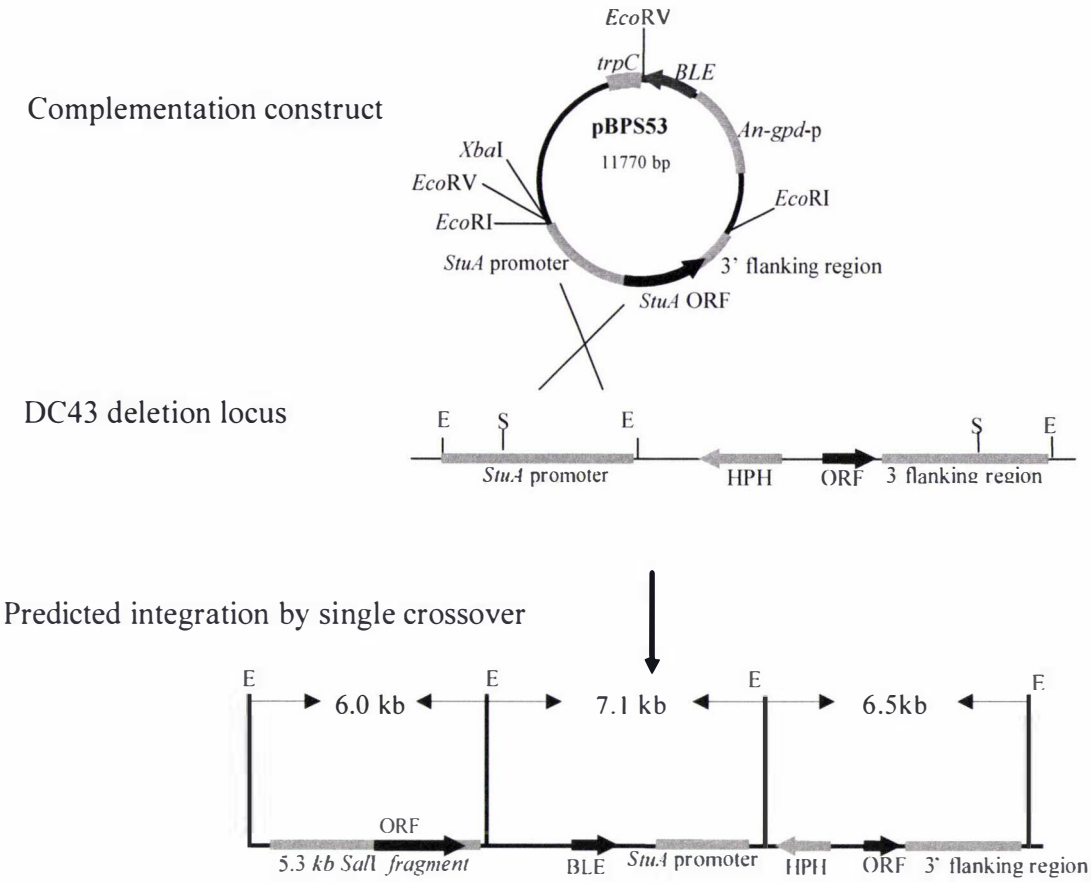


Figure 4.30 First complementation construct and a predicted integration event

A complementation vector was constructed by cloning a 5.3 kb *Sal*I restriction fragment containing the *StuA* gene and 2.25 kb of the promoter into the plasmid pBC-phleo. This plasmid also contained the BLE gene coding a protein product conferring phleomycin resistance. Homologous recombination by a single crossover event could occur between the *StuA* promoter region in the complementation vector and the *StuA* promoter present in the deletion mutant. A map of the resulting genomic DNA is shown. If DNA from such a transformant was digested with the restriction enzyme *Eco*RV and Southern blotted with the probe Sal53, three hybridising bands (6.0 kb, 7.1 kb and 6.5 kb, respectively) would be observed. Abbreviations used are as follows: *An-gpd-p. Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter; *trpC*, *Aspergillus nidulans trpC* terminator; HPH, hygromycin B resistance gene; E, *Eco*RV; S, *Sal*I.

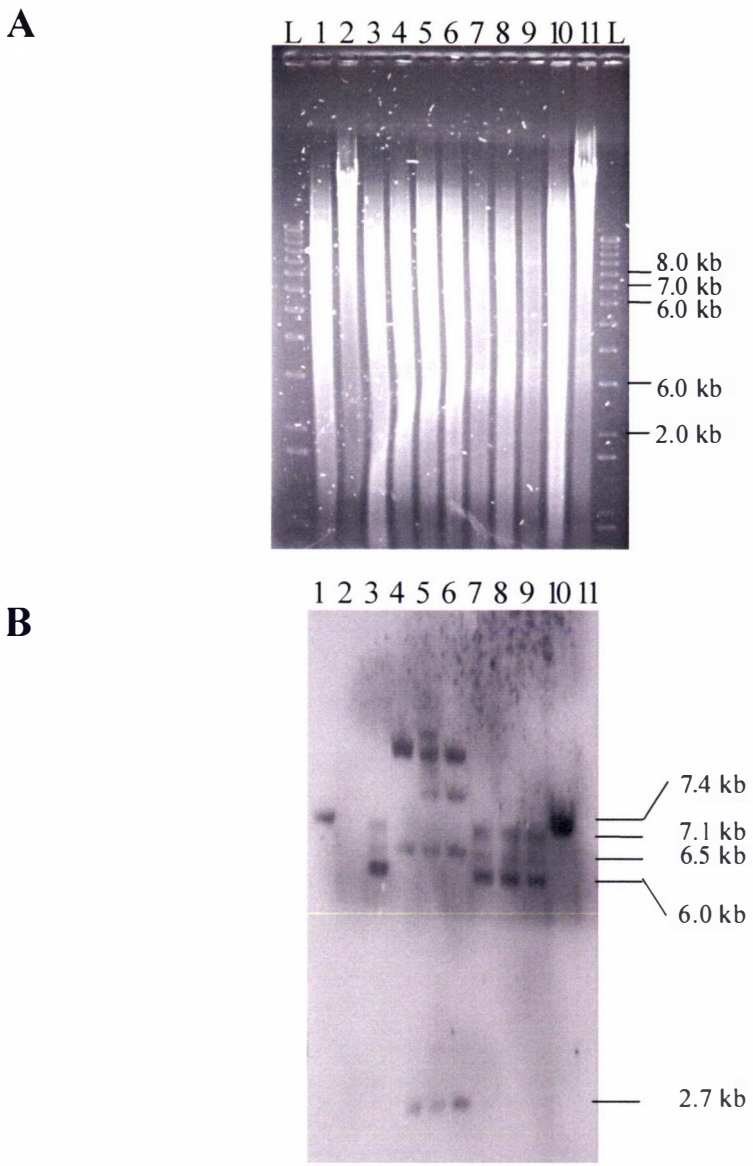


Figure 4.31 Southern blot analysis of the complementation mutants

Panel A: Agarose gel electrophoresis of *Eco*RV digested genomic DNA from the wild type (lanes 1 and 10), the deletion mutant DC43 (lanes 2 and 11), and the complementation mutants R532-2 (lane 3), R532-4 (lane 4), R532-6 (lane 5), R532-8 (lane 6), R532-9 (lane 7), R532-10 (lane 8), and R532-11 (lane 9). Lanes L are the 1 kb plus DNA ladder as size marker. Due to the poor DNA digestion, the hybridised bands on lanes 2 and 11 were not visible.

Panel B: Autoradiograph of a Southern blot of the gel shown in panel A probed with Digoxigenin-11-dUTP labeled probe Sal53.

Transformants R532-4, R532-6 and R532-8 that looked like the deletion mutant were also included in this Southern blot analysis. The 6.5 kb and 2.7 kb hybridizing bands were observed for these three transformants, indicating that the *StuA* deletion locus remained unchanged. The extra bands seen for these three transformants (one in R532-4, two in R532-8 and three in R532-6) indicated that random insertion of the complementation vector had occurred. It seemed that *StuA* function was not restored in these transformants because their mycelium was flat and white just like that of the deletion mutant. These results suggested that the *StuA* gene could only function when it was reintroduced into its own locus or that the vector was inserted via non-homologus integration via *StuA* sequences thereby inactivating *StuA*. The latter possibility could be clarified by a Southern blot analysis but was not pursued further.

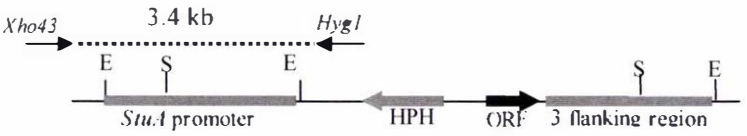
Transformants R532-2, R532-9, R532-10 and R532-11 displayed similar growth rate, conidia production, formation of aerial hyphae and wetability as the wild type (Appendix 4). However, it was noticed that the aerial hyphae collapsed and mycelium became wetable after 7-10 days growth on PDA plates, suggesting that reversion to the deletion mutant had occurred. This was probably due to instability of the single crossover integration event. A PCR reaction was performed to test for the presence of a reformed deletion mutant. DNA was extracted from four transformants R532-2, R532-9, R532-10 and R532-11 from mycelia grown for 7 days on PDA plates with or without phleomycin. PCR was performed using the primer pair xho43 and hyg1, which would amplify a 14.2 kb product from the restored *StuA* locus (Fig 4.32), and a 3.4 kb fragment from the deletion mutant. Under the conditions used for the PCR, no product was expected from the restored *StuA* locus. The 3.4 kb PCR product was observed for all four complementation mutants, indicating that reversion to the genotype of the deletion mutant had occurred.

4.5.2 Double crossover strategy for restoration of the *StuA* gene

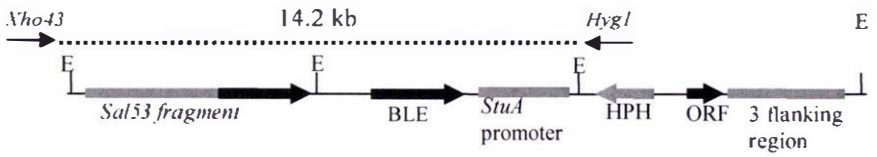
A second restoration vector was designed to replace the hygromycin resistance cassette in the deletion mutant DC43 with the *StuA* gene by means of a double crossover event.

A

DC43 deletion locus



Complementation mutants



B

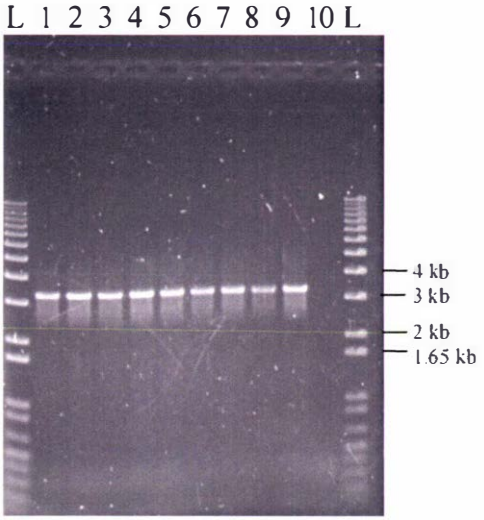


Figure 4.32 PCR analysis of reversion of the complementation mutants

Panel A: Predicted PCR products from the complementation mutant and deletion mutant. E = *Eco*RV; S = *Sal*I.

Panel B: PCR products amplified with the primer pair xho43 and hygl using genomic DNA from phleomycin supplemented cultures of R532-2 (lane 1), R532-9 (lane 2), R532-10 (lane 3), R532-11 (lane 4); and from non-phleomycin supplemented culture of R532-2 (lane 5), R532-9 (lane 6), R532-10 (lane 7), R532-11 (lane 8). The genomic DNA from the deletion mutant DC43 was used as a positive control (lane 9). Lane 10 was the PCR reaction with no template as a negative control.

It was anticipated that this would avoid the instability observed in the complementation mutants obtained by single crossover integration. The fragment containing the *Aspergillus nidulans gpd* promoter and the BLE gene (encoding resistance to phleomycin) was excised, by *Dra*I/*Eco*RV double digestion, from the plasmid pBC-phleo and cloned into the *Stu*I site of the plasmid pXHE (Fig 4.33). Orientation of this fragment was determined by restriction enzyme mapping. The selected plasmid was designated pXHE-phleo-R. It was linearised with restriction enzyme *Eco*RI and transformed into protoplasts prepared from conidia of the deletion mutant strain DC43 as described for the first complementation experiment. Eighty-seven phleomycin resistant colonies were obtained in one transformation, and twenty five colonies were transferred to fresh PDA plates for further growth. Twelve colonies displayed a wild type phenotype. Two of these (NR5 and NR13) were chosen for Southern blot analysis.

Genomic DNA from the restoration transformants NR5 and NR13, along with the wild type strain and the deletion mutant DC43, was digested with either *Xho*I or *Hin*dIII. The DNA digests were size-fractionated by electrophoresis, transferred to a nylon membrane and Southern blotted with the probe Sal53 (Fig 4.34). The hybridization pattern for the *Xho*I digests was used to determine the integrity of the integration event at the 5' end of the *StuA* locus (Fig 4.35). In the wild type, a 4.1 kb *Xho*I fragment and a 2.6 kb *Xho*I fragment hybridized to the probe as expected. For the deletion mutant DC43, while the 2.6 kb *Xho*I fragment was still present, a 6.0 kb *Xho*I fragment was observed because the hygromycin resistance cassette (HPH) was 1.9 kb longer than the *StuA* fragment it replaced. When the *StuA* gene was restored by the complementation cassette, the 4.1 kb *Xho*I fragment was regained and since a new *Xho*I site had been introduced on the 3' side of the *StuA* ORF a 2.85 kb hybridizing band was observed instead of the 2.6 kb fragment. A downstream *Xho*I fragment (2.2 kb) contained a 275 bp region homologous to the probe Sal53, however, this fragment was not expected to be seen because the probe binding would be too weak over such a small region. These results confirmed that the restoration cassette had integrated correctly at the 5' end of the *StuA* locus.

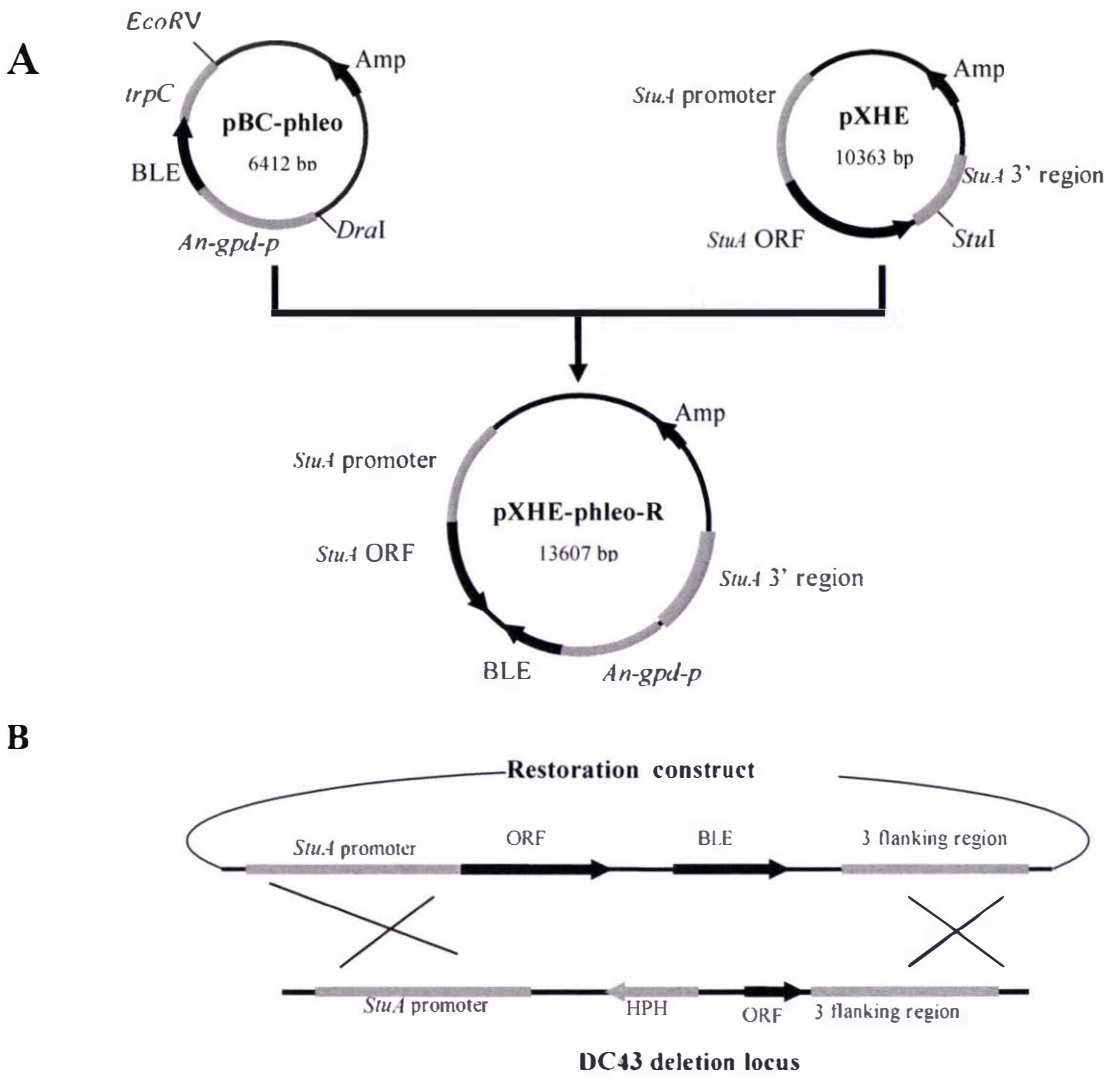


Figure 4.33 Construction of the second restoration vector and its predicted integration into the genome of the deletion mutant

Panel A: Restoration vector construction. The fragment containing the *Aspergillus nidulans gpd* promoter and the BLE gene was excised by *DraI*/*EcoR*V double digestion of the plasmid pBC-phleo and cloned into the *StuI* site of the plasmid pXHE, resulted the plasmid pXHE-phleo-R.

Panel B: Predicted recombination event. After transformation of the deletion mutant DC43, the hygromycin resistance cassette would be replaced with the restoration cassette by homologous recombination via double crossover event.

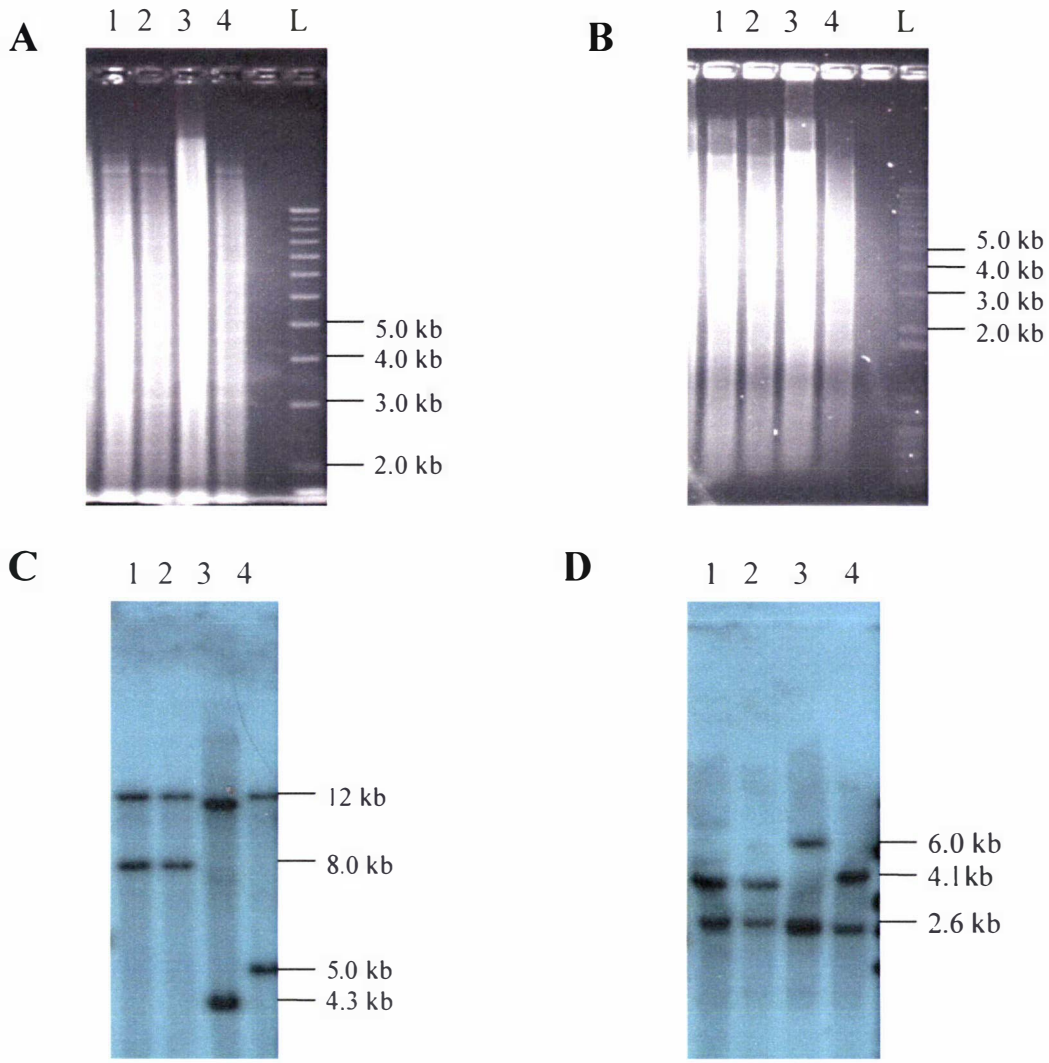


Figure 4.34 Southern blot analysis of the second restoration mutants

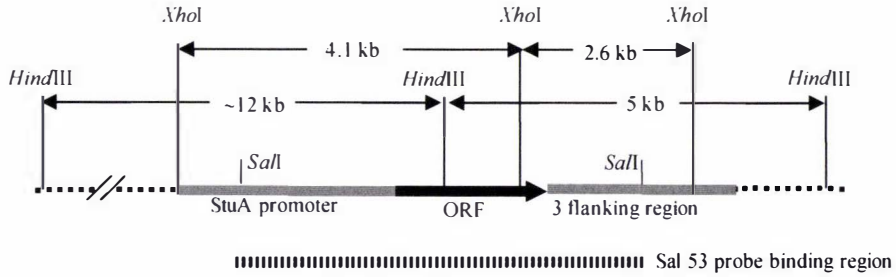
Panel A: Agarose gel electrophoresis of the *Hin*dIII digested genomic DNA of the restoration mutants NR5 (lane 1), NR13 (lane 2), the deletion mutant DC43 (lane 3) and the wild type (lane 4).

Panel B: Agarose gel electrophoresis of the *Xho*I digested genomic DNA of restoration mutants NR5 (lane 1), NR13 (lane 2), the deletion mutant DC43 (lane 3) and the wild type (lane 4).

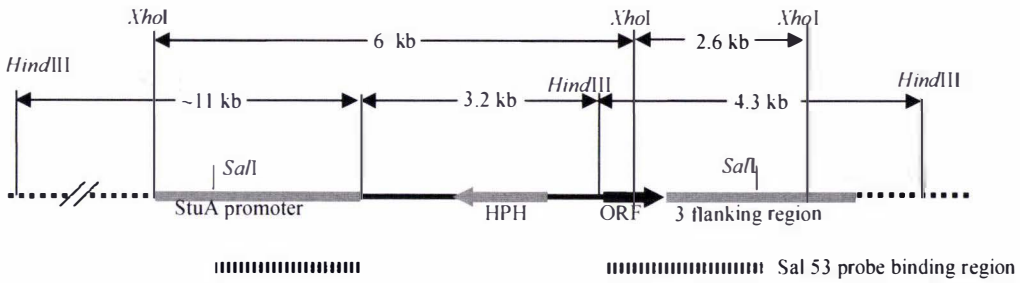
Panel C: Autoradiograph of a Southern blot of the gel shown in panel A probed with Digoxigenin-11-dUTP labelled probe Sal53.

Panel D: Autoradiograph of a Southern blot of the gel shown in panel B probed with Digoxigenin-11-dUTP labelled probe Sal53.

A



B



C

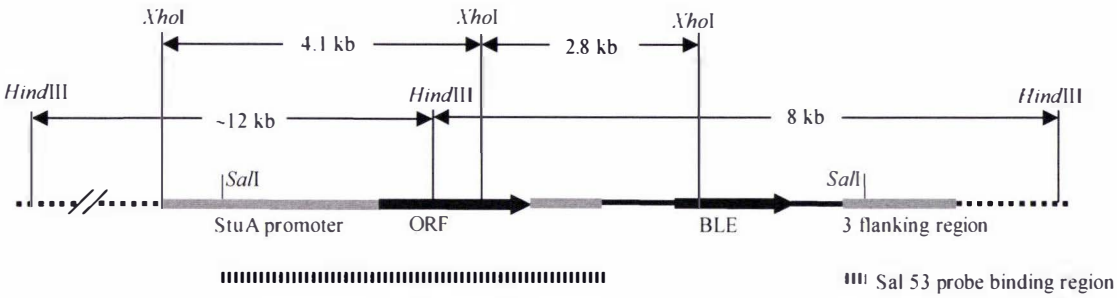


Figure 4.35 Predicted hybridisation pattern for the wild type, the deletion mutant and the restoration mutants

Panel A is the predicted hybridisation pattern for the wild type strain. Panel B is the predicted hybridisation pattern for the deletion mutant DC43. Panel C is the predicted hybridisation pattern for the restoration mutants. The striped horizontal bars indicate the regions to which the probe Sal53 would hybridise. The size of the *Xho*I and *Hind*III fragments is shown. The figure is not to scale.

The hybridization pattern for the *Hin*dIII digests was used to determine the integrity of the integration event at the 3' end of the *StuA* locus in the deletion mutant (Fig 4.35). In the wild type, a *Hin*dIII site was present in the *StuA* gene. The previous Southern analysis with the StuA595 probe (Fig 4.7) suggested that in the wild type a 5.0 kb *Hin*dIII fragment was downstream of this *Hin*dIII site. The probe Sal53 covered this *Hin*dIII site and would hybridize to the 5.0 kb *Hin*dIII fragment and another fragment (12 kb) upstream of this *Hin*dIII site (Fig 4.7). In the deletion mutant, the *Hin*dIII fragments that would hybridize to the probe were shorter (~11 kb and 4.3 kb), because the region of the *StuA* gene containing the *Hin*dIII site was replaced by a 3.2 kb hygromycin resistance cassette which was cut out by *Hin*dIII and would not itself hybridize to the probe Sal53. When the deletion cassette was replaced by the restoration cassette, the 12 kb fragment was restored and because the 3.2 kb phleomycin resistance cassette did not contain any *Hin*dIII sites, a downstream 8 kb fragment hybridized to the probe (Fig 4.34). These results confirmed that the restoration cassette had integrated correctly at the 3' end of the *StuA* locus.

As shown in Fig 4.34, the two restoration transformants (NR5 and NR13) displayed exactly same hybridization pattern. They were then used in the further characterization of the function of the *StuA* gene.

4.6 Characterisation of the *StuA* mutant phenotype

To examine the function of the *StuA* gene in *G. cingulata*, a systematic study of the phenotype was carried out for the wild type, the deletion mutants DC42 and DC43, the restoration mutants NR5 and NR13.

4.6.1 Mycelium

4.6.1.1 Radial growth rate

Radial growth rates for the wild type, deletion mutants and restoration mutants were measured on PDA plates and on agar medium containing bovine serum albumin (BSA)

as the sole nitrogen source as described in Section 2.11.1. All plates were incubated at 25^0C in the dark.

No sectoring was observed in either the deletion mutants or the restoration mutants, indicating that they were genetically stable. The wild type strain grew at a rate of 0.47 ± 0.09 mm/hr on PDA plates at 25^0C in the dark. Both deletion mutants (DC42: 0.48 ± 0.11 mm/hr; DC43: 0.45 ± 0.12 mm/hr) and the restoration mutants (NR5: 0.59 ± 0.2 mm/hr; NR13: 0.56 ± 0.19 mm/hr) showed no significant difference (Student's t-test, $P > 0.1$, $n = 6$) in growth rate compared to the wild type, indicating that the *StuA* gene was not required for vegetative growth.

The wild type strain grew at a rate of 0.43 ± 0.05 mm/hr on the plates containing BSA as sole nitrogen source at 25^0C in the dark. Neither restoration mutant showed any significant difference (Student's t-test, $P > 0.1$, $n = 6$) in growth rate (NR5: 0.45 ± 0.07 mm/hr; NR13: 0.46 ± 0.08mm/hr) compared to the wild type. But a significantly slower growth rate was observed in the deletion mutants DC42 (0.37 ± 0.02 mm/hr, $P < 0.05$, $n = 6$) and DC43 (0.34 ± 0.01 mm/hr, $P < 0.01$, $n = 6$). This suggested that the *StuA* gene was required for utilization of protein. However, a clear halo in the medium around the edge of the mycelium was observed for the deletion mutants, the wild type strain and the restoration mutants. There were no differences in the relative size of the halo between strains. The halo is formed as the protein in the medium is degraded by secreted peptidases. Observation of the halo suggested that the deletion mutants must still be able to express secreted peptidases. For *G. cingulata*, growth on a medium using protein as the sole nitrogen source may require, besides secreted peptidase, enzymes involved in transport and utilisation of amino acids. It is possible that the *StuA* gene positively regulates the expression of some of them that are responsible for the slow growth on the BSA containing plate.

4.6.1.2 Mycelial morphology

Mycelium of the wild type, the deletion mutants and the restoration mutants was examined both macroscopically and microscopically. After 36-48 hours growth in the

dark on PDA plates inoculated with a single conidium, differences in hyphal branching between the wild type and the deletion mutants were observed (Fig 4.36). Similar results were observed for cultures grown under a light/dark cycle (Fig 4.37). In the deletion mutants, hyphae were less branched and more compact compared with the hyphae of the wild type and the restoration mutants which were branched and formed an open network. After 3-4 days growth on PDA inoculated with a 0.5 cm agar plug and incubated in the dark or under a 12 hr light/dark cycle, differences in mycelium morphology could be observed macroscopically. Mycelium of the wild type and the restoration mutants showed cottony, fluffy aerial hyphae when growing in the dark (Fig 4.38), or shorter aerial hyphae, that was pink in colour, and an orange mass of conidia scattered outwards from the colony centre when growing under a 12 hr light/dark cycle (Fig 4.39). Mycelium of the deletion mutants showed stunted aerial hyphae which had collapsed at the centre of the colony following growth under both conditions. This was obvious in a 7 day old culture. Mycelium of the deletion mutants was sometimes pink in colour when grown under a 12 hr light/dark cycle, but the orange mass of conidia was never observed. When mycelia of the wild type and restoration mutants was grown under a 12 hr light/dark cycle and examined under the microscope (Fig 4.40), numerous conidiophores and conidia were observed. In the deletion mutants, conidiophores were not observed. A single conidium was only occasionally seen at a hyphal tip. This is consistent with the quantification of conidia production reported in the following section. After 7-10 days growth on PDA plates, cultures of the wild type and the restoration mutants started to turn grey to black in colour (Fig 4.41, Fig 4.42). Young perithecia could be seen under a light microscope (Fig 4.43). Following maturation of perithecia, the wild type and the restoration mutant cultures became a dark black colour (Figure 4.44). Mature perithecia could be seen on these plates under microscope, and asci within perithecia could be observed by methylene blue staining of smashed perithecia (Figure 4.44). Colonies of the deletion mutants remained white (growing in dark, Fig 4.41), or pink (growing under a light/dark cycel, Fig 4.42 and Figure 4.44), but never became grey or black. Perithecia were never observed with the deletion mutants (Fig 4.43). These results suggest that in *G. cingulata* the *StuA* gene is required for the formation of perithecia and has an important role in the formation of the conidiophores.

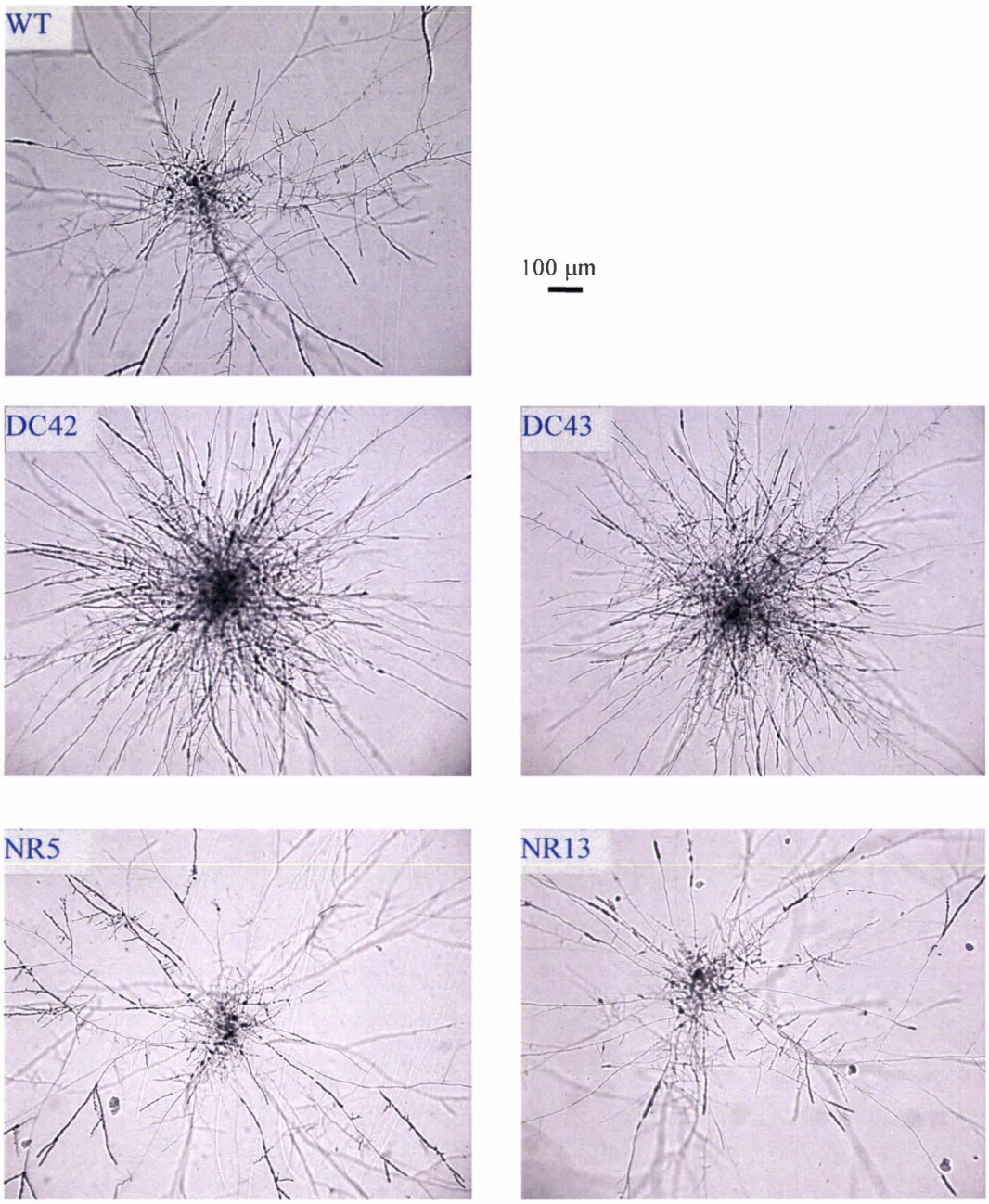


Figure 4.36 Morphology of mycelium grown in the dark for 2 days

About 10 conidia were spread on a PDA plate and incubated at 22^0C in the dark for 48 hours. Colonies were observed with a light microscope. Hyphae of the deletion mutants were less branched, and the colony more compact; than for either the wild type or the restoration mutants.

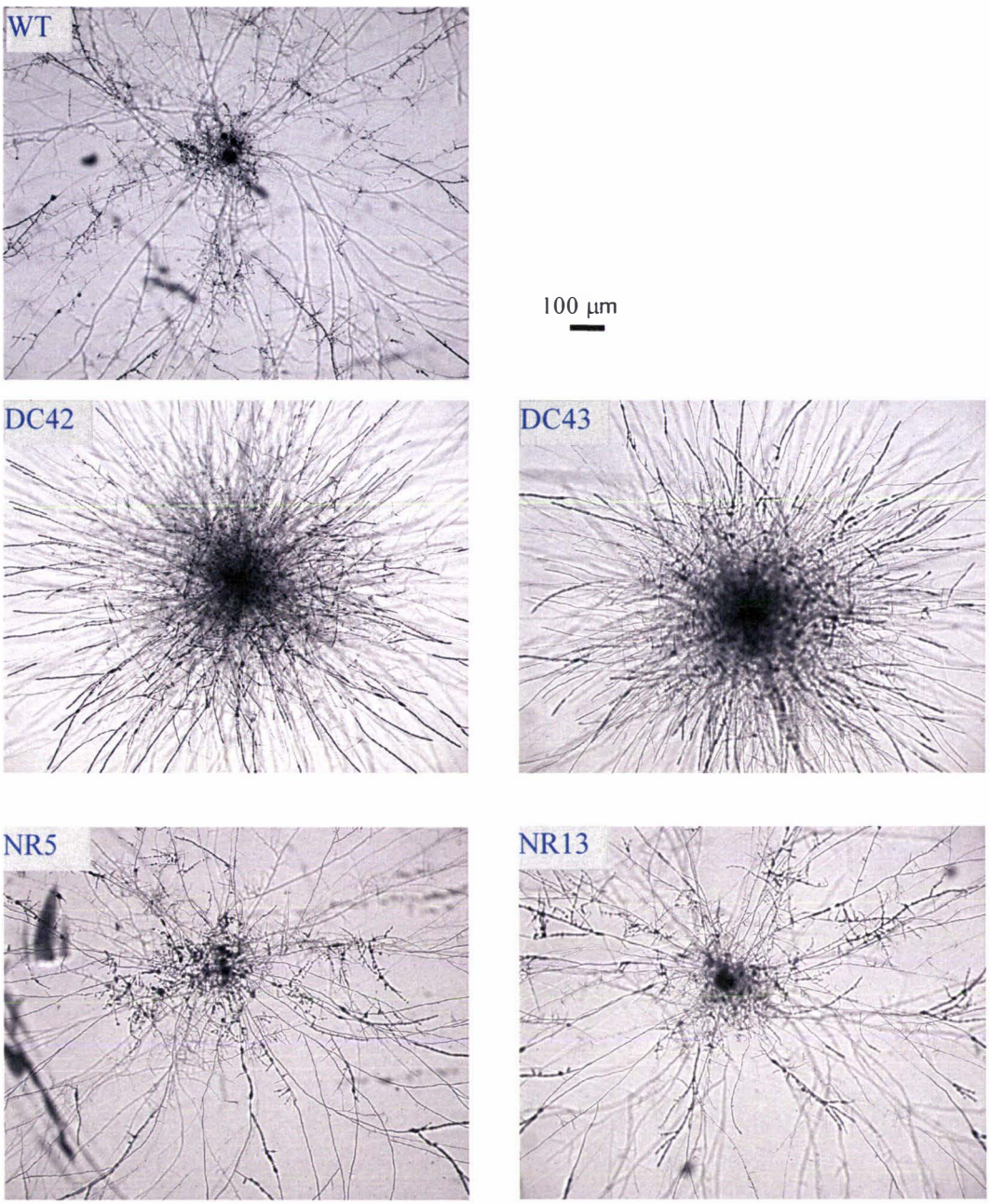


Figure 4.37 Morphology of mycelium grown under a light/dark cycle for 2 days

About 10 conidia were spread on a PDA plate and incubated at 22^0C under a light/ dark cycle for 48 hours. Colonies were observed with a light microscope. Hyphae of the deletion mutants were less branched, and the colony more compact; than for either the wild type or the restoration mutants.

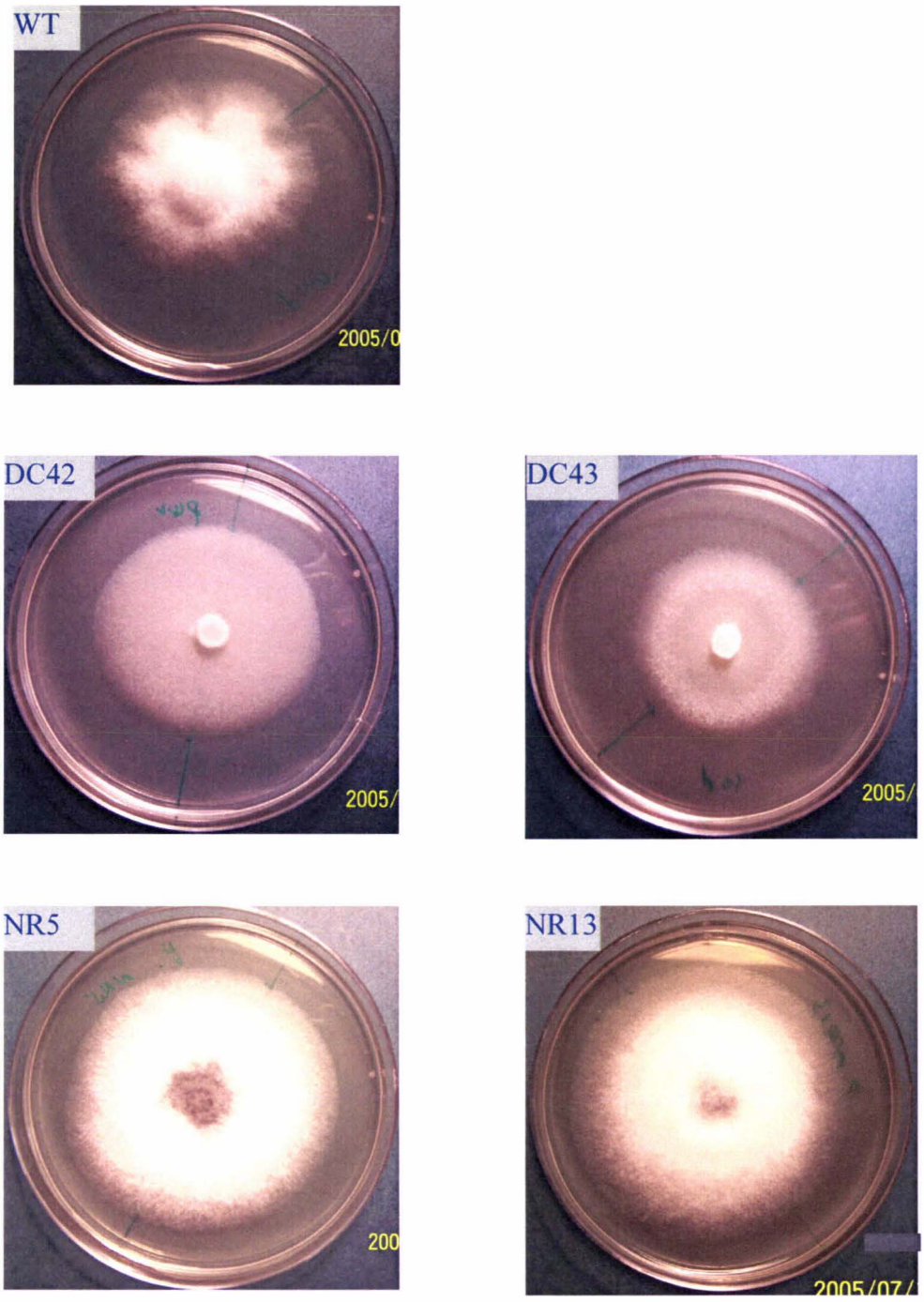


Figure 4.38 Morphology of mycelium grown in the dark for 4 days

PDA plates were inoculated with a 5 mm agar plug from the growing edge of a colony and incubated at 25^0C in the dark. The wild type and the restoration mutants (NR5 and NR13) displayed cottony, fluffy aerial hyphae. The deletion mutants (DC42 and DC43) displayed stunted aerial hyphae.

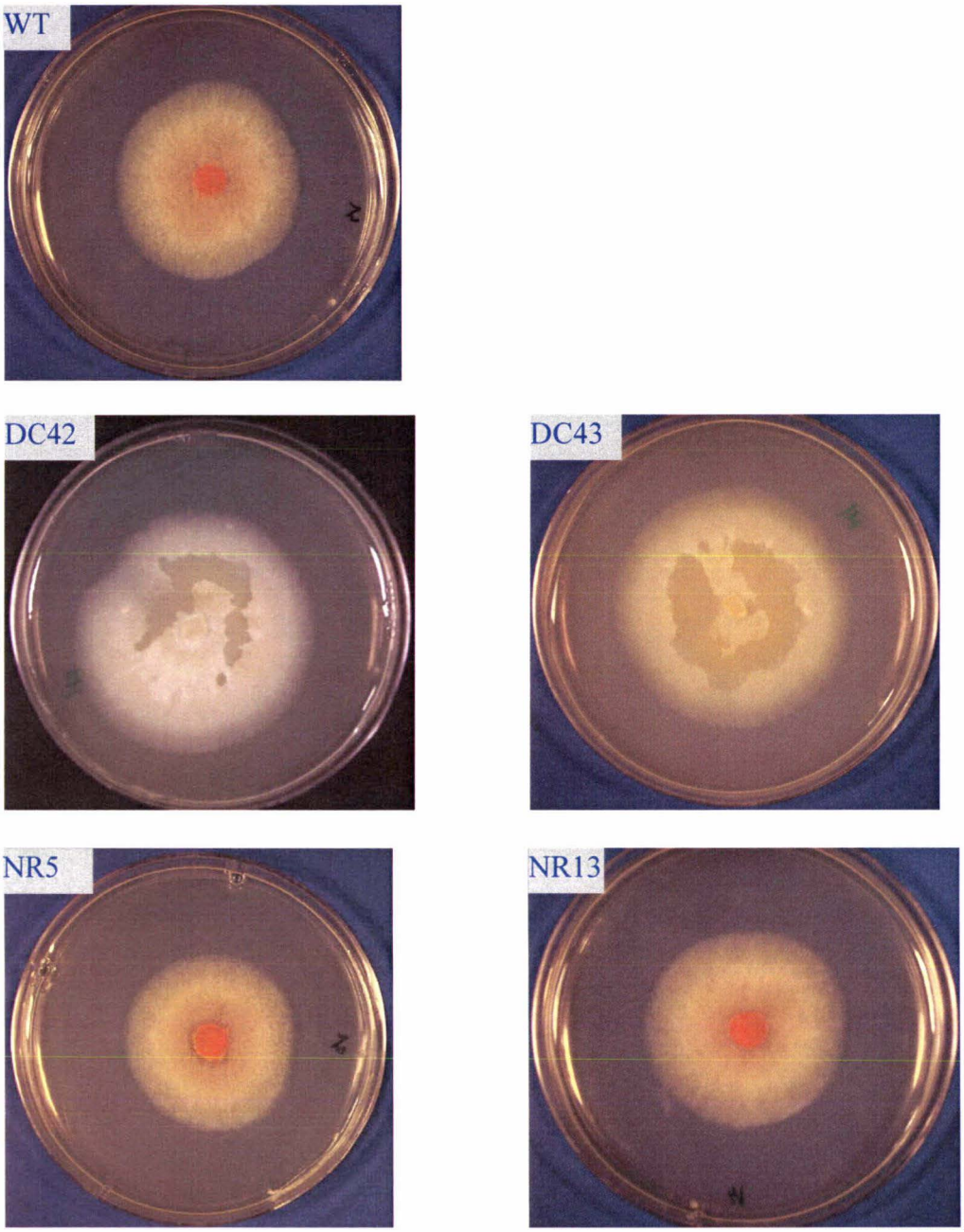


Figure 4.39 Morphology of mycelium grown under a light/dark cycle for 4 days

PDA plates were inoculated with a 5 mm agar plug from the growing edge of a colony and incubated at 22^0C under a light/dark cycle. The wild type and the restoration mutants (NR5 and NR13) displayed cottony, fluffy aerial hyphae. Conidiation had occurred and the colony was a characteristic pink colour. The deletion mutants DC42 and DC43 displayed stunted aerial hyphae which had collapsed in the centre of the colony.

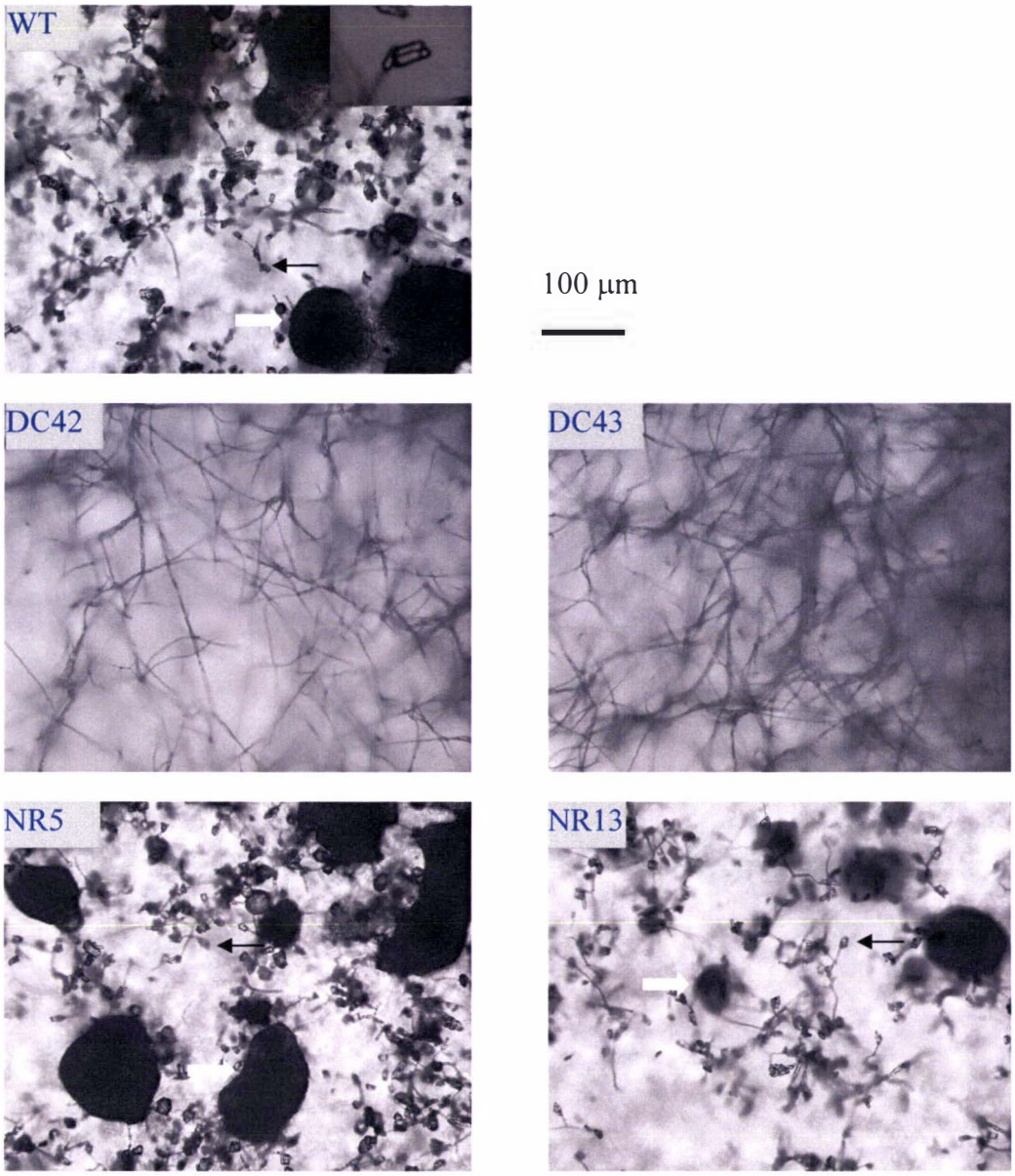


Figure 4.40 Microscopic observation of mycelium grown under a light/dark cycle for 4 days

The plates shown in Figure 4.39 were examined with a light microscope. Numerous conidiophores and their associated conidia were observed in the wild type and the restoration mutants. In the deletion mutants, conidia/conidiophores were rarely observed. Small black arrows indicate a conidiophore and large white arrows indicate a mass of conidia.

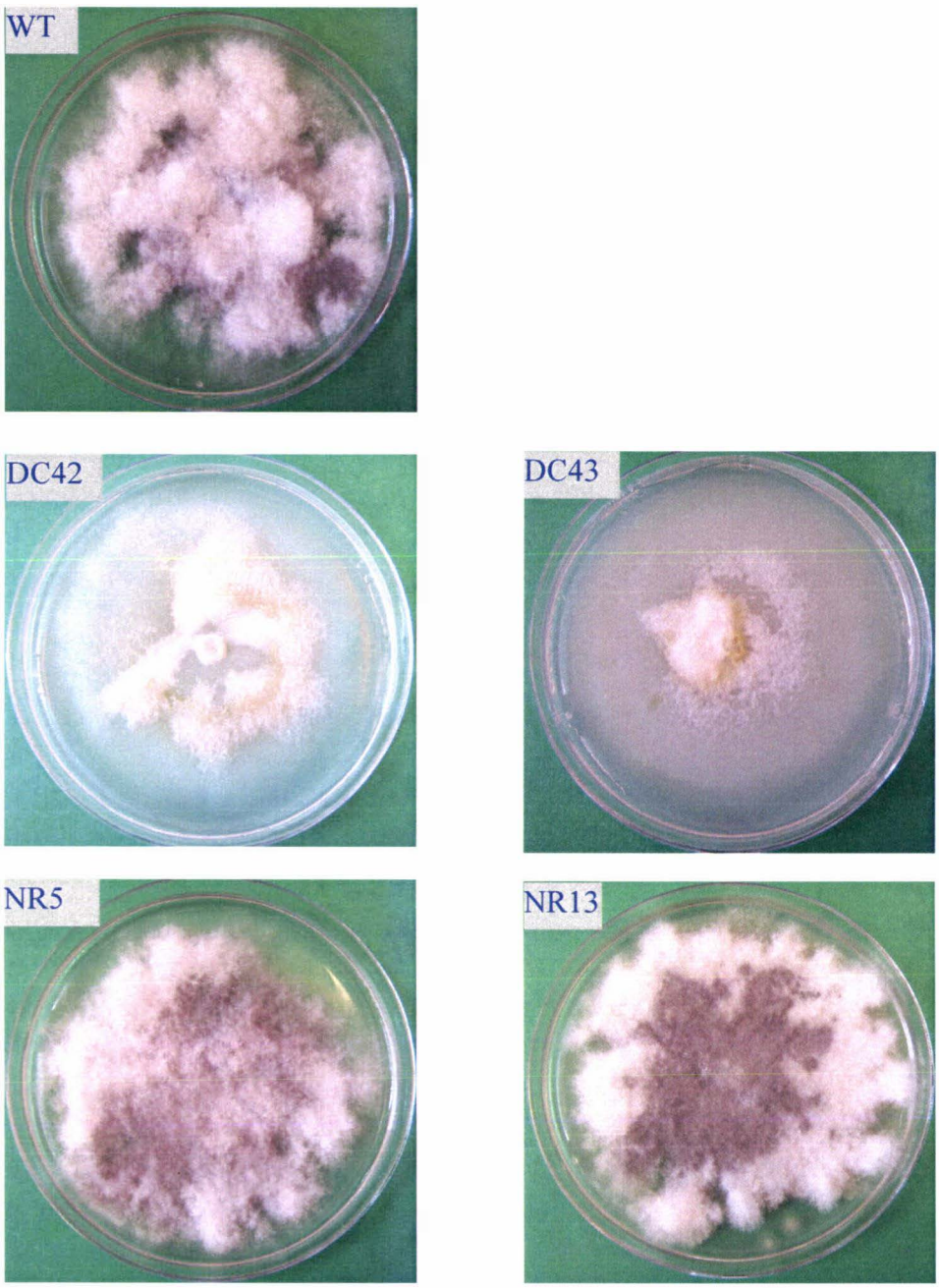


Figure 4.41 Morphology of mycelium grown in the dark for 7 days

PDA plates were inoculated with a 5 mm agar plug from the growing edge of a colony and incubated at 25^0C in the dark. The wild type and the restoration mutants (NR5 and NR13) displayed cottony, fluffy aerial hyphae and the colony started to appear melanized. The deletion mutants (DC42 and DC43) displayed stunted aerial hyphae and the colony was not melanized.

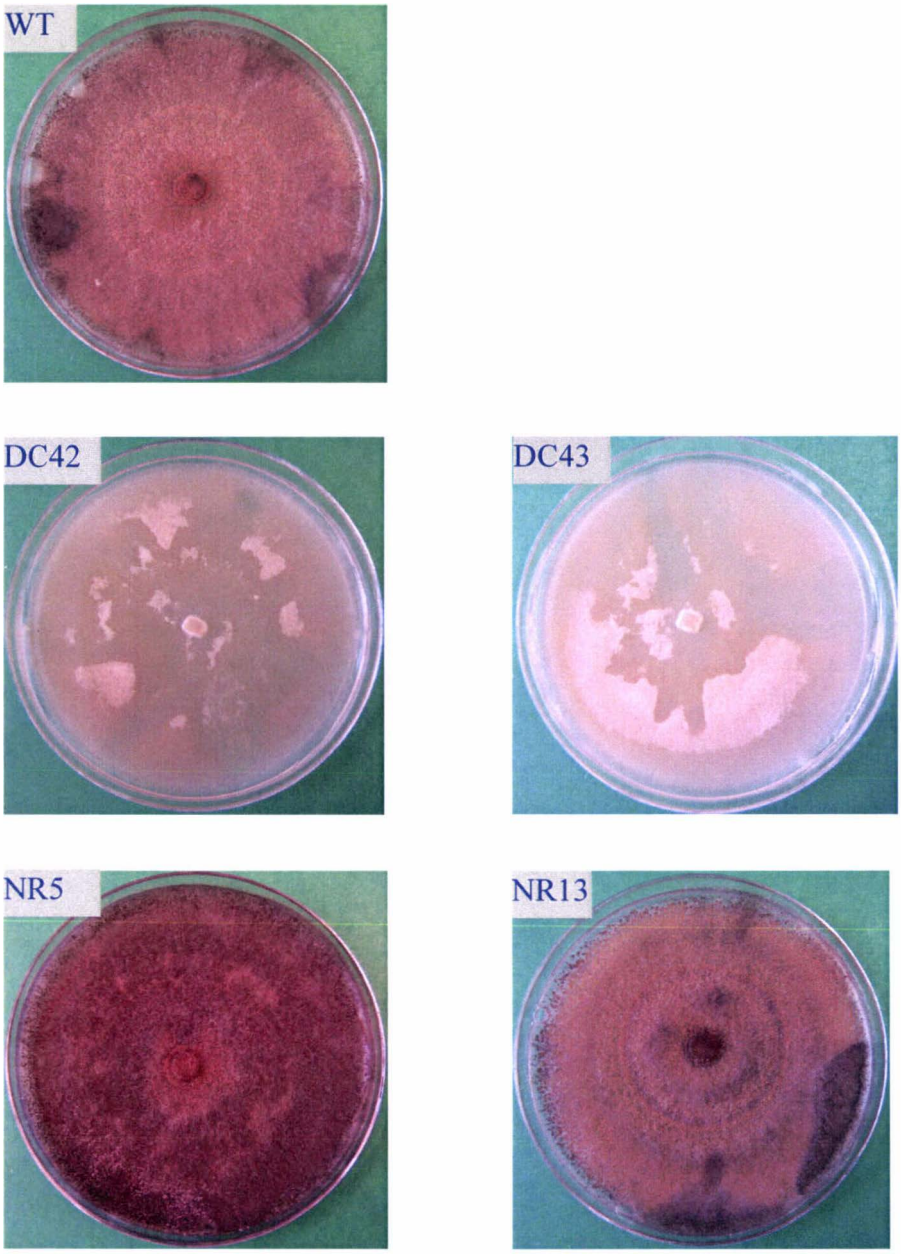


Figure 4.42 Morphology of mycelium grown under a light/dark cycle for 7 days

PDA plates were inoculated with a 5 mm agar plug from the growing edge of a colony and incubated at 22^0C under a light/dark cycle. Conidiation was induced in the wild type and the restoration mutants (NR5 and NR13). Conidia covered the colony which had a pink appearance. The deletion mutants DC42 and DC43 display stunted aerial hyphae which had collapsed in the centre of colony. Although the colony had a pink appearance, no conidiation was observed.

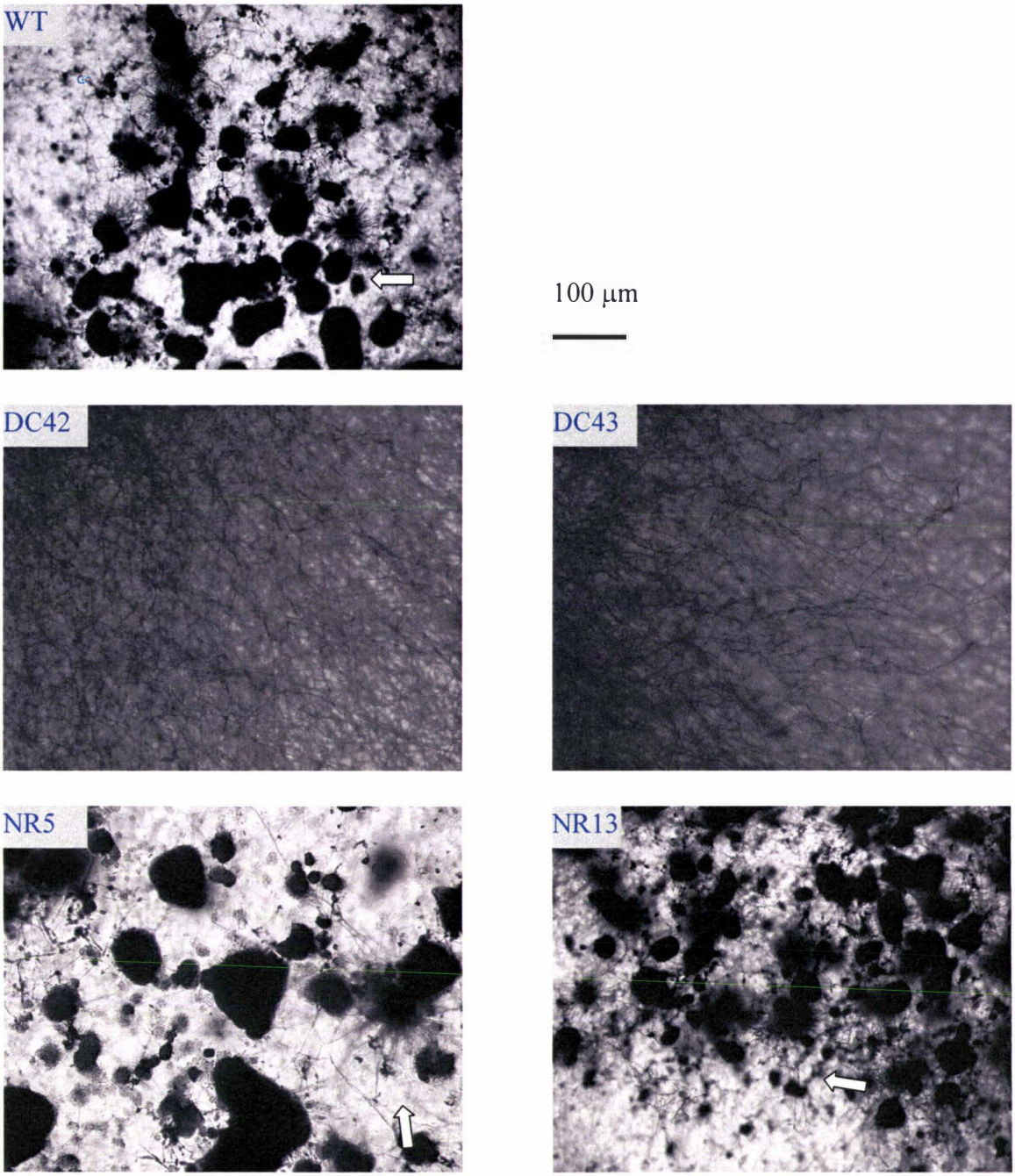


Figure 4.43 Microscopic observation of mycelium grown under a light/dark cycle for 7 days

The plates shown in Figure 4.42 were examined with a light microscope. Conidiophores and associated conidia as well as young perithecia (arrows) were visible on the wild type and the restoration mutant plates. In the deletion mutants, neither conidiophores nor perithecia were observed.

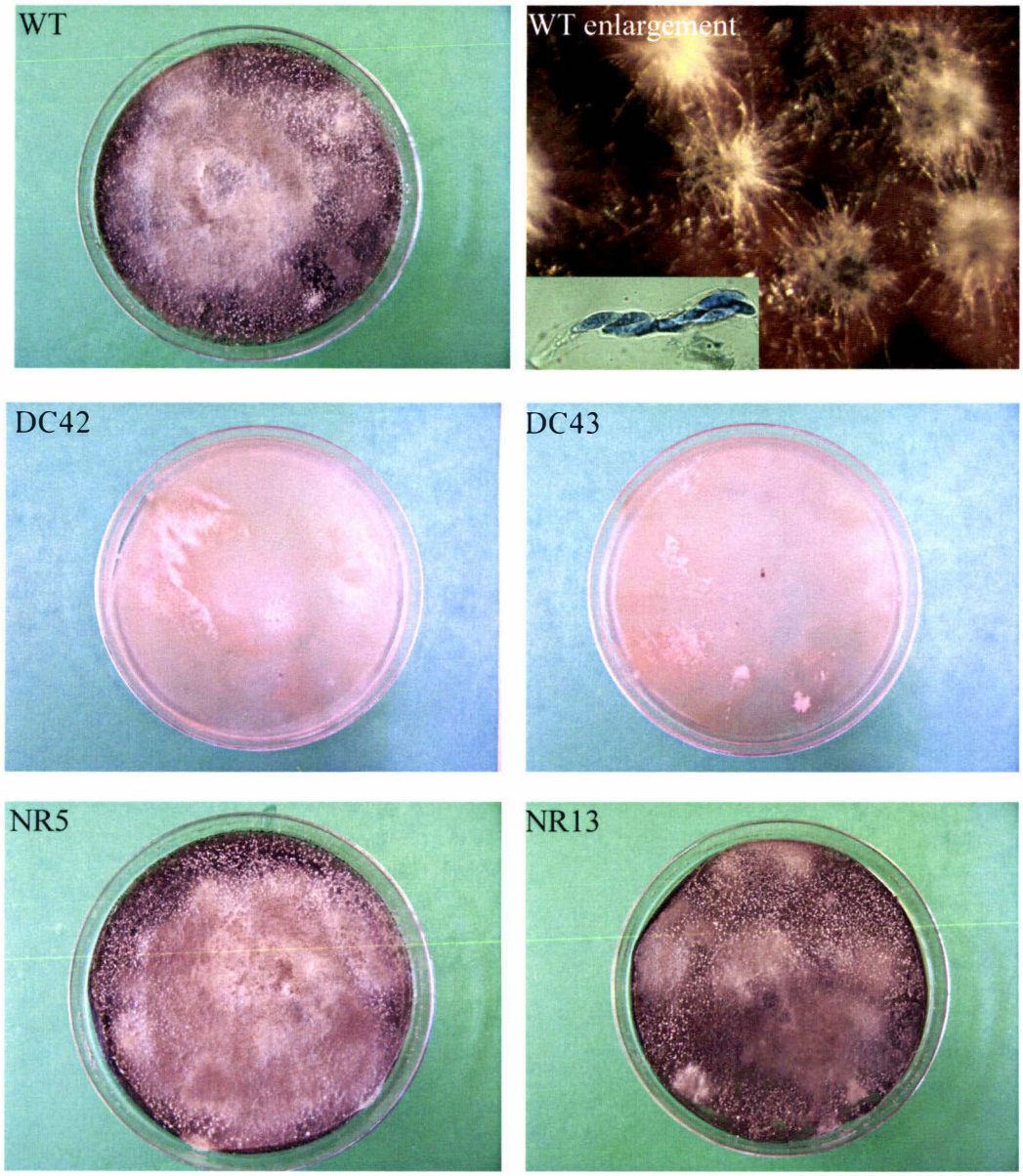


Figure 4.44 Morphology of mycelium grown under a light/dark cycle for 14 days

PDA plates were inoculated with a 5 mm agar plug from the growing edge of a colony and incubated at 22^0C under a 12 hour light/dark cycle. After 14 days incubation, black perithecia were observed both macroscopically and microscopically on the wild type and the restoration mutant plates, but not on the deletion mutant plates. The panel labelled WT enlargement shows the perithecia observed under a light microscope. These were also observed on the restoration mutant plates (data not shown). Eight-spore asci were observed by methylene blue staining of smashed perithecia from the wild type (insert) and the restoration mutant plates (data not shown).

4.6.1.3 Wettability of mycelium

Aerial mycelium is usually not "wettable", because hyphae are coated with a layer of the protein called hydrophobin, which makes the surface hydrophobic. During the growth of mycelium on PDA plates, small beads of water were observed on the aerial hyphae of both wild type *G. cingulata* and the restoration mutants, but they were not seen on the deletion mutant. The "wettable" phenotype is characteristic of fungi carrying a mutation in the hydrophobin genes. Interestingly, stunted aerial hyphae were also associated with mutations in the hydrophobin gene *MPG1* in *M. grisea* (Talbot *et al.*, 1996). When a drop of water was applied to the mycelium of a wild type *G. cingulata* colony or the restoration mutant colony, the water drop stood still on the mycelium surface (Figure 4.45) and remained that way for hours or longer. In contrast, when a drop of water was applied to the mycelium of the *G. cingulata StuA* deletion mutants, the water dispersed immediately. The deletion mutant mycelium is therefore described as "wettable". This observation suggests that the *StuA* gene may control expression of the hydrophobin genes in *G. cingulata.*

4.6.2 Conidia and appressoria

4.6.2.1 Conidium shape

Conidium shape and size are important features used in the classification of fungi. Conidia from 7 day old PDA cultures of the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13) were examined under a microscope and classified into three categories on the basis of their shape. The number of conidia in each category was counted (Table 4.4). One hundred conidia for each strain were counted and their size was measured. Cylindrical conidia were most commonly seen for all strains. Oval and cylindro-ovoid shapes were also observed (Fig 4.46). In the deletion mutants, cylindrical conidia were also predominantly seen, but 3% (DC42) and 4% (DC43) abnormal conidia with a large size and irregular shapes were observed (Fig 4.46).

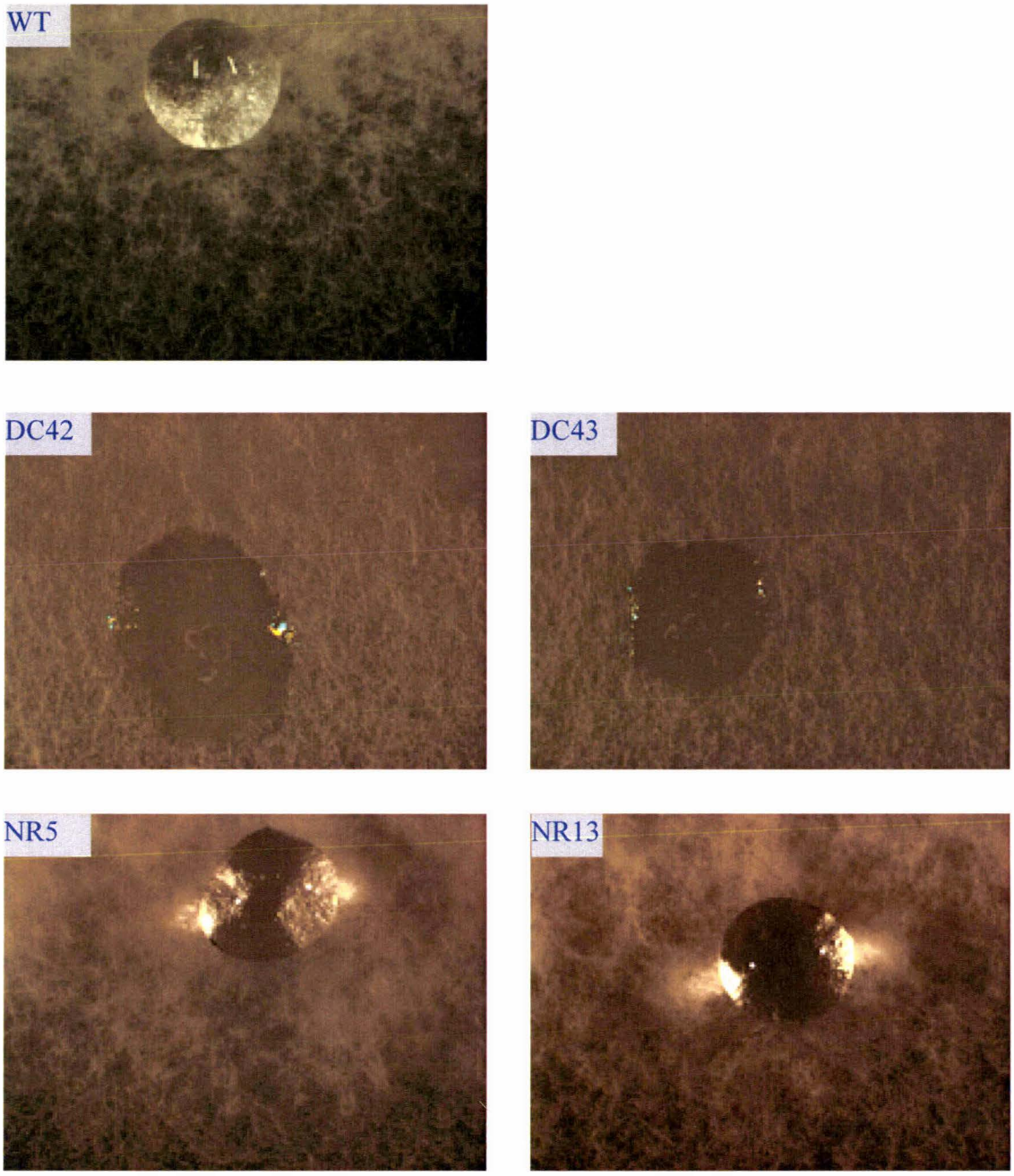
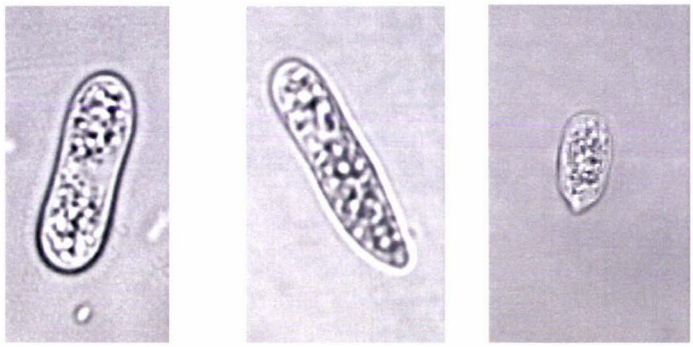


Figure 4.45 Wettability of the mycelium

Wettability of mycelium was tested by applying a drop of water onto the surface of 4 day old mycelium growing on a PDA plate. The water drop stood still on the surface of the mycelium for the wild type and the restoration mutants, but dispersed immediately in the case of the deletion mutants.

Normal conidial shapes



Abnormal conidial shapes observed in the deletion mutants

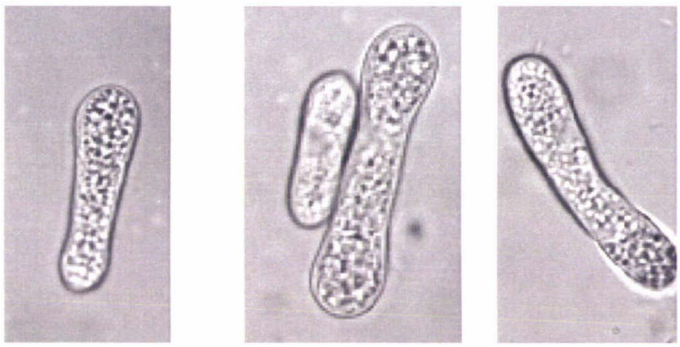


Figure 4.46 Conidial shape

Conidia were harvested from 7 day old PDA cultures of the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13). Conidial shapes were classified into the categories illustrated.

Table 4.4 Shape and size of conidia

Strains	Mean size of conidia (μm)		Shape of conidia			
	Length	**Width**	**Cylindrical**	**Cylindro -ovoid**	**Oval**	**Abnormal**
Wild type	17.62 ± 4.23*	4.33 ± 0.86	91%	2%	7%	0%
DC42	16.33 ± 6.22	4.02 ± 1.23	79%	3%	13%	3%
DC43	15.66 ± 5.68	3.93 ± 1.58	78%	3%	15%	4%
NR5	16.87 ± 4.55	4.23 ± 1.07	87%	1%	12%	0%
NR13	17.34 ± 3.89	4.54 ± 0.89	82%	2%	16%	0%

Conidia were harvested from 7 day old PDA cultures of the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13). The number of conidia in each shape category was counted under a light microscope. One hundred conidia for each strain were counted and their size was measured.

* Values are the mean ± the standard deviation.

4.6.2.2 Production of Conidia

Significantly lower production of conidia by the deletion mutant was observed on PDA medium both macroscopically and microscopically during routine manipulations. A quantitative study was therefore conducted on both solid medium and liquid medium. Conidia were collected from 7 day old cultures grown on PDA plates under a 12 hr light/dark cycle and counted. As shown on Table 4.5, the deletion mutants produced about 10^3 fold fewer conidia than the wild type or the restoration mutant (Student's t-test, $P < 0.01$, $n = 10$).

Conidia production was also examined in liquid PDB culture. Conidia freshly harvested from a PDA plate were inoculated into 50 ml of liquid PDB medium at a final concentration at 10^5/ml and incubated at 25°C with shaking at 150 rpm for 7 days. Mycelia were removed by filtering the culture through 8 layers of nappy liner and conidia were collected by centrifugation. Conidia production in the deletion mutants DC42 and DC43 was lower than in the wild type or the restoration mutants. The difference was smaller (~30 fold) but still significant (Student's t-test, $P < 0.01$, $n = 5$).

In order to obtain enough conidia from the deletion mutant for transformation experiments, an induction medium was developed that would stimulate conidia production. High glucose concentration was shown to stimulate conidiation in *Penicillium* and other fungi (Roncal and Ugalde, 2003). Therefore, conidia production in this induction medium was quantified. As shown in Table 4.5, conidia production by the deletion mutants was induced (~25 fold, Student's t-test, $P < 0.01$, $n = 5$) in comparison with non-induction medium (growth in PDB liquid medium), however, it still did not reach the level of the wild type. In the induction medium, conidia production by the wild type was still much higher than the deletion mutants (~70 fold, Student's t-test, $P < 0.01$, $n = 5$). This suggested that the induction medium was not able to fully complement the endogenous deficiency caused by deletion of the *StuA* gene. Conidia production by the wild type and the restoration mutants also increased in the induction medium but was to a lesser extent. These results suggest that the *StuA* gene is required for efficient conidiation.

Table 4.5 Conidia production on PDA plates

Strains	Conidium production on PDA plate* (10^{-5} x number of conidia/plate)		Conidium production on PDB liquid medium** (10^{-5} x number of conidia/ml)	Conidium production on liquid induction medium*** (10^{-5} x number of conidia/ml)
	Experiment 1	Experiment 2		
WT	2300 ± 1600 (10)	2100 ± 1300 (10)	97 ± 20 (5)	558 ± 137 (5)
DC42	1.4 ± 0.97 (10)	1.4 ± 0.86 (10)	3.7 ± 0.68 (5)	86.6 ± 17.3 (5)
DC43	1.0 ± 0.63 (10)	1.1 ± 0.76 (10)	3.0 ± 0.71 (5)	74.6 ± 13.8 (5)
NR5	1900 ± 1600 (10)	2700 ± 2100 (10)	109 ± 21 (5)	570 ± 153 (5)
NR13	1900 ± 1100 (10)	2000 ± 1500 (10)	98 ± 20 (5)	604 ± 164 (5)

* PDA plates were incubated at 22^0C under a 12 hr light/dark cycle for 7 days. Values shown are the mean ± the standard deviation for 10 plates.

** Conidia were inoculated into 50 ml of liquid PDB medium at 10^5/ml and incubated at 25°C with shaking at 150 rpm for 7 days. Mycelium was removed by filtration through 8 layers of nappy liner and conidia were collected by centrifugation. Values shown are the mean ± the standard deviation for 5 different cultures.

*** Cultures were incubated with shaking at 22^0C in the dark. Growth media contained 12.5% glucose to induce conidia production as described in Section 2.10.1.

4.6.2.3 Adhesion of conidia to a hydrophobic surface

Adhesion of conidia to the plant surface is the first step in the infection process. Hydrophobicity of the plant surface favours this process. Hydrophobic polystyrene surfaces have been used to mimic the plant surface in conidia adhesion experiments for many fungi including *Colletotrichum* species (Sela-Buurlage *et al.*, 1991). Adhesion of conidia from the wild type, the deletion mutants and the restoration mutants was compared on polystyrene Petri dishes as described in Section 2.11.5. Three independent experiments were performed (Table 4.6). In each case, the ability to adhere to the polystyrene Petri surface was the same for the wild type strain, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13). This suggests that deletion of the *StuA* gene did not affect adhesion of conidia to hydrophobic surfaces.

4.6.2.4 Germination and appressorium formation

Germination and appressorium formation for all strains were examined on glass slides coated with apple wax, polystyrene Petri dishes and onion epidermal peels as described in Section 2.11.4. Washed conidia from 7 day old cultures growing on PDA plates under a 12 hr light/dark cycle were used in these experiments. The percentage of conidia that germinated and formed appressorium was determined after 16 hours incubation at 22^0C on glass slides and polystyrene Petri dishes, and after 24 hours incubation on onion epidermal peels. As shown in Table 4.7, Table 4.8 and Table 4.9, most of the conidia (>80%) were able to germinate and form appressorium under these conditions. Both the percentage of conidia that germinated and the percentage of conidia that formed appressorium were similar for the wild type, the deletion mutants and the restoration mutants (Student's t-test, $P > 0.1$ for all groups). The size, shape and level of melanization of the appressoria for all strains were indistinguishable (Fig 4.47). These results suggested that the *StuA* gene is not essential for either conidium germination or appressorium formation.

Table 4.6 Conidium adhesion assay

Inoculum	Adhered conidia (%)	Total conidia counted
Wild type	98.03% ± 1.1* (3)	1175
DC42	97.73% ± 1.0 (3)	1206
DC43	97.13% ± 1.7 (3)	1211
NR5	96.97% ± 0.6 (3)	1245
NR13	98.23% ± 1.0 (3)	1215

Conidia were harvested from the 7 day old PDA plates and suspended in water. The suspension (50μl of 10^5 conidia/ml) was incubated on polystyrene Petri dishes at 22^0C for 2 hours. Conidia in a marked area were counted before and after washing the plate with water on a reciprocal shaker at 50 rpm for 5 min. Three areas containing at least 100 conidia each were counted. The percentage of adhered conidia was calculated from the number of attached conidia after the wash and the total number of conidia before wash. Data was collected for three independent experiments, each performed as described above. No statistically significant differences were observed between the wild type and the deletion mutants (Student's t-test, $P > 0.1$, $n = 3$), or the wild type and the restoration mutants (Student's t-test, $P > 0.1$, $n = 3$).

* Each value represents the mean and standard deviation of the percentage of conidia that adhered to the surface of polystyrene Petri dishes from three independent experiments.

Table 4.7 Appressorium formation on wax coated glass slides

Inoculum	**Appressorium/ total conidia (%)**	**Appressorium/ germinated conidia (%)**	**Germinated conidia/ total conidia (%)**	**Total number of conidia**
Wild type	93.6 ± 0.8*(3)	97.4 ± 0.9 (3)	96.1 ±1.6 (3)	1075
DC42	87.4 ± 4.5 (3)	91.0 ± 5.8 (3)	96.1 ± 2.1 (3)	1217
DC43	89.3 ± 4.9 (3)	93.4 ± 7.6 (3)	95.7 ± 3.6 (3)	1231
NR5	96.5 ± 0.4 (3)	99.6 ± 0.7 (3)	96.9 ± 1.1 (3)	879
NR13	96.2 ±1.0 (3)	99.9 ± 0.2 (3)	96.3 ± 0.9 (3)	890

Conidia were harvested from 7 day old PDA plates and suspended in water. The suspensions (50 μl of 10^5 conidia/ml) were inoculated on to a glass slide coated with apple wax and incubated at 22^0C for 16 hours. Appressoria and germinated conidia were counted under a light microscope. Three areas containing about 100 conidia each were counted. Data from three independent experiments was analysed by Student's t-test. The percentage of germination and appressorium formation is not significantly different between the wild type and the deletion mutants ($P > 0.1$), the wild type and the restoration mutants ($P > 0.1$), or the deletion mutants and the restoration mutants ($P > 0.1$).

* Each value represents the mean and standard deviation of three independent experiments.

Table 4.8 Appressorium formation on polystyrene Petri dishes

Inoculum	Appressorium/ total conidia (%)	Appressorium/ germinated conidia (%)	Germinated conidia/ total conidia (%)	Total number of conidia
Wild type	91.3 ± 1.4*(3)	95.6 ± 2.7 (3)	95.6 ± 4.1 (3)	322
DC42	90.6 ± 5.1 (3)	97.1 ± 2.5 (3)	93.3 ± 5.5 (3)	336
DC43	89.5 ± 2.5 (3)	95.4 ± 4.6 (3)	93.9 ± 4.0 (3)	315
NR5	90.7 ± 5.1 (3)	95.8 ± 2.4 (3)	94.1 ± 3.8 (3)	320
NR13	92.9 ± 3.8 (3)	97.0 ± 2.0 (3)	95.8 ± 2.4 (3)	309

Conidia were harvested from 7 day old PDA plates and suspended in water. The suspensions (50 µl of 10^5 conidia /ml) were inoculated on to a polystyrene Petri dish and incubated at 22^0C for 16 hours. Appressoria and germinated conidia were counted under a light microscope. Three areas containing at least 100 conidia each were counted. The percentage of germination and appressorium formation is not significantly different between the wild type and the deletion mutants (Student's t-test, $P > 0.1$), the wild type and the restoration mutants (Student's t-test, $P > 0.1$), or the deletion mutants and the restoration mutants (Student's t-test, $P > 0.1$).

* Each value represents the mean and standard deviation of three randomly selected regions of the plate.

Table 4.9 Appressorium formation and penetration on onion epidermal cells

Inoculum	Appressorium/ total conidia (%)	Appressorium/ germinated conidia (%)	Germinated conidia/ total conidia (%)	Penetrated appressoria (%)	Total number of conidia
Wild type	92.5 ± 1.8* (3)	93.8 ± 1.5 (3)	98.6 ± 1.3 (3)	7.33 ± 0.58 (3)	345
DC42	90.7 ± 1.8 (3)	95.4 ± 2.1 (3)	95.1 ± 2.3 (3)	0 ± 0 (3)	366
DC43	89.6 ± 3.8 (3)	94.3 ± 0.6 (3)	94.9 ± 3.8 (3)	0 ± 0 (3)	353
NR5	92.3 ± 2.4 (3)	94.8 ± 1.4 (3)	98.9 ± 1.3 (3)	7.67± 0.58 (3)	336
NR13	93.1 ± 2.3 (3)	94.2 ± 2.3 (3)	98.9 ± 1.3 (3)	6.67 ± 1.5 (3)	344

Appressorium formation and penetration was assessed on onion epidermal peels. Conidia were harvested from 7 day old PDA plates and suspended in water. Conidia suspensions (50 μl of 10^5/ml) were inoculated on onion epidermal peels and incubated at 22°C. Appressoria and germinated conidia were counted under a light microscope after 24 hours incubation. Appressorium penetration was examined under a light microscope after 48 hours incubation. The percentage penetration is an underestimate because only when a hypha underneath the epidermal cells could be tracked back to an appressorium were they counted. Three areas and at least 100 conidia for each area were examined. Percentage of germination and appressorium formation was not significantly different between the wild type and the deletion mutants (Student's t-test, $P > 0.1$), the wild type and the restoration mutants (Student's t-test, $P > 0.1$). Penetration was not significantly different between the wild type and the restoration mutants (Student's t-test, $P > 0.1$), but was significantly different between the wild type and the deletion mutants (Student's t-test, $P < 0.01$), or between the restoration mutants and the deletion mutants (Student's t-test, $P < 0.01$).

* Each value represents the mean and standard deviation for three randomly selected regions of the onion epidermal peel.

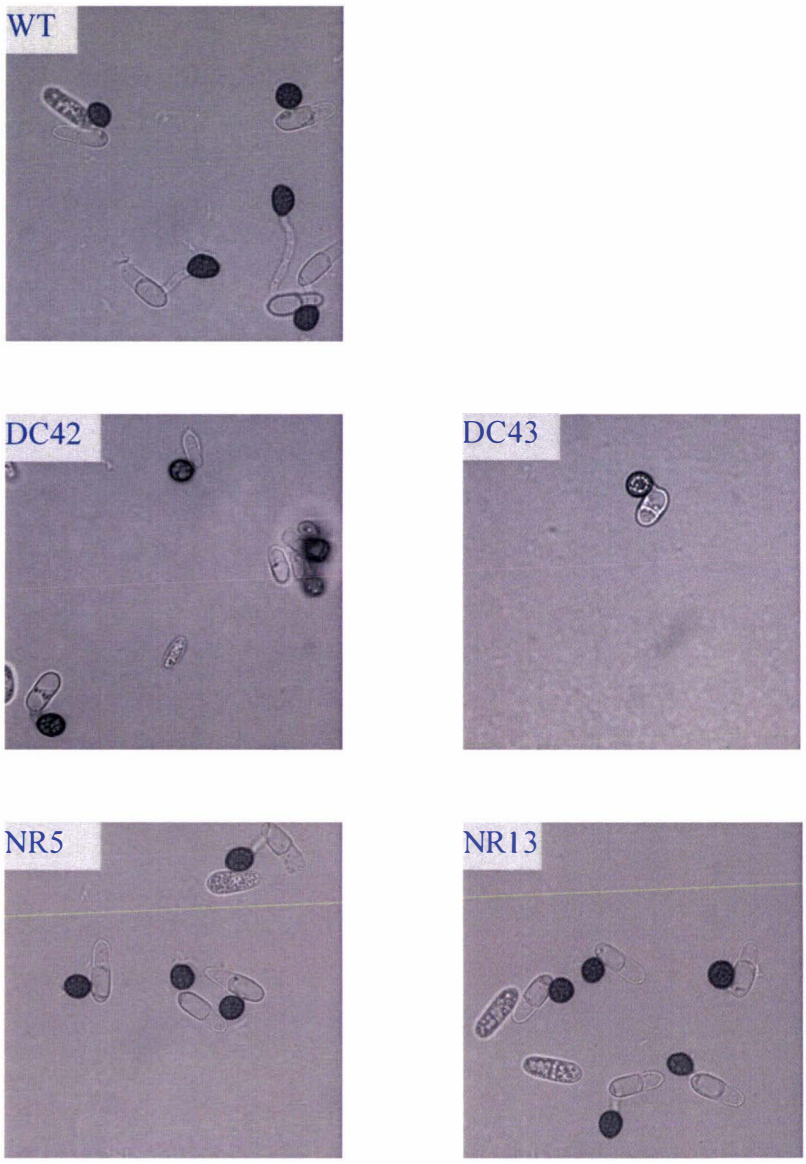


Figure 4.47 Appressorium morphology

Appressoria induced on glass slides coated with apple wax were examined under the light microscope. Appresssorial shape, size, and melanization were indistinguishable between the wild type, the deletion mutants and restoration mutants.

4.6.2.5 Mobilisation of glycogen and lipid

The synthesis of glycerol in eukaryotic cells can occur via several routes. Glycogen and triacylglycerol were shown to be the major reserves in conidia and are mobilised to the appessorium as the substrate for glycerol production in *M. grisea* (Thines *et al*., 2000). Therefore, the mobilisation of glycogen and lipid was examined during appressorium differentiation for the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13). Conidia suspended in water were inoculated on to polystyrene Petri dishes and incubated at 22^0C. Glycogen staining was performed at 0, 6, 12 and 24 hours after inoculation and the yellowish-brown glycogen deposits were observed with bright-field microscopy. As shown in Fig 4.48, glycogen was present in abundance in ungerminated conidia for all strains. After 6 hours incubation, appressoria had developed from the most of the conidia. Glycogen deposits could be seen within both conidia and appressoria, indicating that mobilisation of glycogen deposits from conidia to appressoria had began. Following appressorium maturation (12 hour and 24 hour time points), glycogen deposits were greatly decreased in one cell followed by the other cell in the conidia. However, glycogen deposits were not visible in appressorium at these time points, possibly because glycogen deposits were used up during appressorium maturation, or the melanized appressorial wall prevented the visualisation of glycogen deposits. The distribution of glycogen deposits displayed the same pattern in all five strains.

Nile red staining for lipid was performed at the same time points (Fig 4.49). Lipid droplets, visible as intense red fluorescence, were present in ungerminated conidia for all strains. After 6 hours incubation, the intensity of fluorescence decreased in conidia. Fluorescence was detected in young appressoria with poorly melanised appressorial walls. Fluorescence was not detected in those appressoria with highly melanised walls at 12 hour (Fig 4.49) and at 24 hour (data not shown). It is not clear whether lipid droplets were used up during appressorium maturation, or the melanized appressorial wall prevented the visualisation of lipid droplets. The distribution of lipid droplets displayed the same pattern in all five strains. These results suggested that the *StuA* gene was not required for the mobilisation of glycogen or lipid during appressorium differentiation.

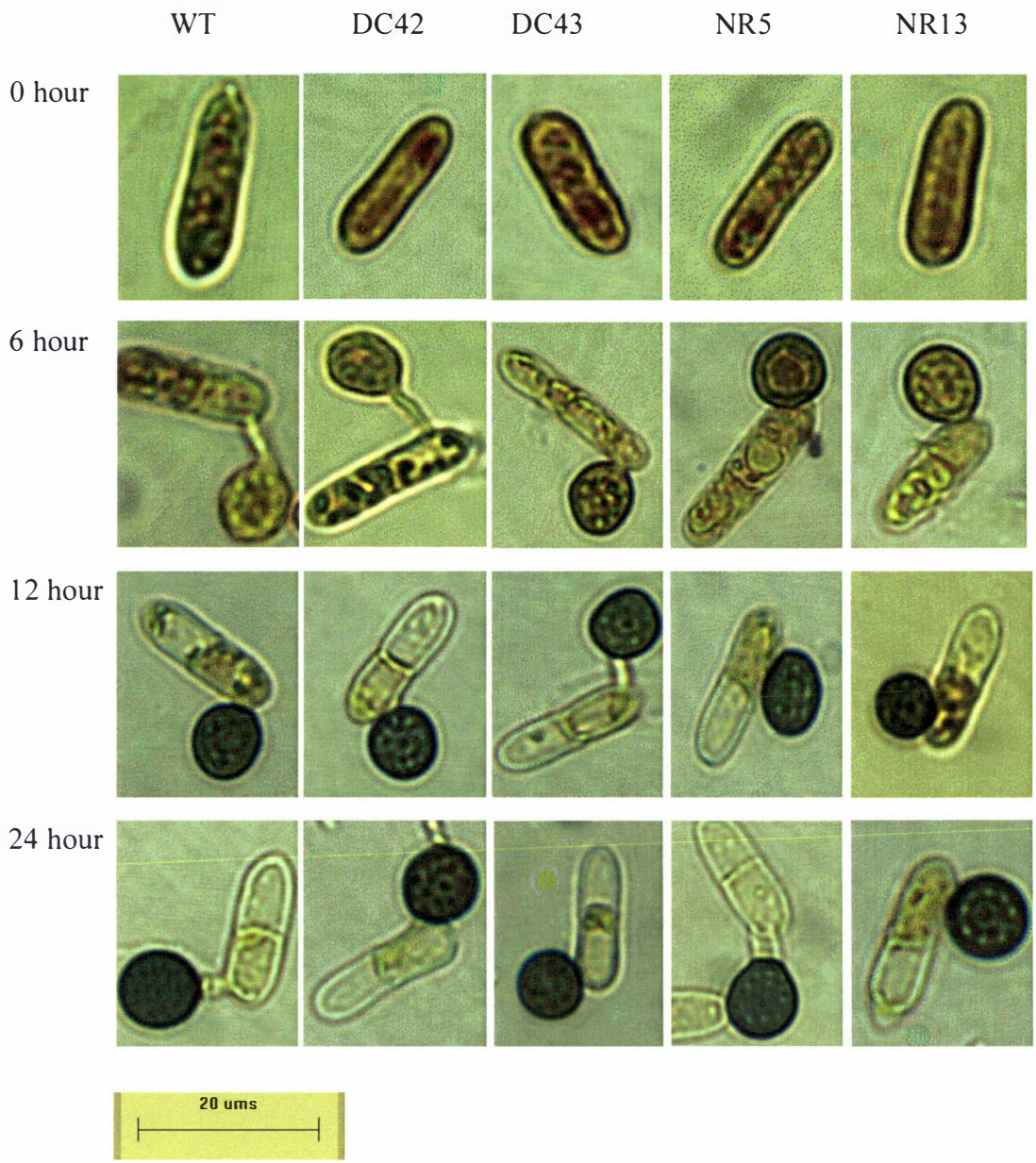


Figure 4.48 Mobilisation of glycogen during appressorium development

Conidia of the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13) were inoculated on to polystyrene Petri dishes and incubated at 22^0C. At 0, 6, 12, and 24 hours, glycogen deposits were stained yellowish-brown with potassium iodide.

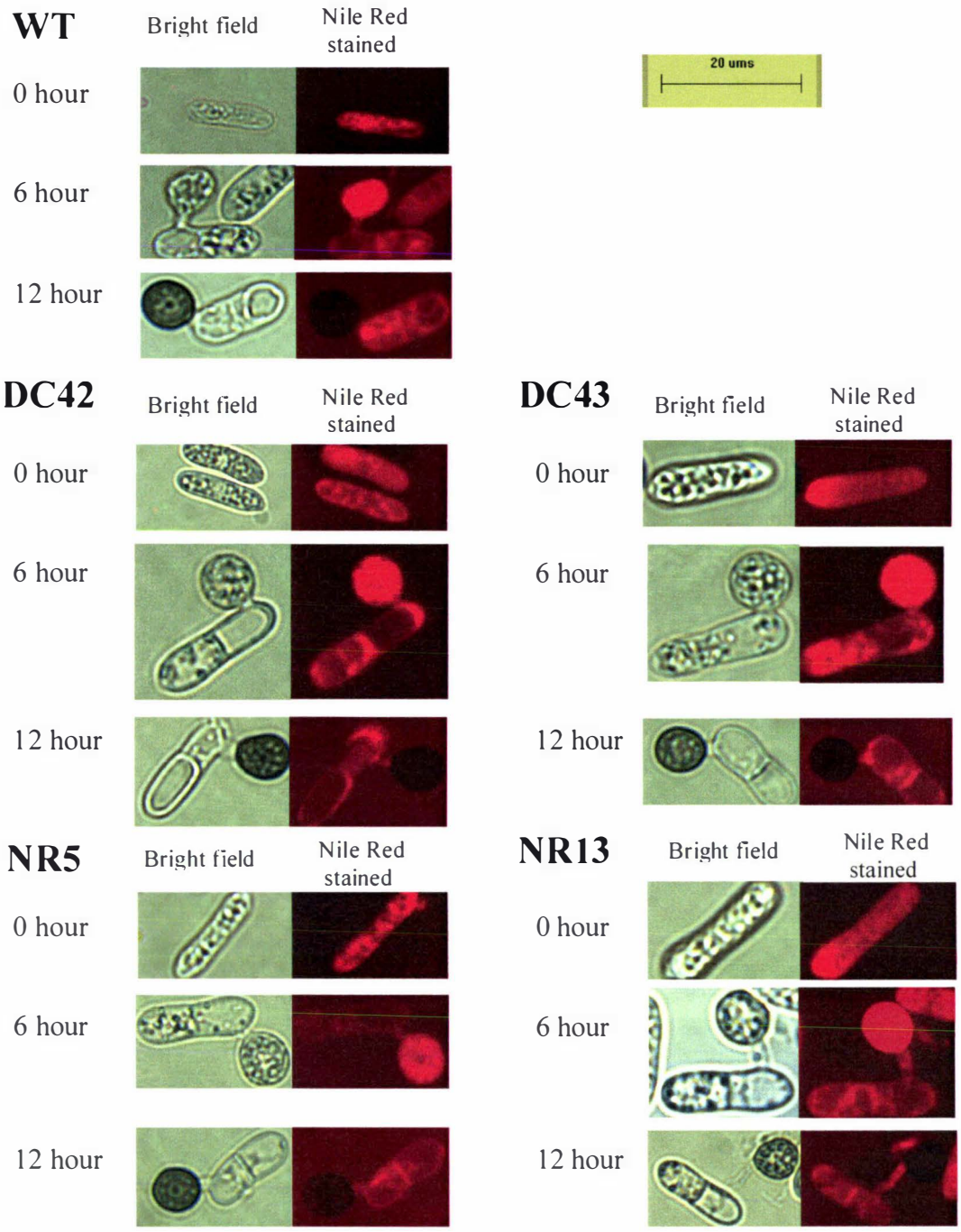


Figure 4.49 Mobilisation of lipid during appressorium development

Conidia of the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13) were inoculated on polystyrene Petri dishes and incubated at 22^0C. At 0, 6, and 12 hour, lipid bodies were stained with Nile Red. Fluorescence of the stained lipids was observed with a fluorescence microscope (excitation at 450 to 490 nm, 505 nm dichroic mirror and 520 nm barrier filter). Fluorescence of the sample stained at 24 hour was similar to that stained at 12 hour (data not shown).

4.6.2.6 Cytorrhysis assay

Estimation of appressorial turgor pressure was performed by an incipient cytorrhysis assay previously used with *M. grisea* (Howard *et al.*, 1991; Jong *et al.*, 1997). Exogenous hyperosmotic solute that is unable to cross the appressorial wall can draw water from an appressorium and cause it to collapse. Conidia suspended in water were inoculated onto polystyrene Petri dishes and incubated at 22^0C. After 12 or 24 hours incubation, the water was replaced by glycerol at concentrations ranging from 1 M to 4 M. The number of collapsed appressoria was determined with a microscope. Three independent experiments were performed and the percentage of the collapsed appressoria is shown in Table 4.10. With 2 M glycerol, more than 60% of the deletion mutant appressoria collapsed whereas only about 10% of the wild type and the restoration mutant appressoria collapsed. This suggested that the appressoria turgor pressure was significantly decreased in the deletion mutants compared to the wild type and the restoration mutant. Collapsed appressoria are shown in Fig 4.50.

4.6.2.7 Penetration assay

The ability of appressoria to penetrate cells was assayed on onion epidermal peel as described by Xu *et al.* (1997). Conidia were inoculated on to onion epidermal peel and incubated at 22^0C for 24-72 hours in humid conditions. Appressorium formation was examined 24 hours after inoculation. Appressoria could form on onion epidermal peel as efficiently as on apple wax coated glass slides. Appressorium penetration was examined 48 hours after inoculation. By adjusting the plane of focus, it could be established whether fungal structures were above or below the onion epidermal cells. As shown in Fig 4.51 (for wild type), Fig 4.52 (for the restoration mutant NR5) and Fig 4.53 (for NR13), appressoria and hyphae could be seen on the surface of the onion epidermal cells. Some hypha could only be seen under the onion epidermal cells and these could be tracked back to an appressorium, suggesting that this hypha was derived from the appressorium which had penetrated the cells. When onion epidermal peels were examined after 72 hours of incubation, a hyphal net could be observed under the onion epidermal cells, though it was hard to track the individual hypha back to an

Table 4.10 Cytorrhysis assay

A: Percentage of collapsed appressoria after 12 hours incubation

[Glycerol] (M)	Percentage of collapsed appressoria				
	WT	**DC42**	**DC43**	**NR5**	**NR13**
0.0	0 ± 0* (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)
1.0	4.2 ± 2.2 (3)	28.8 ± 6.3 (3)	28.4 ± 3.8 (3)	2.9 ± 1.0 (3)	2.7 ± 0.7 (3)
2.0	7.4 ± 2.2 (3)	63.2 ± 4.9 (3)	65.3 ± 4.2 (3)	6.3 ± 1.8 (3)	6.2 ± 1.3 (3)
3.0	27.2 ± 4.6 (3)	74.2 ± 4.3 (3)	65.7 ± 2.3 (3)	19.1 ± 4.5 (3)	23.4 ± 1.9 (3)
4.0	42.9 ± 3. 8 (3)	73.4 ± 3.5 (3)	80.8 ± 1.5 (3)	28.7 ± 3.3 (3)	37.4 ± 6.6 (3)

B: Percentage of collapsed appressoria after 24 hours incubation

[Glycerol] (M)	Percentage of collapsed appressoria				
	WT	**DC42**	**DC43**	**NR5**	**NR13**
0.0	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)
1.0	3.0 ± 0.6 (3)	6.0 ± 2.4 (3)	24.3 ± 8.1 (3)	3.7 ± 1.7 (3)	3.8 ± 2.3 (3)
2.0	6.6 ± 2.2 (3)	48.9 ± 4.1 (3)	58.9 ± 7.0 (3)	7.2 ± 3.4 (3)	10.4 ± 3.4 (3)
3.0	22.8 ± 4.2 (3)	62.1 ± 6.5 (3)	69.1 ± 3.6 (3)	18.0 ± 4.0 (3)	19.0 ± 2.3 (3)
4.0	38.0 ± 4.8 (3)	78.0 ± 3.2 (3)	73.7 ± 2.6 (3)	28.5 ± 1.6 (3)	25.6 ± 3.3 (3)

Conidia were harvested from the 7 day old PDA plates and suspended in water. Conidia (50 µl at 10^5/ml) were inoculated on to a polystyrene Petri dish and incubated at 22^0C. The water droplet was then replaced with glycerol at concentrations ranging from 1 M to 4 M after 12 (table A) or 24 hours (table B) incubation. Appressorial collapse was observed under a light microscope. Three independent experiments were performed and the percentage of collapsed appressoria is shown. The percentage of collapsed appressoria was significantly different between the wild type and the deletion mutants (Student's t-test, $P < 0.01$, $n = 3$), and between the restoration mutants and the deletion mutants (Student's t-test, $P < 0.01$, $n = 3$) when the glycerol concentration was at or above 2 M. Appressoria that were counted as collapsed had the appearance shown in Fig 4.50.

* Each value represents the mean and standard deviation of three independent experiments.

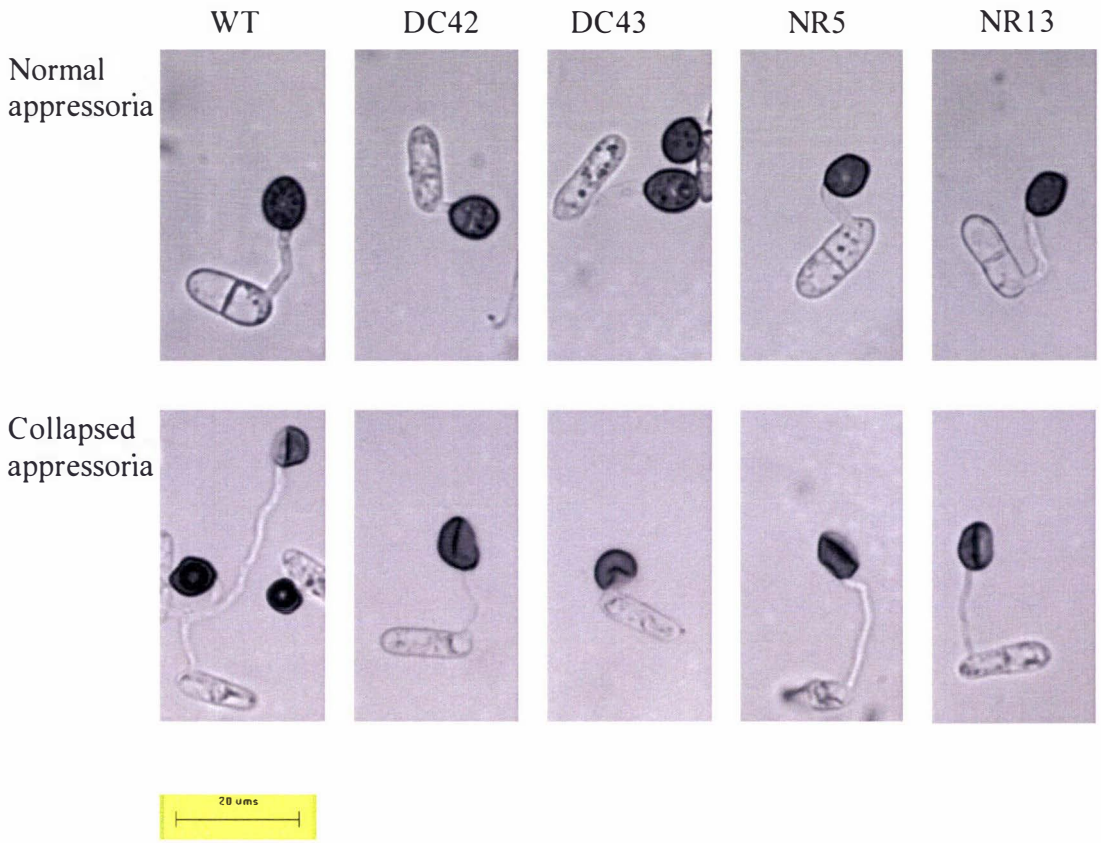


Figure 4.50 Collapsed appressoria

Conidia harvested from 7 day old PDA plates were inoculated on to polystyrene Petri dishes and incubated at 22^0C for 12 or 24 hours. The water droplet was then replaced with glycerol of different concentrations. Appressorial collapse was observed under a light microscope. The percentage of collapsed appressoria for each strain is shown in Table 4.10.

Upper panel: appressoria before application of glycerol.
Lower panel: appressoria after application of glycerol (4 M).

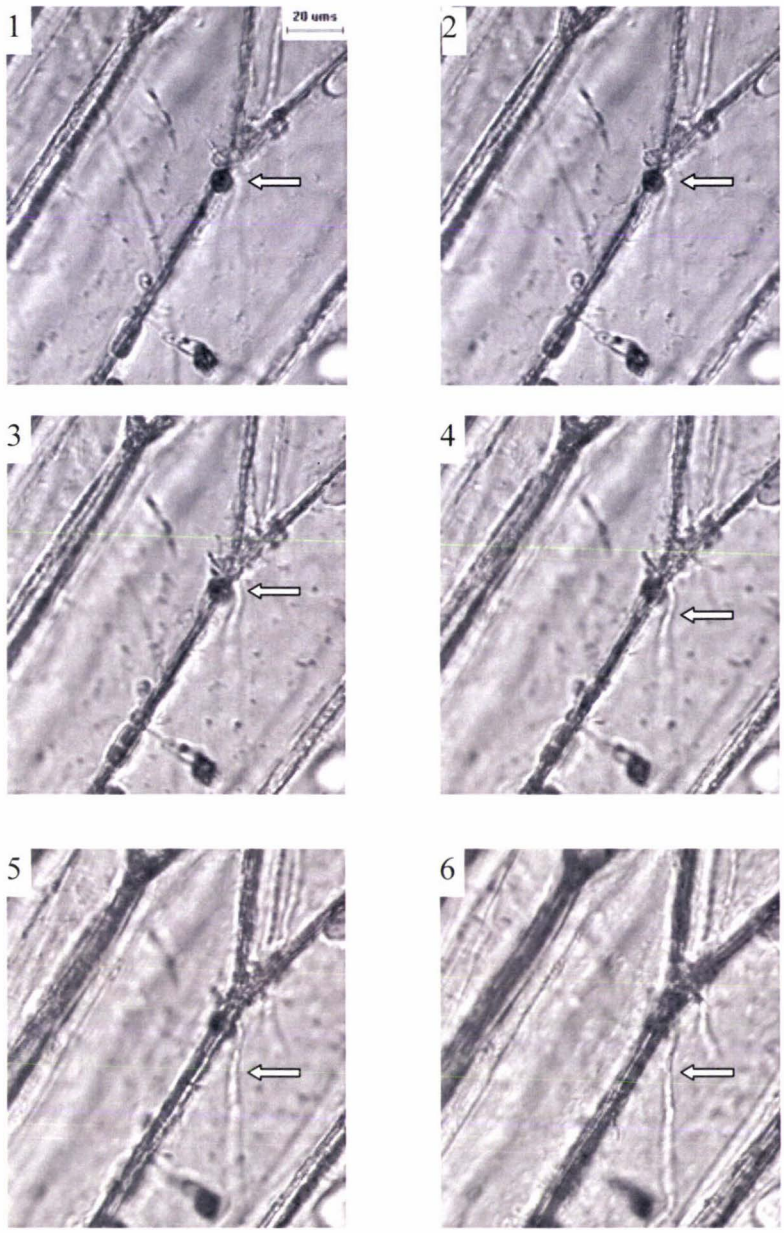


Figure 4.51 Wild type appressorium penetration of onion epidermal cells

Wild type conidia were inoculated onto onion epidermal peels and examined under a light microscope after 48 hours incubation at 22^0C. Photos were taken at a series of focal planes (number 1-6) to show the fungal structures above and below the onion epidermal cells. A hypha, derived from an appressorium, which had penetrated through the epidermal cells and developed underneath the cell is indicated by arrows.

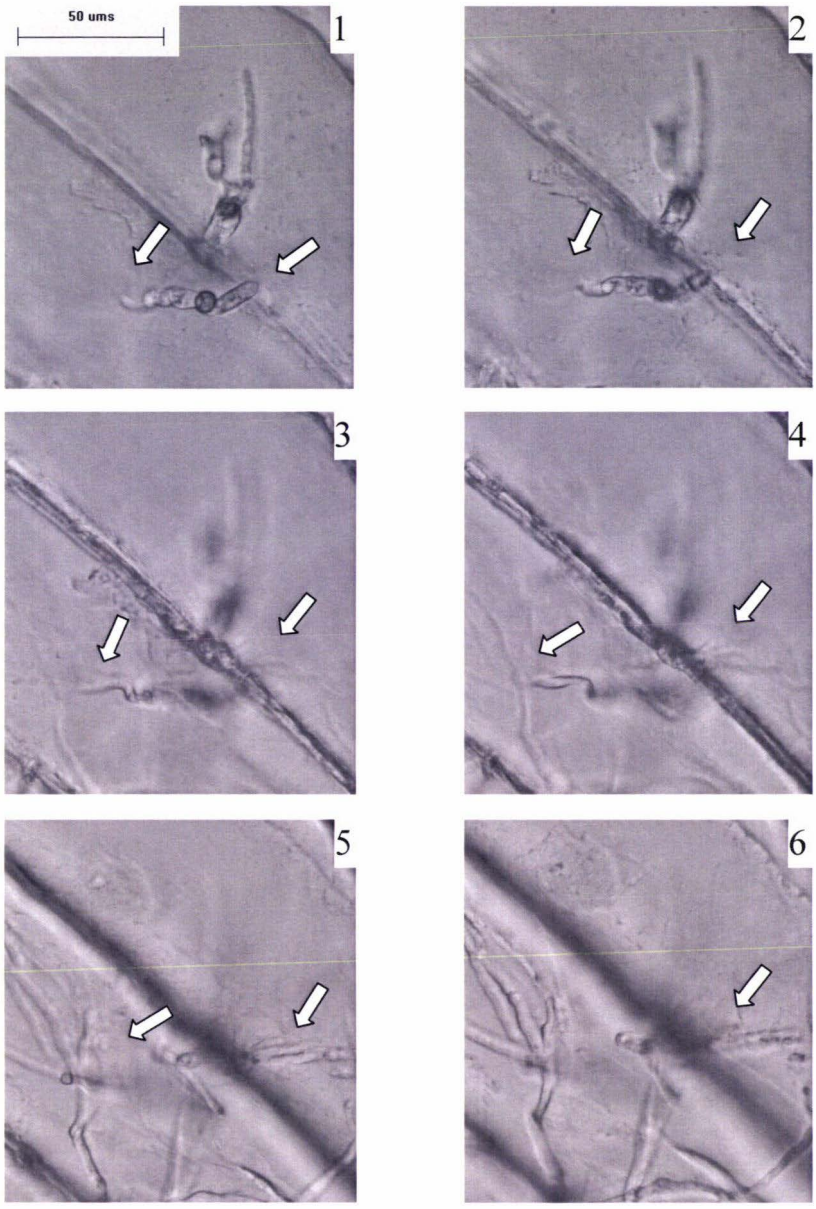


Figure 4.52 Penetration of onion epidermal cells by the restoration mutant NR5

Conida from the restoration mutant NR5 were inoculated on to onion epidermal cells and examined under a light microscope after 48 hours incubation at 22^0C. Photos were taken at a series of focal planes (number 1-6) to show the fungal structures above and below the onion epidermal cells. A hypha, derived from an appressorium, which had penetrated through the epidermal cells and developed underneath the cell is indicated by arrows.

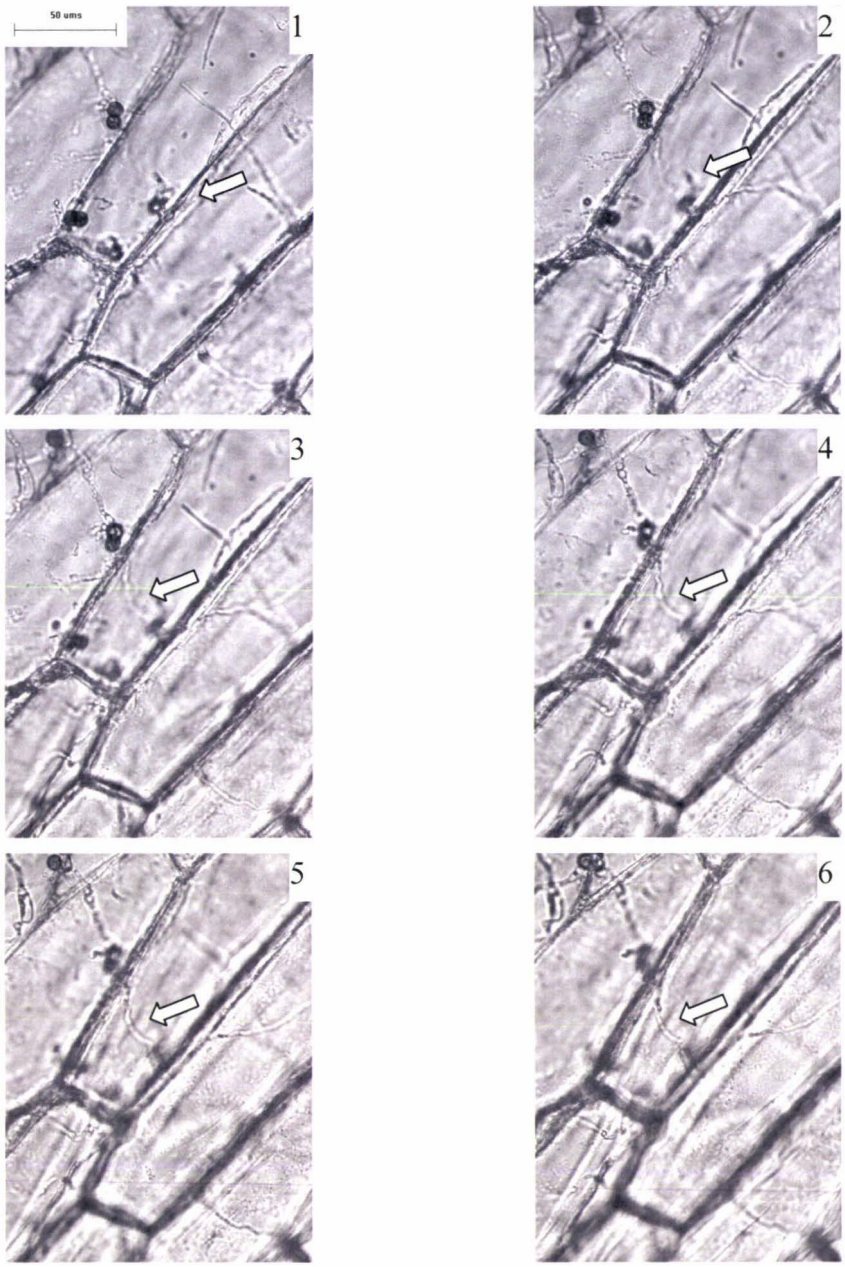


Figure 4.53 Penetration of onion epidermal cells by the restoration mutant NR13

Conida from the restoration mutant NR13 were inoculated on to onion epidermal cells and examined under a light microscope after 48 hours incubation at 22^0C. Photos were taken at a series of focal planes (number 1-6) to show the fungal structures above and below the onion epidermal cells. A hypha, derived from an appressorium, which had penetrated through the epidermal cells and developed underneath the cell is indicated by arrows.

appressorium. However, on the onion epidermal peels inoculated with the deletion mutant conidia (Fig 4.54 and Fig 4.55), appressoria and hyphae were seen on the cell surface but never under the cells, suggesting that the deletion mutant was deficient in penetration.

4.6.3 Pathogenicity assay

The pathogenicity assay was conducted by inoculating conidia onto Granny Smith apples. This variety of apple was chosen because the wild type *G. cingulata* strain was isolated from Granny Smith apples. Four independent assays were performed following the method described by Brook (1977). Conidia from the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13) were inoculated onto unwounded, surface sterile apples. Lesions started to emerge on the apple surface around 7 days after inoculation. New lesions continued to emerge after this. Adjoining lesions merged together after prolonged incubation. Therefore, 12 days after inoculation was chosen as the time point to record the number and diameter of lesions (Table 4.11). Lesions appeared on almost all apples inoculated with the wild type and the restoration mutant conidia, but not with the deletion mutant or water even after a further 13 days incubation. This suggested that the *StuA* gene of *G. cingulata* is required for pathogenicity on apple. Combined with the results from the appressorium penetration assay, it is speculated that loss of the pathogenicity for the deletion mutant is due to the failure of appressorium penetration.

To test whether the deletion mutant was capable of colonizing and developing lesions on apple if the penetration step was by-passed, apples were inoculated by injection of conidia into the apple tissue. The deletion mutants (DC42 and DC43), as well as the wild type and the restoration mutants (NR5 and NR13), were able to develop similar size lesions on wounded apples in the same period (Table 4.12). However, differences were observed between lesions caused by the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13). With the wild type and the restoration mutants as the inoculum, a brown lesion started to appear around the inoculation point and extended outwards. Sequentially concentric zones of light and

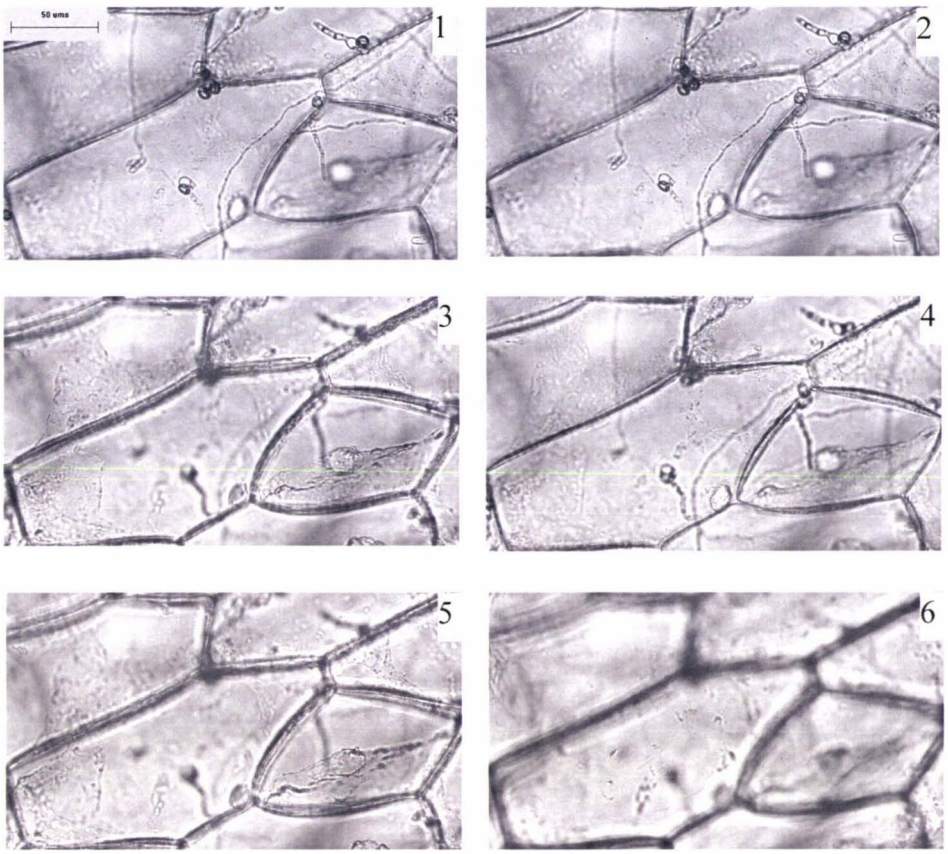


Figure 4.54 Appressorium development of the deletion mutant DC42 on onion epidermal cells

Conida from the deletion mutant DC42 were inoculated onto onion epidermal peels and examined under a light microscope after 48 hours incubation at 22^0C. Photos were taken at a series of focal planes (number 1-6) to show the fungal structures above and below the onion epidermal cells. Hyphae derived from appressoria could be observed on the surface of the onion cells but no hypha could be observed underneath the epidermal cells even if the incubation was continued for 96 hours.

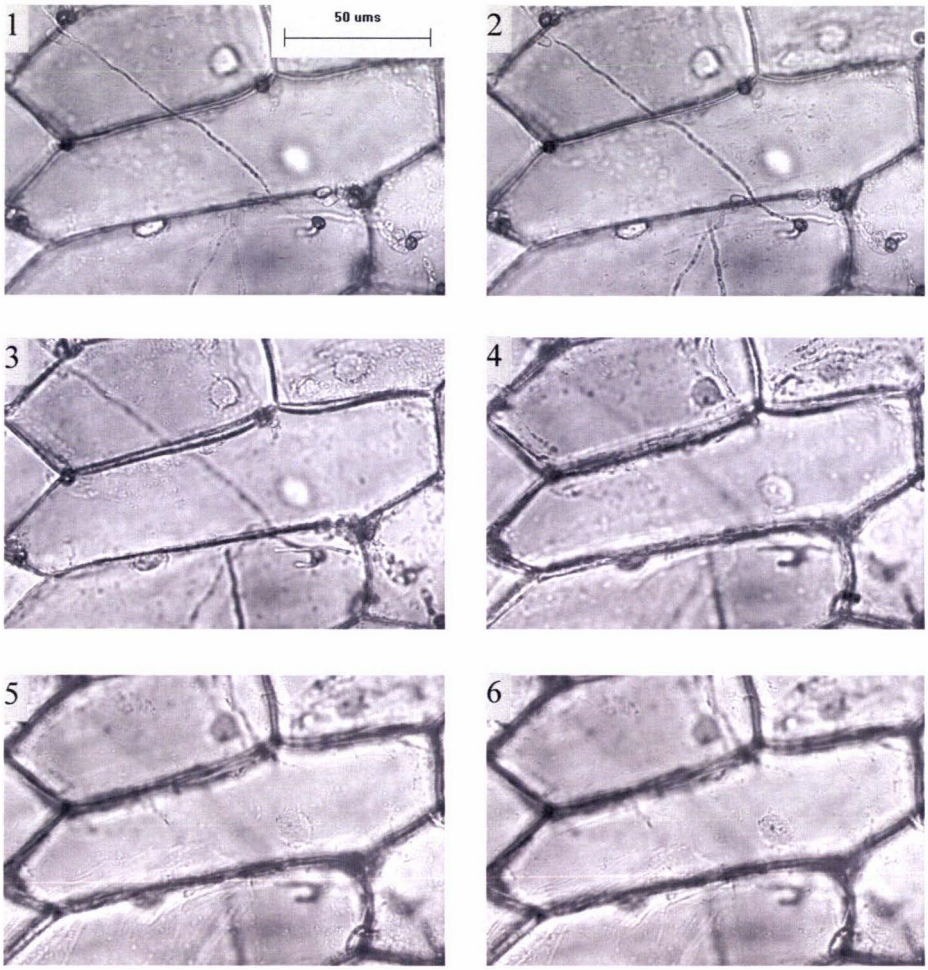


Figure 4.55 Appressorium development of the deletion mutant DC43 on onion epidermal cells

Conida from the deletion mutant DC43 were inoculated onto onion epidermal peels and examined under a light microscope after 48 hours incubation at 22^0C. Photos were taken at a series of focal planes (number 1-6) to show the fungal structures above and below the onion epidermal cells. Hyphae derived from appressoria could be observed on the surface of the onion cells but no hypha could be observed underneath the epidermal cells even if the incubation was continued for 96 hours.

Table 4.11 Pathogenicity assays on unwounded apples

Inoculum	Number of lesions per apple	Diameter of lesion (cm)
Wild type	2.25 ± 1.50 (40)*	1.97 ± 1.26 (90)**
DC42	0 ± 0 (16)	0 ± 0 (0)
DC43	0 ± 0 (24)	0 ± 0 (0)
NR5	1.83 ± 1.24 (24)	1.74 ± 1.09 (44)
NR13	1.94 ± 1.25 (16)	1.72 ± 1.42 (33)
Control	0 ± 0 (40)	0 ± 0 (0)

Pathogenicity assays were conducted by inoculating conidia from the wild type, the deletion mutants (DC42 and DC43), the restoration mutants (NR5 and NR13) on unwounded apples. Water was used as inoculum for the negative controls. Lesions were counted and their diameter measured 12 days after inoculation. The ability to infect apple was significantly different between the wild type and the deletion mutants, or between the restoration mutants and the deletion mutants when the number of lesions on apples was compared (Student's t-test, $P < 0.01$ for lesion numbers). While the number of lesions and the diameter of lesions caused by the wild type and the restoration mutants were not significantly different (Student's t-test, $P > 0.1$ for both lesion numbers and diameters).

* Each value represents the mean and standard deviation for the number of apples (shown in parenthesis) used in the experiments.

** Each value represents the mean and standard deviation for the number of lesions (shown in parenthesis).

Table 4.12 Pathogenicity on wounded apples

Inoculum	Diameter of lesion (cm)
Wild type	3.12 ± 0.46 (22)*
DC42	3.35 ± 0.46 (8)
DC43	3.37 ± 0.55 (14)
NR5	3.26 ± 0.39 (14)
NR13	3.13 ± 0.52 (8)
Control	0 ± 0 (22)

Pathogenicity assays were conducted by inoculating conidia (50 μl of 10^6/ml) from the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13) on to wounded apples. Water was used as inoculum for the negative controls. All apples formed a lesion except the apples inoculated with water. The diameter of the lesion was measured 14 days after inoculation. The lesion diameter was not significantly different among the wild type, the deletion mutants and the restoration mutants (Student's t-test, $P > 0.1$ for all groups).

* Each value represents the mean and standard deviation for the number of apples used in the experiments.

dark brown tissue emerged on the lesion surface. These were from acervuli, which developed in the apple tissue, became swollen, ruptured the cuticle and exposed a gelatinous mass of pink conidia on the lesion surface (Fig 4.56). With prolonged incubation, the lesion continued to grow and black perithecia developed (15-30 days) on the surface of lesion (Fig 4.57). The deletion mutants caused lesions of similar size to that produced by the wild type and the restoration mutants, but the lesion surface was smooth, no acervuli or perithecia were visible either on the surface of the apple or inside the apple (Fig 4.56, Fig 4.57). It was also noticed that the lesion caused by the deletion mutants was light grey in colour rather than the dark black lesions seen for the wild type and the restoration mutants (Fig 4.57), consistent with the differences in melanization observed for cultures growing on PDA plates.

Another difference between lesions formed on wounded apples was that those caused by the deletion mutants were softer than those caused by the wild type. A firmness assay was conducted as described in Section 2.11.11. Fourteen days after inoculation of conidia onto the wounded apples, diameters of lesions were measured (Table 4.13). These showed no statistically significant differences (Student's t-test, $P > 0.1$, $n = 8$). The lesion caused by the deletion mutants was significantly softer (Student's t-test, $P < 0.01$, $n = 8$) than that caused by the wild type. This was possibly because of the up-regulation of some degradative enzymes in the deletion mutant, e.g pectin lyase and secreted peptidases (next section).

4.6.4 Gene expression

Northern blot analysis has been widely used in gene expression studies and was therefore carried out to study the expression of 10 genes during vegetative growth and appressorium formation for the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13). This study could provide a good overview of the differences, but not accurate changes, in gene expression. Real-time PCR could provide more precise data in study of gene expression but has not been adapted due to time restriction.

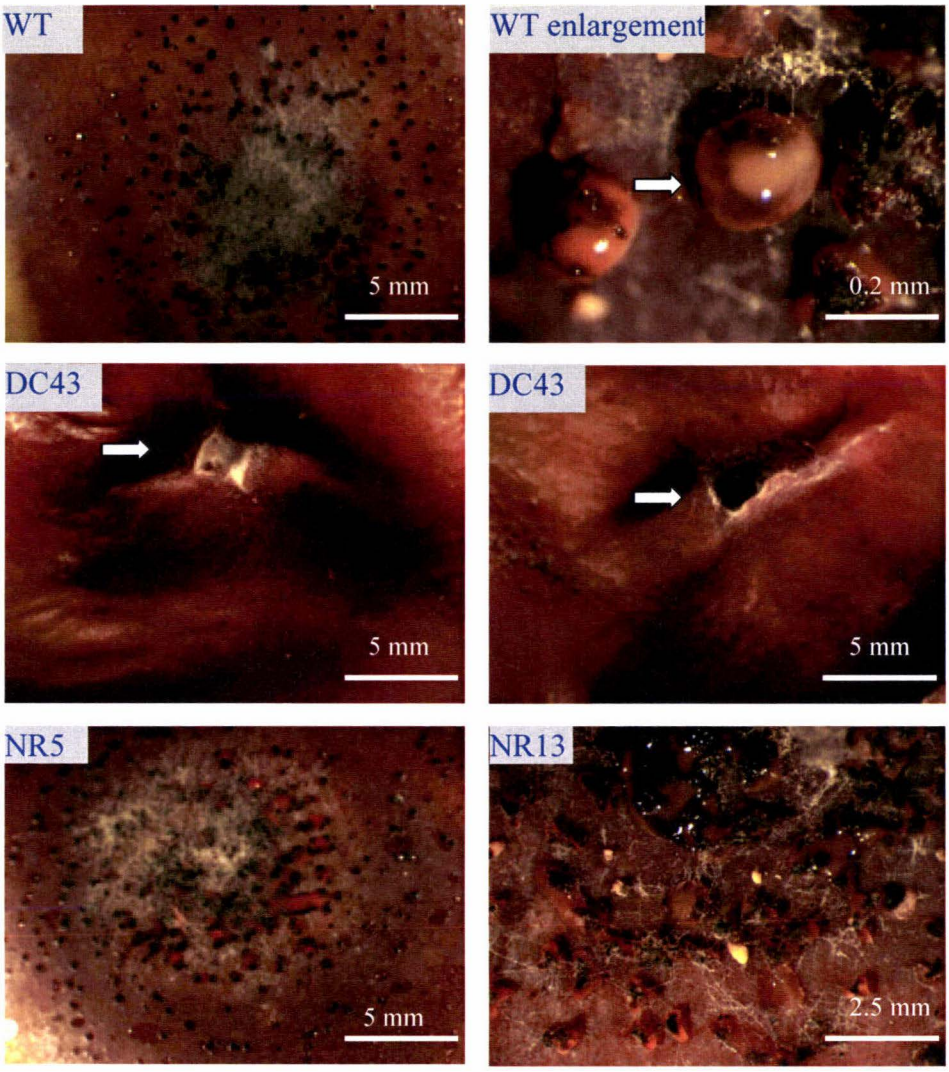


Figure 4.56 Surface of apple lesions

The surface of 7 day old lesions formed on wounded apples inoculated with conidia from the wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13) is shown. Conidia erupting from acervuli under the apple cuticle were observed on the apples inoculated with the wild type and the restoration mutants but not the deletion mutants. In the two middle panels, the arrow indicates the inoculation hole. On the apples inoculated with wild type and the restoration mutants, the hole was covered by mycelium. In top right panel (WT enlargement), the arrow indicates a mass of conidia.

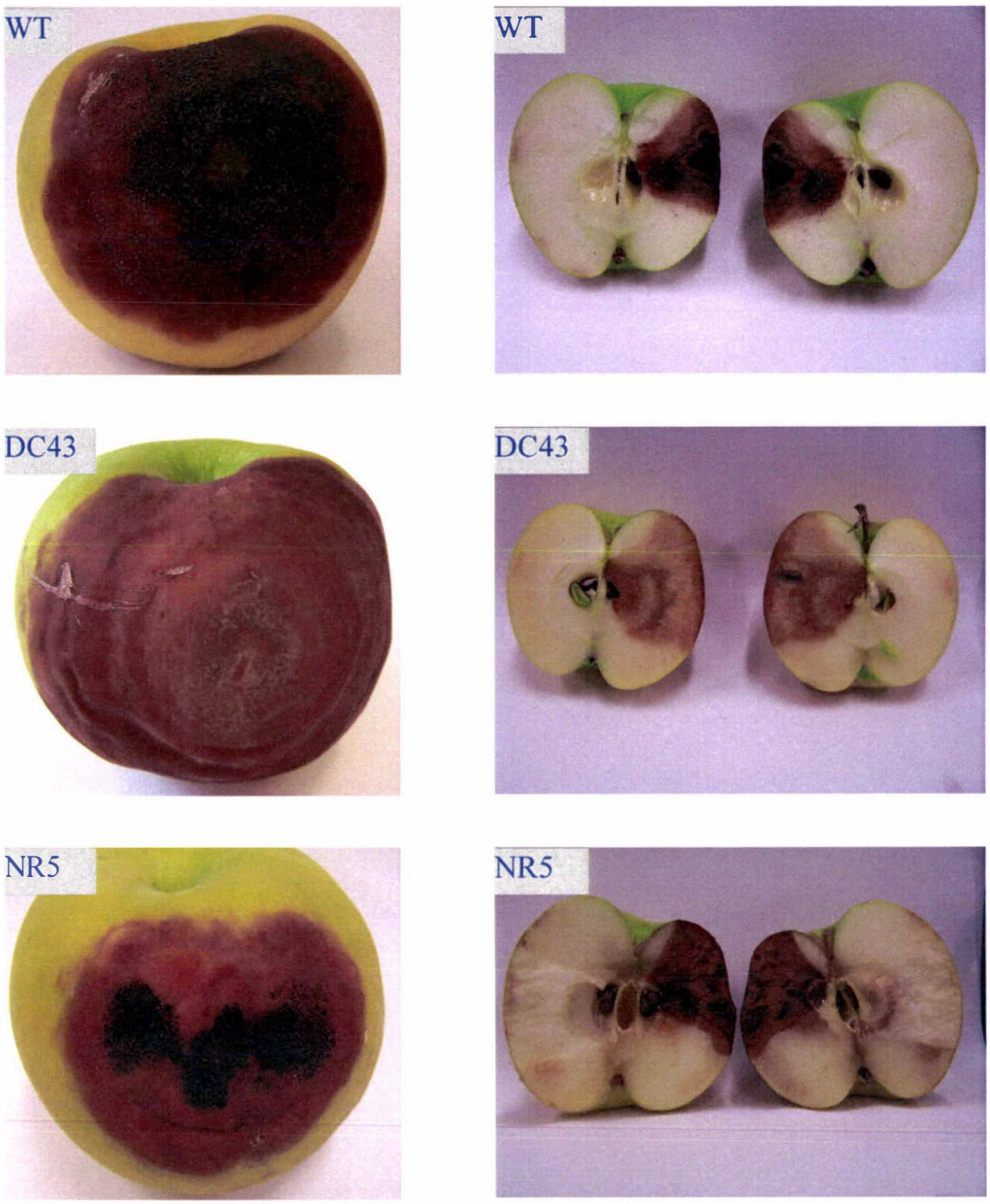


Figure 4.57 Apple lesions

After 15-30 days inoculation, perithecia were observed on the surface of apples inoculated with the wild type and the restoration mutants, but not that inoculated with the deletion mutants. A cross-section through the lesion was light grey in colour on the apple inoculated with the deletion mutant DC43 (and DC42, data not shown); whereas it was dark black on the apple inoculated with wild type and the restoration mutant NR5 (and NR13, data not shown).

Table 4.13 Softness of apple lesions

Strains	Diameter of lesion (cm)	Force required to puncture lesion (kg)
Wild type	2.88 ± 0.32 (8)*	1.13 ± 0.26 (8)
DC42	3.18 ± 0.35 (8)	0.50 ± 0.15 (8)
DC43	2.94 ± 0.3 (8)	0.56 ± 0.13 (8)
NR5	3.03 ± 0.35 (8)	1.05 ± 0.21 (8)
NR13	3.23 ± 0.47 (8)	1.09 ± 0.27 (8)
Control	0 ± 0 (8)	8.4 ± 1.82 (8)

Wounded apples were inoculated with conidia (50 μl of 10^6/ml) from wild type, the deletion mutants (DC42 and DC43) and the restoration mutants (NR5 and NR13). Water was used as inoculum for the negative controls. The diameter of the lesion was measured and the softness of the lesion was measured using a penetrometer 14 days after inoculation. The lesions caused by the deletion mutant (DC42 and DC43) were significantly softer than the lesions caused by the wild type (Student's t-test, $P < 0.01$ for both DC42 and DC43) or the restoration mutants (Student's t-test, $P < 0.01$, for both DC42 and DC43). Softness of lesions caused by the wild type was not significantly different from the lesions caused by the restoration mutants (Student's t-test, $P > 0.5$ for both NR5 and NR13).

* Each value represents the mean and standard deviation for the number of apples used in the experiments.

Gene fragments used as probes were either obtained by restriction digestion of cloned genes or by PCR amplification from genomic DNA. Design of specific or degenerate primers for PCR was based on the information available in the GenBank database. PCR products were purified, sequenced and the identity of the sequences was confirmed by alignment with known sequences from *G. cingulata* or of homologous genes from other organisms.

RNA was extracted from mycelium grown on PDA plates and appressoria induced by apple wax for 4 or 16 hours as described in Section 2.11.4. The RNA concentration was determined by using a spectrophotometer (Section 2.9.2). The relative quantities of RNA estimated by this method and by ethidium bromide fluorescence were consistent. RNA samples were size-fractionated by electrophoresis in a formaldehyde agarose gel, blotted onto a nylon membrane for hybridisation with α-^{32}P- labelled probe. Ribosomal RNA is commonly used as the loading control in northern analysis, therefore sample loading was adjusted on the basis of the intensity of the 28S and 18S rRNA bands in this study. However, the quantification in these ways may not be accurate enough and this may interfere the interpretation of the northern blot results. Real-time PCR is an alternative way which may provide more accurate quantitative information in study of gene expression and this may be conducted in future study.

4.6.4.1 Expression of the *G. cingulata gpd*, actin, and β-tublin genes

The glyceraldehyde-3-phosphate dehydrogenase (*gpd*), actin, and β-tubulin genes are classified as housekeeping genes in many fungi. They are expressed constitutively under most growth conditions, and could be a suitable positive control in a northern blot analysis. Design of the primer pair gpd47F/gpd583R (Appendix 3) was based on the *G. cingulata gpd* sequence (Templeton *et al.*, 1992) and used to amplify a 536 bp fragment from the *G. cingulata gpd* gene. Design of the primer pair act474F/act977R (Appendix 3) was based on the *C. gloeosporioides f. sp. malvae* actin gene sequence (Jin *et al.*, 1999). Design of the primer pair Tub2-648F/Tub2-1210R (Appendix 3) was based on the *C. gloeosporioides f. sp. aeschynomene* β-tubulin gene sequence (Buhr and Dickman, 1993). These primers were used in PCR to amplify a 503 bp and a 562 bp fragment, respectively, of the *G. cingulata* actin and β-tubulin genes. In each case the

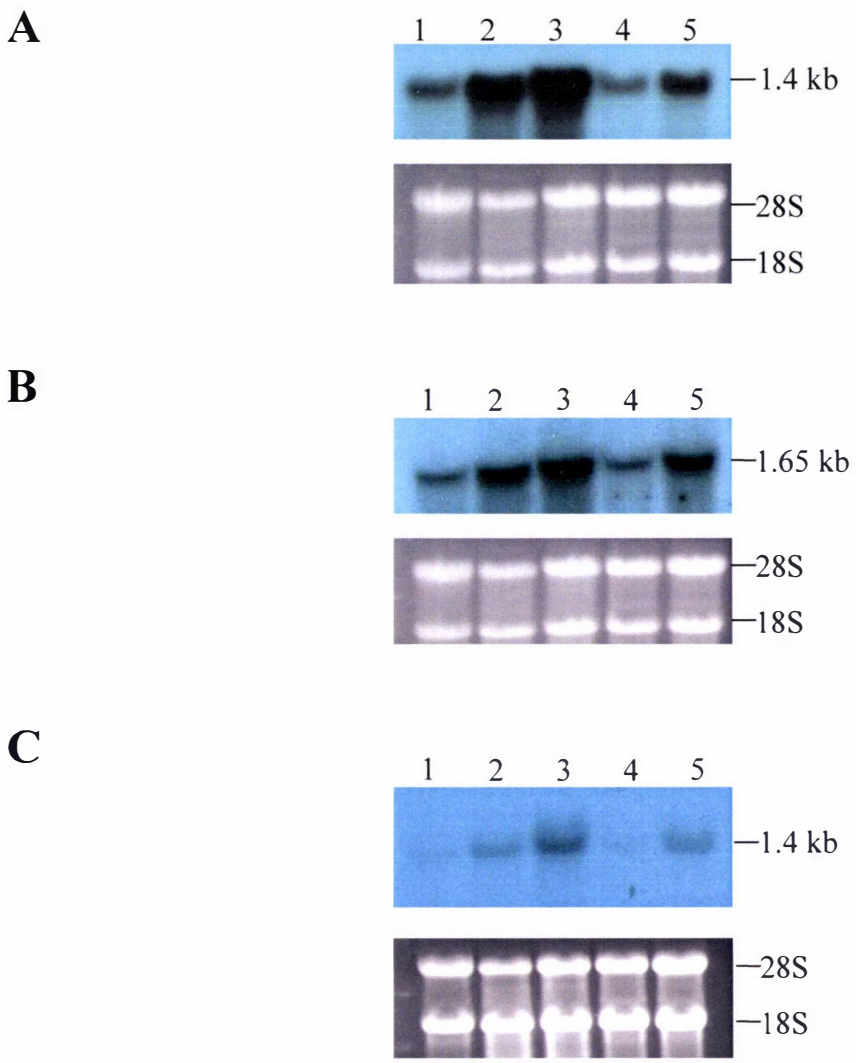


Figure 4.58 Northern blot analysis of the *gpd*, actin and β-tubulin genes

Northern blot analysis was performed with mycelial RNA (grown on PDA plates for 7 days in the dark) from the wild type (lane 1), the deletion mutants DC42 (lane 2), DC43 (lane 3) and the restoration mutants NR5 (lane 4) and NR13 (lane 5). The RNA concentration was determined by using a spectrophotometer (Section 2.9.2). The membrane was hybridised with α^{32}P-dCTP labelled PCR products from the glyceraldehyde-3-phosphate dehydrogenase gene (panel A), actin gene (panel B) and β-tubulin gene (panel C). In each panel, the upper image is an autoradiograph of the northern blot and the lower image is the ethidium bromide stained ribosomal RNA bands.

sequence (Appendix 5) of the PCR product aligned with the appropriate gene from other fungi. These gene fragments were used as probes in northern blot analysis for the RNA extracted from mycelium grown for 7 days on PDA plates. Hybridisation patterns for these three genes were similar as shown in Fig 4.58. They all appeared to be up-regulated in the deletion mutants DC42 and DC43. Compared to the wild type, expression of the *gpd* gene was up-regulated ~4 fold in DC42 and ~7 fold in DC43, expression of the actin gene was up-regulated ~2 fold in DC42 and ~3 fold in DC43, expression of the β-tubulin gene was up-regulated ~2 fold in DC42 and ~6 fold in DC43.

4.6.4.2 Expression of the *G. cingulata PKS1*, *SCD1* and *THR1* genes

Since colonies of the deletion mutants were poorly melanised, three melanin biosynthesis genes were included in the study. These genes were the polyketide synthase gene (*PKS1*), the scytalone dehydratase gene (*SCD1*), and the 1,3,8-trihydroxynaphthalene reductase gene (*THR1*). These genes have been studied in *Colletotrichum lagenarium* (Takano *et al.*, 1997) and *Magnaporthe grisea* (Tsuji *et al.*, 2000), but not in *G. cingulata.* Based on the sequence information of these genes from *C. lagenarium* and *M. grisea*, gene specific primers were designed for PCR amplification of these genes from *G. cingulata.* Using the wild type genomic DNA as template, the primer pairs pks558F/pks2537R, scd237F/scd830R, thr742F/thr1239R (Appendix 3) were used in PCR to amplify a 1979 bp, 593 bp and 497 bp fragments, respectively, of the *G. cingulata PKS1*, *SCD1* and *THR1* genes. In each case the sequence (Appendix 5) of the PCR product was aligned with the appropriate gene from other fungi. These PCR products were used as probes in the northern blot analyses for appressorial RNA and mycelial RNA. As shown in Fig 4.59, all three genes appeared to be down-regulated in the deletion mutant either in mycelial RNA or appressorial RNA. In spite of this, the appressoria were melanised. In vegetative growth, expression of both *PKS1* and *THR1* was down-regulated at ~10 fold in both DC42 and DC43, expression of the *SCD1* was down-regulated at ~2 fold in DC42 and at ~1 fold in DC43, compared with the wild type. During the early stage (4 hour) of appressorium formation, expression of all three genes was down-regulated (*PKS1*, at ~3 fold; *SCD1*, at ~2 fold;

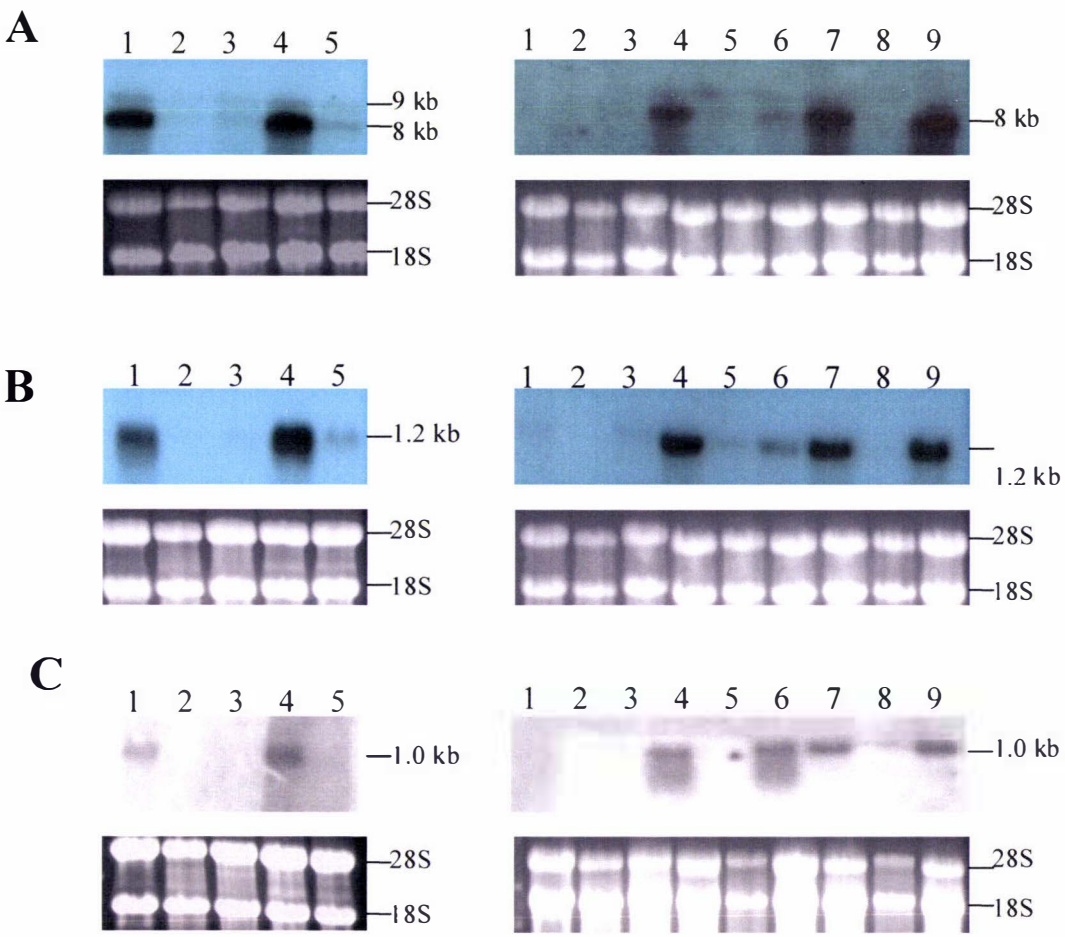


Figure 4.59 Northern blot analysis of the *G. cingulata PKS1*, *THR1* and *SCD1* genes

Northern blot analysis was performed with α^{32}P-dCTP labelled PCR products from the *PKS1* gene (panel A), the *THR1* gene (panel B) and the *SCD1* gene (panel C). For each panel, the top left image is a northern blot using mycelial RNA (grown on PDA plates for 7 days in a light/dark cycle) from the wild type (lane 1), the deletion mutants DC42 (lane 2), DC43 (lane 3) and the restoration mutants NR5 (lane 4) and NR13 (lane 5); the top right image is a northern blot using RNA from conidia (lane 1, wild type; lane 2, DC43; lane 3, NR5), 4 hour appressoria (lane 4, wild type; lane 5, DC43; lane 6, NR5), and 16 hour appressoria (lane 7, wild type; lane 8, DC43; lane 9, NR5). In each panel, the upper image is a northern autoradiograph and the lower image is the ethidium bromide stained ribosomal RNA bands. Appressoria were induced with apple wax.

THR1, at ~7 fold). The same patterns were observed at the later stage (16 hour) of appressorium formation (*PKS1*, at ~7 fold; *SCD1*, at ~3 fold; *THR1*, at ~7 fold).

4.6.4.3 Expression of the *G. cingulata SAP* and *pnlA* genes

Extracellular enzymes that degrade plant cuticle and tissue are thought to have a role in fungal pathogenicity. The apple lesions caused by the deletion mutants were significantly softer than those caused by the wild type (Section 4.5). Therefore the *G. cingulata SAP* gene and pectin lyase A (*pnlA*) gene (Templeton *et al.*, 1994) were chosen for Northern blot analysis. Using the wild type genomic DNA as template, the primer pairs C847/S1723 and pnl593F/pnl1379R (Appendix 3) were used to amplify an 851 bp and a 787 bp fragments of the *G. cingulata SAP* and *pnlA* genes, respectively. In each case the sequence of the PCR product was correct. These PCR products were used as probes in the northern blot analyses. To prepare RNA for hybridisation with the *pnlA* probe, mycelium was grown in liquid PDB medium and then transferred to medium containing 5% pectin as the sole carbon source for the induction of pectin lyase A. To prepare RNA for hybridisation with the *SAP* probe, mycelium was grown on BSA agar plates. RNA was then extracted as described in Section 2.9.1. As shown in Fig 4.60, both the *pnlA* and the *SAP* gene appeared to be up-regulated in the deletion mutant DC42 and DC43. Northern blots to detect their expression in appressoria were not successful and were not repeated due to time restrictions.

4.6.4.4 Expression of the *G. cingulata StuA* and areA genes

Expression of the *G. cingulata StuA* gene was also studied by northern blot analysis. Since the *StuA* gene was involved the regulation of conidiation, RNA was extracted from mycelium of different ages grown on PDA plates in the dark or under a light/dark cycle. A 5.3 kb *Sal*I fragment containing the *StuA* gene was used as the probe. As shown in panel A of Fig 4.61, the *StuA* gene was expressed in mycelium from both growth conditions and different ages. In addition, the only hybridising band is ~3.7 kb, which is longer than the cDNA obtained from the RACE experiment (Section 4.3). This size is close to the size (3.4 kb) of the *A. nidulans StuA* mRNA, which has a 1.9 kb ORF

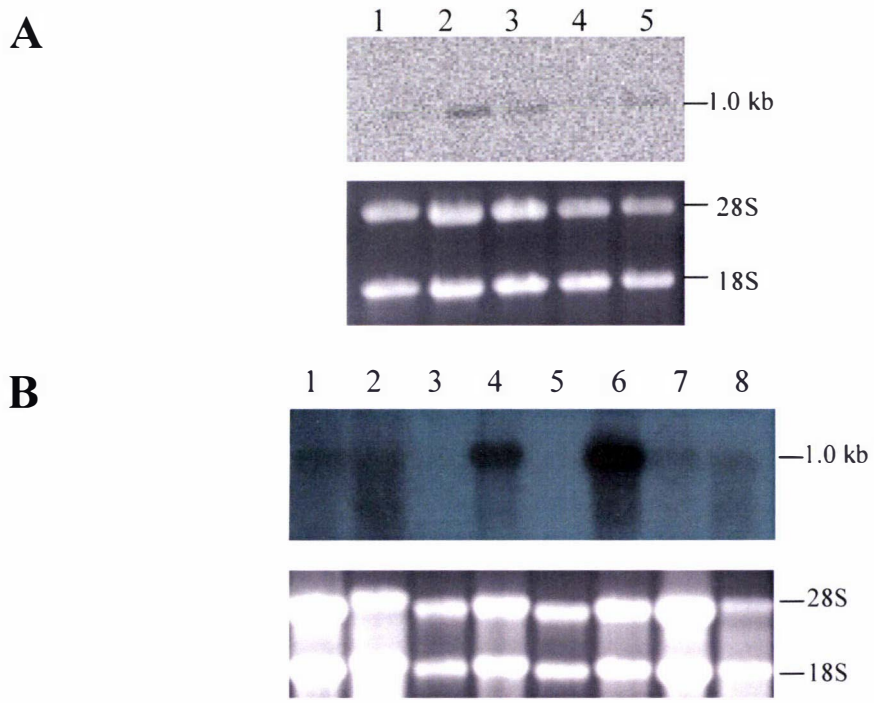


Figure 4.60 Northern blot analysis of the *G. cingulata pnlA* and the*SAP* gene

A: Northern blot analysis of the *G. cingulata pnlA* gene. RNA was extracted from the mycelium grown in pectin lyase induction medium (Section 2.3.2 and 2.9). A PCR product amplified with primer pair pnl593F/pnl1379R from the *pnlA* gene was labelled with α^{32}P-dCTP and hybridised with the membrane containing RNA from wild type (lane 1), the deletion mutants DC42 (lane 2) and DC43 (lane 3), the restoration mutants NR5 (lane 4) and NR13 (lane 5).

B: Northern blot analysis of the *G. cingulata SAP* gene. A PCR product amplified with primer pair C847/S1723 from the *sap* gene was labelled with α^{32}P-dCTP and hybridised with the membrane containing RNA from the wild type (lanes 1 and 2), the deletion mutant DC42 (lanes 3 and 4), DC43 (lanes 5 and 6), the restoration mutants NR5 (lanes 7 and 8). Mycelia for RNA extraction were grown in PDB liquid medium (lanes 1, 3, 5, and 7) or in the medium contained BSA as the sole nitrogen source (lanes 2, 4, 6, and 8).

In each panel, the upper image is a northern autoradiograph and the lower image is the ethidium bromide stained ribosomal RNA bands.

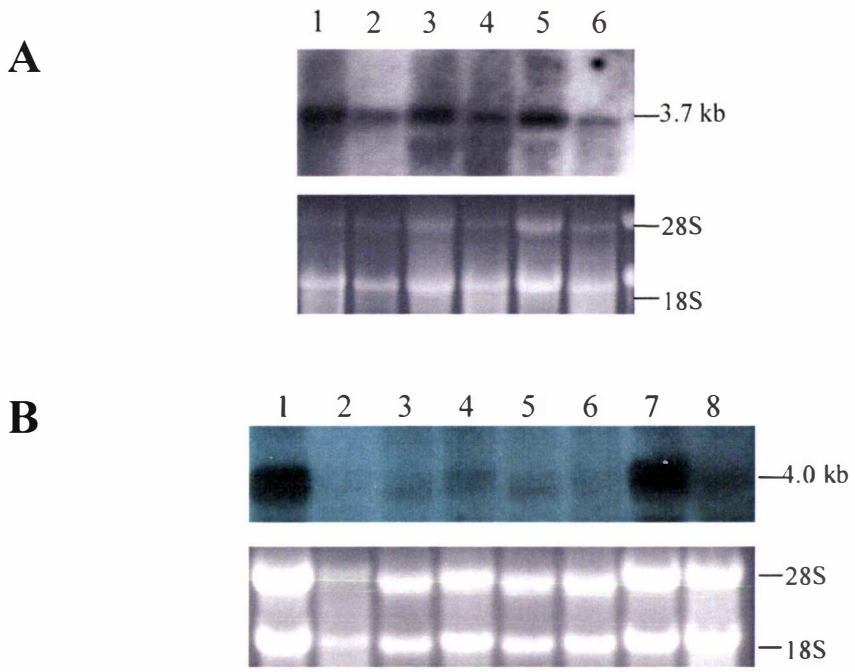


Figure 4.61 Northern blot analysis of the *G. cingulata StuA* and *areA* genes

A: Northern blot analysis of the *G. cingulata StuA* gene. A 5.3kb *Sal*I fragment of the *StuA* gene was labelled with α^{32}P-dCTP and hybridised with a membrane containing RNA from the wild type mycelium grown in the dark for 2 days (lane 1), 4 days (lane 3), and 7 days (lane 5); mycelium grown under a light/dark cycle for 2 days (lane 2), 4 days (lane 4), and 7 days (lane 6).

B: Northern blot analysis of the *G. cingulata areA* gene. A 1.0 kb *Nco*I fragment which contained part of the *areA* ORF was labelled with α^{32}P-dCTP and hybridized with a membrane containing RNA from wild type (lane 1, PDA medium; lane 2, BSA containing medium), the deletion mutant DC42 (lane 3, PDA medium; lane 4, BSA containing medium), DC43 (lane 5, PDA medium; lane 6, BSA containing medium), and the restoration mutant NR5 (lane 7, PDA medium; lane 8, BSA containing medium).

In each panel, the upper image is a northern autoradiograph and the lower image is the ethidium bromide stained ribosomal RNA bands.

and 1.0 kb 5-UTR (Miller *et al.*, 1992). If the mRNA structure of the *G. cingulata StuA* gene was similar to that of the *A. nidulans StuA*, it was possible that the *G. cingulata StuA* mRNA stop its transcription at a site downstream of the site determined by 3' RACE, or start its transcription at a site upstream of the site determined by 5' RACE, or both.

The *areA* gene encodes a transcription factor required for regulation of nitrogen metabolism (Kudla *et al.*, 1990) and pathogenicity (Pellier *et al.*, 2003) in fungi. It was of interest to see whether the *G. cingulata areA* was regulated by the *G. cingulata StuA* gene product. RNA was extracted from mycelium grown in PDA medium and medium containing BSA as the sole nitrogen source. A 1.0 kb *Nco*I fragment which contained part of the *areA* ORF (Hudson *et al.*, unpublished) was used as the probe for the detection of *areA* expression. As shown in panel B of Fig 4.61, the *areA* gene appeared to be down-regulated in the deletion mutants under either growth condition, suggesting that the *G. cingulata StuA* gene product does control the expression of the *G. cingulata areA* gene. For the wild type and the restoration mutants, the *areA* gene was also down regulated in mycelium grown on medium containing BSA as the sole nitrogen source. The mechanism for this down-regulation is not known.

4.7 Summary

To investigate appressorium formation and function in *G. cingulata*, the *StuA* gene was cloned and characterised. The deduced protein sequence showed a high level of identity with members of the APSES family, especially in the APSES domain. Deletion mutants displayed a series of phenotypic changes, including stunted aerial hyphae, wettable mycelium, absence of sexual reproduction structures, and decreased conidia production. Conidium adhesion and appressorium formation by the deletion mutants were not different from the wild type or the restoration mutants. Mobilisation of the major substrates for glycerol production, glycogen and lipid, was not impaired in the deletion mutants. However, appressorial turgor pressure in the deletion mutants was much lower than in the wild type or the restoration mutants. This is possibly the reason that appressorium of the deletion mutants could not penetrate onion epidermal peels, whereas the wild type and the restoration mutants could. Consistent with this, the

deletion mutants failed to cause disease on unwounded apples. However, they are able to cause lesions when conidia were inoculated into wounded apples and the lesions were softer than those caused by the wild type or the restoration mutants. The *SAP* and *pnlA* genes appeared to be up-regulated in the deletion mutants, which may explain the softness of the apple lesions caused by the deletion mutants. Three melanin synthesis genes appeared to be down-regulated under different growth conditions, consistent with the observation of that colonies of the deletion mutants remained unmelanised. Taken together, the *StuA* gene is likely to be a gene that controls the formation of reproduction structures as shown in other fungi. It is the first transcription factor to be characterised that controls appressorial turgor pressure, penetration, and pathogenicity in appressorium forming fungi.

Chapter five

Discussion

This project commenced with the characterization of the transcription start points of the *G. cingulata SAP* promoter, and an attempt to examine expression of *SAP* promoter-GFP reporter constructs in order to determine the promoter fragment responsible for expression of the long *SAP* transcript. Unfortunately, expression of the *SAP* promoter-GFP reporter constructs in *G. cingulata* did not result in fluorescence above that of the wild type. Therefore the gene candidate approach was followed. A *G. cingulata* member of the APSES family of transcription factors was cloned and characterized by analysis of the phenotypic changes in a deletion mutant.

5.1 Characterisation of the *SAP* promoter

RT-PCR was performed to determine the approximate location of the most upstream transcription start point (TSP) of the *G. cingulata SAP* gene. Unexpectedly the long transcript was found to be expressed in the late stage of appressorium formation, and during vegetative growth on protein as the sole nitrogen source. The exact transcription start points of the *SAP* gene were determined by RLM-RACE. Three transcription start points for the short, middle, and long transcripts were determined with RNA from vegetative mycelium; but only two transcription start points, for the short and middle transcripts, were determined for appressorial RNA. The evidence from RT-PCR suggested that the long transcript did exist in appressorial RNA, failure to determine this transcription start point may be due to difficulties during the performance of RLM-RACE. There are many enzymatic reactions in a RLM-RACE procedure, which may cause RNA degradation. There are also three steps that each required using 10-fold less sample in subsequent reaction (10^3 decrease of the sample in three steps together). These may result in the level of the *SAP* transcript falling below detectable in the PCR reaction. In addition, the TSP (-110) for the short transcript was 6 base pairs upstream from that determined in previous work (Clark *et al.*, 1997). Since the sequencing results from 6 independent PCR reactions consistently indicated a TSP at -110, this was considered reliable. The previously reported TSP was also determined by a RACE experiment using an adaptor primer that was ligated to the cDNA (Clark *et al.*, 1997). The shorter cDNA sequence could be the result of limited RNA degradation or failure of the polymerase to reach the end of the RNA during cDNA synthesis.

Although there are no reports to date of multiple transcription start points for other secreted aspartic proteinase genes, the use of multiple promoters and transcription start points is frequently observed in eukaryotes. Alternative promoters can react differently to some signals, resulting in variation in the level of transcription, generation of mRNA isoforms and protein isoforms (Ayoubi and Van De Ven, 1996). However, the significance of the three transcription start points in the *G. cingulata SAP* gene is not clear, since all three transcripts are present in the different growth conditions tested.

Green fluorescence protein (GFP) fusion protein has been successfully used as a reporter in several *Colletotrichum* species (Dumas *et al.*, 1999; Liu and Kolattukudy, 1999; Takano *et al.*, 2001b), and in other fungi (Lorang *et al.*, 2001). However, no significant fluorescence due to GFP was observed either in appressoria or mycelium, grown under *SAP*-inducing conditions, of transformants carrying the GFP coding sequence under the control of either the *G. cingulata* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) or *SAP* promoter. This may be due to the relatively strong fluorescence background of the wild type strain or the relatively weak fluorescence of transformants. Expression of *GFP* transcripts was detected by RT-PCR reactions.

The *GFP* gene used in the reporter construct was a modified version, where Ser65 was replaced with a Thr residue (Chiu *et al.*, 1996). This modified GFP protein has increased fluorescence and solubility and decreased photobleaching in fungi compared to the wild type *Aequorea victoria* GFP. It has been successfully expressed at high levels in a number of filamentous fungi (Spellig *et al.*, 1996; Dumas *et al.*, 1999; Robinson and Sharon, 1999; Sexton and Howlett, 2001). Also, the *G. cingulata* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter-*GFP* reporter was successfully expressed in a plant pathogen *Venturia inaequalis* (Fitzgerald *et al.*, 2003). The integration of the constructs in *G. cingulata* was established by PCR. Although routine RT-PCR does not provide quantitative data, *GFP* expression appeared to be at a reasonable level compared with the control (the *G. cingulata sod* gene). Therefore, the relatively weak fluorescence may have resulted from positional effects, or post-transcriptional effects, i.e. poor translation or inappropriate folding of the protein.

Some important discoveries during the transformation experiments for this phase of the work led to a refined transformation protocol which benefited subsequent work in this

project. Firstly, conidia were found to be a better source than mycelium for protoplast preparation. Contrary to the general idea that the conidial cell wall is hard to digest, digestion of conidia with the cell wall degradation enzyme Glucanex was more efficient than was degradation of the mycelial wall. In addition, protoplasts generated from conidia were uniform, clean and easily purified; all of which may result in more efficient transformation. Protoplasts prepared from mycelium contained a lot of cell debri which could not be separated from the protoplasts. This may interfere with the uptake of DNA by viable protoplasts. The number of real protoplasts prepared from mycelium may be much lower than that prepared from conidia because the large amount of cell debri interferes with counting. Secondly, the concentration of hygromycin in the overlay applied to the regeneration plates was found to be critical for successful selection of the transformants. The optimal concentration of hygromycin B was determined to be 200 μg/ml. A lower concentration (100 μg/ml) resulted in growth of too many untransformed colonies, and a higher concentration (300 μg/ml) resulted in no colonies growing through the agar overlay.

5.2 Cloning and characterization of the *G. cingulata StuA* gene

5.2.1 Cloning and sequence analysis of the *G. cingulata StuA* gene

The 5' region of the *G. cingulata StuA* gene was isolated by PCR on the basis of homology to the conserved APSES domain found in the APSES transcription factor family followed by subgenomic library screening. The 3' region was obtained by plasmid rescue from a *G. cingulata StuA* gene disruption mutant. Southern blot analysis revealed that this is a single copy gene. Although the Southern blot results were obtained from a stringent washing condition, this was consistent with the other studies. The total sequence obtained was 7,432 bp comprising 2.7 kb of the promoter region, the 2.1 kb *StuA* predicted open reading frame and 2.7 kb of 3' flanking region.

In the promoter region, there are four potential binding sites for the *A. nidulans* transcription factor BrlAp. These binding sites were also found in the *A. nidulans StuA* promoter and the expression of the *A. nidulans StuA* has been demonstrated to be controlled by this protein (Wu and Miller, 1997). The presence of these sites in the *G.*

cingulata StuA promoter suggests that this gene may be regulated by a similar mechanism. Two potential binding sites for the *A. nidulans* transcription factor AbaAp was also identified, however, they were not present in the *A. nidulans StuA* promoter and the *A. nidulans StuA* was not regulated by the *A. nidulans* AbaAp. The significance of the AbaAp binding sites present in the *G. cingulata StuA* promoter was not known. In *A. nidulans*, AbaAp and BrlAp cooperate with StuAp to regulate the differentiation of asexual reproductive structures (Andrianopoulos and Timberlake, 1994; Miller *et al.*, 1992; Wu and Miller, 1997). However, homologs of AbaAp and BrlAp have not yet been reported in *G. cingulata.*

In *A. nidulans*, *StuA* expression is enhanced by a regulatory loop in which StuAp positively regulates *StuA* expression, particularly during development (Wu and Miller, 1997). However, no StuAp response elements were found in the *A. nidulans StuA* promoter region; this is also true for the *G. cingulata StuA*. But whether that regulatory loop exists for the *G. cingulata StuA* gene is not known.

There is one potential binding site (TGCTAT, at -364) for the *A. nidulans* AreA protein or the *N. crassa* NIT2 protein (Chiang and Marzluf, 1994; Kudla *et al.*, 1990; Peters and Caddick, 1994), which are the major positive-acting nitrogen regulatory proteins that activate gene expression by binding to DNA elements (which have the core sequence GATA) on the target gene promoter. Two (or more) GATA elements located within 30 bp of each other, facing in the same or opposite directions, constitute a strong binding site (Chiang and Marzluf, 1994; Chiang *et al.*, 1994). This suggested that expression of the *G. cingulata StuA* may not be regulated by the *G. cingulata* homolog of these proteins.

No typical cAMP response element (TGACGTCA) was found in the promoter region, but two CGTCA motifs were identified at -320 and -326 on opposite strands. Such a configuration can function cooperatively to interact with cAMP response element binding proteins in response to cAMP stimulation (Fink *et al.*, 1988; Montminy, 1997), suggesting that the expression of this gene may be regulated by cAMP levels. However, the exact role of these elements remains to be determined by further investigation.

The predicted open reading frame was identified by its high degree of homology to other APSES genes in the APSES domain, suggesting a conserved function in this gene family. For the full length polypeptide sequences in this gene family, the level of identity is not high (varying between 14.1% for the *G. cingulata* StuAp compared to the *S. cerevisiae* PHD1p and 59.9% for the *G. cingulata* StuAp compared to the *N. crassa* Asm1p). But for a 113 amino acid long region that contained the conserved APSES domain, the level of identity is much higher, between 64.6% for the *G. cingulata* StuAp compared to the *S. cerevisiae* PHD1p and 98.2% for the *G. cingulata* StuAp compared to the *N. crassa* Asm1p. The highest identity was observed between the *N. crassa* Asm1p and the *G. cingulata* StuAp.

Using the ScanProsite program (http://www.gene-regulation.com/), a number of phosphorylation sites were found for Protein kinase C (241-243, 484-486, 571-573 and 575-577), Tyrosine kinase (273-279), and cAMP- and cGMP-dependent protein kinase (672-675), suggesting that this protein may be subject to phosphorylation by one or more of these protein kinases. This is consistent with the assumption that the *G. cingulata* StuAp is a downstream target of PKA. However, the interaction of the APSES family with these protein kinases has not been reported and *in vivo* the role of these sites remains to be determined.

5.2.2 Generation of deletion mutants for the *G. cingulata StuA*

Gene inactivation has become a routine procedure in analyses of gene function in fungi. A gene deletion vector containing two gene fragments flanking the selection marker is used in most cases, including *Colletotrichum* species. When homologous recombination by double crossover occurs between the vector and fungal genome it will result in the replacement of the target gene with the selection marker (Hwang *et al.*, 1995; Kim *et al.*, 2000; Takano *et al.*, 2000; Veneault-Fourrey *et al.*, 2005). However, transformation of the *G. cingulata* strain used in this study using a deletion vector was reported to occur with low efficiency and the two deletion mutants obtained also had additional copies of vector integrated ectopically (Bowen *et al.*, 1995). On the other hand, transformation using a disruption vector was reported to be highly efficient at producing single copy integrants, which was desirable for subsequent analysis (Bowen *et al.*, 1995). Another

advantage of using a disruption vector was the ease of vector construction (the use of only a small portion of the gene of interest). Disruption of *G. cingulata StuA* was therefore first attempted using this strategy.

A total of forty transformants were obtained in three transformation experiments. Of the twenty-one disruption mutants only two carried a single copy of the vector integrated at the *StuA* locus. Another 19 disruption mutants contained more than one copy of the vector either tandemly integrated in the *StuA* locus or ectopicly integrated into the genome. It is not clear what caused the difference in the integration pattern compared to the results reported by Bowen *et al* (1995).

It was found that all the disruption mutants displayed flat and white mycelium, whereas the ectopic transformants had the same appearance as the wild type strain. This indicated that the changes in mycelial morphology were due to the disruption of the *StuA* gene. When the two single-copy disruption mutants (SC5 and SC40) were further characterized, it was noticed that sectors resembling the wild type in appearance developed on most plates. Subculturing of these sectors and PCR analysis suggested that reversion to the wild type had occurred.

Reversion of these disruption mutants may have occurred because of the instability of a single crossover integration event. This was also observed in previous work and has been suggested to be an indication of the high rate of homologous recombination in this *G. cingulata* strain (Rikkerink *et al.*, 1994).

Deletion of the *G. cingulata* StuA gene was achieved using the deletion vector pSXH. In one transformation experiment, 280 colonies were obtained with 20 μg plasmid DNA. That all of sixty randomly selected colonies looked like those of the disruption mutants SC5 and SC40, suggests a high frequency of double crossover events in these transformants. Southern blot analysis of twenty of these transformants revealed that in all of them the desired deletion had occurred and only one of them carried an additional ectopic insertion event. The rate of homologous recombination with single copy integration was higher than the previous work (2 deletion mutants/5 transformants/7 transformations) as reported by Bowen (1995). However, the strategies used in this

transformation were the same as that used in the previous one. The reason for the different outcome between these two experiments is not clear.

Reintroduction of the *StuA* gene into the deletion mutant was performed to confirm the *G. cingulata StuA* gene function. Two strategies were used. Initially, a 5.3 kb *Sal*I fragment containing the *StuA* ORF plus 1.9 kb of 5' flanking and 1.2 kb of 3' flanking sequence was cloned into pBC-phleo, which harbors a phleomycin resistant cassette, resulted in the complementation vector pBPS53. Protoplasts of the deletion mutant DC43 were transformed with a mixture of circular and linearised pBPS53. Seven transformants were chosen for a Southern blot analysis to determine the vector integration pattern. *StuA* gene function, as assessed by the colony morphology, was not restored in those transformants where the complementation vector (pBPS53) had integrated into the genome by a random insertion event, but was restored in those transformants where the complementation vector (pBPS53) had integrated into the *StuA* locus by a single crossover event. These results suggested that the *StuA* gene could only function when it was reintroduced into its own locus. However, instability of the single crossover integration event was once again a problem. After 7-10 days growth on a PDA plate, wild type like aerial hyphae collapsed, and the mycelium became flat and wettable. It resembled the deletion mutants in appearance, indicating that reversion had occurred.

Proper transcription of the *StuA* gene is essential for restoration of function. The *StuA* promoter is possibly as complex as its homolog in *A. nidulans*. This species requires a 3.5 kb promoter region for properly controlled transcription (Wu and Miller, 1997). Complex promoters with multiple protein binding sites are typical in all eukaryotic organisms, and efficient transcription generally requires the combinatorial and synergistic action of activator proteins that function at long and variable distances from the mRNA initiation site. Perhaps the length of the *G. cingulata StuA* promoter (1.9 kb) that integrated into random sites in the genome was not enough to include all the *cis* elements required for proper *G. cingulata StuA* transcription.

A second restoration vector was designed to replace the hygromycin resistance cassette in the deletion mutant DC43 with the *StuA* gene by means of a double crossover event. The restoration vector contained a phleomycin resistant cassette downstream of the *StuA*

ORF. The proper integration of this vector in the deletion mutant should restore both the *StuA* promoter and ORF in full. Protoplasts of the deletion mutant DC43 were successfully transformed with this vector. The restoration mutants produced showed the restored colony morphology and were stable.

In interpreting the results of these experiments it should be noted that there is a possibility that a partially deleted gene could produce an abnormal *StuA*-related product that gave rise to the phenotypes, rather than loss of the *StuA* gene product giving rise to the phenotypes.

5.2.3 The *G. cingulata StuA* protein controls mycelium morphology, sexual and asexual development

The Stunted protein (StuAp) is an important regulator of both asexual and sexual reproduction in *A. nidulans* (Miller *et al.*, 1992). The *StuA* null mutant formed aerial hyphae that were much shorter than those of the wild-type. The asexual reproductive structure, the conidiophore, lacked the uninuclear cell types, the metulae and phialides. Limited numbers of single, abnormally shaped spores differentiated either from short peg-like structures or directly from buds upon the vesicle (Wu and Miller, 1997). StuAp was also shown to be required for the sexual reproductive cycle. The *StuA* null mutant failed to initiate ascosporogenesis or to develop any of the cell and tissue types characteristic of the *A. nidulans* sexual cycle (Wu and Miller, 1997). StuAp functions by moderating expression of key regulatory genes such as *abaA* and *brlA* by interaction with StuAp response elements (StREs) in their promoter regions (Dutton *et al.*, 1997). Deletion of *Asm-1*, the *N. crassa* homologue of *StuA*, also resulted in stunted hyphae, abnormal conidiation and defects in formation of the protoperithecium, the sexual reproductive structure (Aramayo *et al.*, 1996). In organisms lacking sexual reproduction, *Penicillium marneffei* and *Fusarium oxysporum*, StuAp also regulated asexual reproduction (Borneman *et al.*, 2002; Ohara and Tsuge, 2004).

The *G. cingulata StuA* deletion mutants have fewer and stunted aerial hyphae, resembling the observation made in *A. nidulans StuA* and the *N. crassa Asm-1* null mutants. This resulted in a flat appearance to the mycelial mat on PDA plates. The

asexual structure, the conidiophore, was rarely observed in cultures growing on PDA plates. As a consequence, conidium production was about 10^3 fold less than for the wild type. Another form of the asexual reproduction structure, the acervulus, was never observed on infected apple surfaces. Whereas on apple surfaces infected by the wild type and the complementation mutants, acervuli and numerous clumps of conidia were observed. The sexual reproductive structure (the perithecium) was observed both on aged PDA plates and infected apple surfaces for the wild type and the complementation mutants, but was never seen with the deletion mutants. These observations suggest that the *G. cingulata* StuAp controls both asexual and sexual reproduction, consistent with the role of its homologs in *A. nidulans* and *N. crassa.*

In addition, cultures of the deletion mutant, in either liquid or solid media or on apple tissue, showed a light grey or pink colour, but never had the black colour seen with older wild type cultures. However, the level of melanisation of the deletion mutant appressoria was not distinguishable from that of wild type appressoria and those of the complementation mutants. A similar observation was made for the mutants of another transcription factor Cmr1 in *C. lagenarium* and Pig1p in *M. grisea* (Tsuji *et al.*, 2000). This suggested that the *G. cingulata StuA* controls the expression of the genes involved in melanin synthesis during vegetative growth. This phenotype has not been reported for the deletion mutants of other members of the APSES family.

5.2.4 The *G. cingulata StuA* may control hydrophobin expression

It was noticed that the deletion mutant mycelium was "wettable". This phenotype is characteristic of a mutation in the hydrophobin gene of other filamentous fungi, including *M. grisea* (Talbot *et al.*, 1993), *A. nidulans* (Stringer *et al.*, 1991) and *N. crassa* (Bell-Pedersen *et al.*, 1992). Hydrophobins are a group of small proteins that confer water repellent properties to conidia, hyphae and fruiting bodies. They form amphipathic arrays whose hydrophobic side is exposed to the exterior whilst the hydrophilic surface is bound to the cell wall polysaccharide. This greatly lowers the surface tension of the aqueous environment and allows hyphae to grow into the air (Wosten *et al.*, 1999). Hydrophobins are ubiquitous in filamentous fungi and more than 50 hydrophobins have been isolated to date. However, direct evidence for

transcriptional regulation of hydrophobin genes has been reported only in *N. crassa* (Rerngsamran *et al.*, 2005). The *N. crassa fluffy* gene encodes a Gal4p-type C_6 zinc cluster protein which regulates hydrophobin gene expression and is also required for conidiophore development (Bailey-Shrode and Ebbole, 2004; Bailey and Ebbole, 1998). Neither a hydrophobin gene nor its regulatory gene has been reported in *Glomerella* species to date. From the "wettable" phenotype of the deletion mutant, StuAp seems to be the regulator of hydrophobin gene expression in *G. cingulata.* Isolation and characterisation of hydrophobins in *G. cingulata* would help to confirm this interpretation of the "wettable" phenotype. However, due to the low level of sequence conservation among the fungal hydrophobins, it was not possible to clone the hydrophobin genes from *G. cingulata* based on sequence similarity. An unsuccessful attempt was made to identify the hydrophobin gene using two ESTs from a HortResearch database. This was not pursued further.

5.2.5 The *G. cingulata StuA* is dispensible for conidia adhesion and appressorium formation

Conidium adhesion to plant surfaces was shown to be involved in an initial passive phase of adhesion followed by a second active phase involving secretion of an extracellular matrix (Perfect *et al.*, 1999). The initial passive phase involved hydrophobic interactions between the conidial surfaces and the plant surfaces. In *M. grisea*, it was shown that hydrophobin MPG1 mediates this interaction. The *Mpg1* deletion mutant showed reduced conidium adhesion to a host surface or highly hydrophobic artificial substrates (Beckerman and Ebbole, 1996). Since no difference in the percentage of conidia that adhered to polystyrene Petri dishes was observed between the wild type, the *G. cingulata StuA* deletion mutants and the complementation mutants, the role of *StuA* in regulation of hydrophobin expression might be limited to the mycelial phase. A hydrophobin in the Basidiomycete *Pleurotus ostreatus* has been shown to be exclusively present in the hyphae (Penas *et al.*, 1998; Penas *et al.*, 2002). Alternatively, conidia may contain more than one hydrophobin as in the case of *Aspergillus fumigatus* (Paris *et al.*, 2003) with overlapping functions in mediating conidium adhesion.

Appressorium formation was initially performed on glass slides coated with apple wax (Clark, 1998). During the conidium adhesion assay and appressorium penetration assay, it was noticed that appressorium formation on polystyrene Petri dishes and onion epidermal peels was as efficient as on a glass slide coated with apple wax. Therefore, appressorium formation was tested on all three surfaces. No difference in the percentage of appressorium formation was observed among the wild type, the deletion mutants and the complementation mutants.

Appressorium formation in *Colletotrichum* species is induced by physical and chemical signals from the environment (Section 1.3.2). Some signals are species specific. For example, the surface wax of the host, avocado (*Persea americana*) fruit, induced germination and appressorium formation of *Colletotrichum gloeosporioides* spores. Waxes from non-host plants did not induce appressorium formation in this fungus, and avocado wax did not induce appressorium formation in most *Colletotrichum* species that infect other hosts (Podila *et al.*, 1993). This was the reason that the apple wax coated glass slide was initially used in this assay. However, the observation that appressorium could efficiently form on polystyrene Petri dishes and onion epidermal peel suggested that appressorium formation in *G. cingulata* is induced mainly by surface contact, possibly its hydrophobic property rather than specific plant signals since the surface of a polystyrene Petri dish is chemically inert.

At the molecular level, appressorium formation has been shown to be controlled mainly by the MAPK and cAMP signal transduction pathways in *Colletotrichum* species, *M. grisea* and other fungi. Inactivation of the genes in the major MAPK signal transduction pathway resulted in a defect in appressorium formation in these fungi (Section 1.3.4). However, inactivation of the transcription factors *MST12* (in *M. grisea*) and *CST1* (in *C. lagenarium*), that were presumptively downstream targets of a MAP kinase, has no effect on appressorium formation (Park *et al.*, 2002; Tsuji *et al.*, 2003). This suggested that there must be another transcription factor(s) responsible for appressorium formation. Inactivation of the genes in the cAMP signal transduction pathways also caused reduced appressorium formation in these fungi (Section 1.3.4), however, this seems to be a result of reduced conidium germination.

5.2.6 The *G. cingulata StuA* is required for appressorium penetration

In both *Colletotrichum* species and *M. grisea*, appressorium turgor pressure is the major force that enables the penetration hypha to enter the host tissue (Bechinger *et al.*, 1999; Howard *et al.*, 1991). The generation of appressorium turgor pressure in *M. grisea* is mainly from the accumulation of molar concentrations of glycerol (Jong *et al.*, 1997). Since conidia germinate and form appressoria on the plant surface without external nutrients, glycerol and other solutes within the appressorium must be accumulated *de novo* from storage products present in the conidia. Analysis of conidia has revealed that they contain lipids, glycogen and the disaccharide trehalose as the predominant storage products (Foster *et al.*, 2003; Thines *et al.*, 2000). During appressorium formation, lipid bodies and glycogen granules are mobilized and moved into the appressorium. This mobilisation requires the function of the MAP kinase PMK1 (Foster *et al.*, 2003; Thines *et al.*, 2000). Subsequently, lipid bodies coalesce and are taken up by vacuoles, by a process that resembles autophagocytosis. This was observed both in *M. grisea* and *C. graminicola* (Schadeck *et al.*, 1998; Weber *et al.*, 2001). Lipid degradation occurs in vacuoles. Triacylglycerol lipase activity was induced and is shown to be controlled by the cAMP signal transduction pathway (Thines *et al.*, 2000). In a *cpkA* mutant, which lacks the catalytic subunit of PKA, lipase activity was greatly reduced and lipid bodies failed to coalesce or be degraded during appressorium maturation (Thines *et al.*, 2000). In contrast, appressorial lipid degradation occurred more rapidly in a regulatory subunit PKA mutant (SUM1)(Adachi and Hamer, 1998; Thines *et al.*, 2000). The same observation has been made with the *C. lagenarium rpk1* (encoding the regulatory subunit of PKA) and *cpk1* (encoding the catalytic subunit of PKA) mutants (Yamauchi *et al.*, 2004).

In *G. cingulata*, both lipid bodies and glycogen were abundant in ungerminated conidia and young appressoria (6 hours after induction) in the wild type, the deletion mutants and the complementation mutants. This suggested that the mobilisation of lipid and glycogen was not impaired by deletion of the *StuA* gene. Six hours after the induction of appressorium formation, some appressoria were melanised, and neither lipid bodies nor glycogen were visible in these melanised appressoria. The same was true for appressoria 12 or 24 hours after induction. It is not clear whether lipid bodies and glycogen were

used up during appressorium maturation, or the melanized appressorial wall prevented visualization of the stain. Nevertheless, these results suggested that the *StuA* gene was not required for the mobilisation of glycogen or lipid during appressorium differentiation.

A cytorrhysis assay was carried out to estimate the turgor pressure generated by appressoria. At both 12 and 24 hours after induction of appressoria, more than 60% of appressoria from the deletion mutants collapsed in 2 M glycerol whereas less than 10% of appressoria from the wild type and the complementation mutants collapsed. This suggested that the turgor pressure in appressoria of the deletion mutants was much lower than that in appressoria of the wild type and the complementation mutants. Since the mobilization of lipid bodies and glycogen was normal in the deletion mutants, the lower turgor pressure was possibly due to alterations in the metabolism of these solutes.

Appressorium penetration was tested on onion epidermal peels. Appressoria formed on this surface as efficiently as on the glass slide coated with apple wax and on polystyrene Petri dishes, but this needed a longer incubation time. The low percentage of penetration (5-10% for the wild type and the complementation mutants) may be due to the time point of examination, because a dense hyphal net, which would not be derived only from 5-10% of appressoria, could be seen below the onion epidermal cells when they were examined 72 hours after incubation. However, at this time point, it was not possible to track each hypha to an appressorium and perform a quantitative analysis. On the other hand, appressorium penetration may have occurred at a low rate because onion epidermal peel is not the normal host for *G. cingulata*. However, appressorium penetration was never observed for either of the two deletion mutants even after examination of a few hundred appressoria 96 hours after incubation.

Defects in appressorium penetration have been reported following the inactivation of some genes in the cAMP and MAPK signal transduction pathways, including *CTG-1* (encoding a G-protein □ subunit) and *Ct-PKAC* (encoding a PKA catalytic subunit) in *C. trifolii* (Truesdell *et al.*, 2000; Yang and Dickman, 1999), *CPK1* (encoding a PKA catalytic subunit) and *RPK1* (encoding a PKA regulatory subunit) in *C. lagenarium* (Takano *et al.*, 2001a; Yamauchi *et al.*, 2004), *CPKA* (encoding a PKA catalytic subunit) and *MPS1* (encoding a MAPK) in *M. grisea* (Xu *et al.*, 1997; Xu *et al.*, 1998). It was

demonstrated that the low PKA activity in the *C. lagenarium cpk1* did not affect lipid mobilisation, but led to a defect in lipid degradation in the appressoria. This resulted in low glycerol generation and presumably a consequently low turgor pressure in the appressoria. Although the appressorium turgor pressure was not measured in these mutants, the defect in appressorium penetration was suggested to result from low appressorium turgor pressure (Yamauchi *et al.*, 2004). In the *G. cingulata StuA* mutants, mobilisation of lipid bodies and glycogen to the appressorium was not affected, but the process of their degradation was not observed due to the melanised appressorial wall. However, the appressorium turgor pressure was low and this is possibly the reason that these appressoria failed to penetrate onion epidermal cells or cause lesions on unwounded apples.

Appressorium penetration is a complicated process and involves other proteins besides the components of the signal transduction pathways. *MST12* in *M. grisea* and *CST1* in *C. lagenarium* (both encoding a transcription factor downstream of a MAPK pathway) also control appressorium penetration (Park *et al.*, 2004; Park *et al.*, 2002; Tsuji *et al.*, 2003). It was demonstrated that the defect in appressorium penetration of the *mst12* mutant was due to a defect in microtubule reorganisation (Park *et al.*, 2004). Another small membrane protein, tetraspanin, was also shown to be required for penetration pore positioning and penetration peg formation in both *C. lindemuthianum* and *M. grisea* (Clergeot *et al.*, 2001; Veneault-Fourrey *et al.*, 2005). The exact role of the *G. cingulata StuA* in generation of appressorium turgor pressure and whether it has any other role in appressorium penetration requires further investigation.

5.2.7 The *G. cingulata StuA* is required for pathogenicity

Since the deletion mutants were defective in appressorium penetration, it was not surprising that the deletion mutants were not able to invade unwounded apples, whereas the wild type and the complementation mutants infected unwounded apple easily. This suggested that the *G. cingulata StuA* gene was required for pathogenicity. When wounded apples were inoculated with the deletion mutants, lesions formed and the lesion diameters were similar to those caused by the wild type and the complementation mutants, suggesting that colonisation of apple tissue itself was not impaired. However,

the surface of lesions formed by the deletion mutants was smooth and neither acervuli nor perithecia were formed. These reproductive structures were observed on the surface of apples infected by either the wild type or the complementation mutants. Therefore the defect in formation of reproductive structures occurred not only in culture but also in the host tissue. This would greatly reduce the ability of the deletion mutant to infect neighboring apples.

5.2.8 Gene expression in the *G. cingulata StuA* deletion mutants

Gene expression was studied during vegetative growth, and the early and later stages of appressorium formation. Glyceraldehyde-3-phosphate dehydrogenase (*gpd*), actin, and the □-tubulin gene are classified as housekeeping genes in many fungi and *Colletotrichum* species (Buhr and Dickman, 1993; Mahe *et al.*, 1992; Templeton *et al.*, 1992). They are expressed constitutively in most conditions, and could be a suitable positive control in a northern blot analysis. However, results from the northern blot showed that all of these three genes appeared to be up-regulated during vegetative growth in the deletion mutants. The mechanism of this up-regulation is not clear. Variation in the expression of these housekeeping genes has been reported in other fungi in different growth conditions (Delp *et al.*, 2003; Greene *et al.*, 2003; Jin *et al.*, 1999; Mahe *et al.*, 1992; Puyesky *et al.*, 1997; Rhody *et al.*, 2003; Tarkka *et al.*, 2000), but a general mechanism was not able to be deduced. On the other hand, there is no report of the regulation of these genes' expression by the APSES gene family.

Three genes that are involved in melanin biosynthesis, the *G. cingulata PKS1, SCD1* and *THR1* genes, appeared to be down-regulated during vegetative growth in the deletion mutants, consistent with the white/grey appearance of the colonies. It was a surprise that these three genes also appeared to be down regulated at both early and later stages of appressorium formation in the deletion mutants, because the appressoria were melanised just as in the wild type. It is possible that there is more than one melanin biosynthesis pathway in *G. cingulata.* In *Colletotrichum* species and *M. grisea*, three melanin synthesis genes have been cloned and characterised (Section 1.3.6). A transcription factor, Cmr1p in *C. lagenarium* and Pig1p in *M. grisea*, has been shown to control the expression of these melanin biosynthesis genes in mycelium but not in

appressorium (Tsuji *et al.*, 2000), although direct evidence for binding of these transcription factors to the target promoter was not established. It remains to be elucidated whether the *G. cingulata StuA* directly regulates the expression of these melanin biosynthesis genes or via an interaction with the Cmr1p homolog, or other proteins.

Extracellular enzymes that degrade the plant cuticle and tissue are thought to have an important role in fungal pathogenicity. The *G. cingulata SAP* gene and pectin lyase A (*pnlA*) gene appeared to be up-regulated in the deletion mutants during vegetative growth. This may explain why the lesions caused by the deletion mutants on apples were significantly softer than the lesions caused by the wild type or the complementation mutants.

The *AreA* gene is a transcription factor required for regulation of nitrogen metabolism (Kudla *et al.*, 1990) and pathogenicity (Pellier *et al.*, 2003). The *G. cingulata AreA* gene appeared to be down regulated in the two deletion mutants grown on either PDA medium or a medium containing BSA as the sole nitrogen soure, suggesting that the *G. cingulata StuA* gene controls the expression of the *G. cingulata AreA* gene. For wild type and the complementation mutants, *AreA* also appeared to be down-regulated when mycelium was grown on the medium containing BSA as the sole nitrogen source. However the mechanism for this down-regulation is not clear.

Expression of the *G. cingulata StuA* gene was also studied in mycelium. Expression of the *G. cingulata StuA* gene was fairly constant during vegetative growth either without conidiation (mycelium grown in the dark) or with conidiation (mycelium grown in a light/dark cycle).

It would be interesting to see if the expression of the genes encoding the enzymes involved in degradation of lipid and glycogen was altered. However, sequence information for the *G. cingulata* genes was not available.

5.2.9 Conclusion

From the studies decribed above, *StuAp* appeared to be a global regulator in *G. cingulata*. It regulates formation of asexual and sexual reproductive structures. It controls hyphal hydrophobicity and melanisation of the fungal culture. It may also

regulate degradation of lipids and glycogen for the generation of glycerol in appressoria, thereby generating the turgor pressure, which is required for appressorium penetration and fungal pathogenicity. It would be of interest to measure the enzymatic activity of the proteins that are involved in glycogen and lipid degradation during appressorium development in both *G. cingulata* wild type and the *StuA* deletion mutants. The phenotype of the *StuA* deletion mutants (deficient in appressorium penetration but normal mobilization of lipids and glycogen) was similar to that of PKA mutants, suggesting the *G. cingulata StuA* protein may function in the same pathway as PKA, possibly downstream of PKA as is the case for the homologous transcription factor *EFG1p* in *C. albicans*. In the future, it would also be interesting to identify the upstream proteins in the signal transduction pathway that interacts with the *G. cingulata* StuAp, and the downstream proteins regulated by the *G. cingulata* StuAp and their roles in the phenotypic changes seen in the *G. cingulata StuA* deletion mutant. Production of a soluble recombinant *G. cingulata StuA* protein would be the first step towards achieving this aim. The *G. cingulata StuA* protein could be used in PKA phosphorylation assays to test the interaction between these two proteins. Pull down assays might also be conducted to discover other proteins that interact with the *G. cingulata StuA* protein. DNase I footprinting and electrophoretic mobility shift assays (EMSA) could be used to establish the interactions of the *G. cingulata StuA* protein with the promoters of the genes shown to be regulated by StuAp in this study (three melanin biosynthesis genes, *pnlA* and the *SAP* gene). Furthermore, a subtractive cDNA library has been made from the *G. cingulata* wild type and *StuA* deletion mutant. This could be used to discover other genes which might be downstream targets of the *G. cingulata StuA* by transformation the *StuA* deletion mutant with the library and screening the transformants for complementation of the mutant phenotype.

Appendix

Appendix 1 Media, common solutions and buffers

Appendix 1.1 Growth Media

All media were prepared with MQ water and autoclaved at 121^{0}C for 15 min unless stated otherwise. Solid media were prepared by adding 15 g of agar (Life Technologies) per litre of liquid media before autoclaving.

LB medium contained 0.5% (w/v) NaCl; 1% (w/v) tryptone (Difco); 0.5% (w/v) yeast extract (Difco). The pH was adjusted to 7-7.5.

LB agar contained LB base with 1.5% agarose (BDH).

SOB broth contained 2% tryptone (w/v), 0.5% yeast extract (w/v), 0.05% NaCl (w/v), 2.5 mM KCl, and 10 mM $MgCl_2$.

TB buffer contained 10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl.

PDA (Potato Dextrose Agar) contained 3.9% (w/v) dehydrated potato dextrose agar (Difco). The pH was adjusted to 6.5.

PDB (Potato Dextrose broth) contained 2.4% (w/v) dehydrated potato dextrose broth (Difco). The pH was adjusted to 6.5.

Minimum medium for *G. cingulata* growth contained 10 ml of **10x salts,** 10 ml of 10% $(NH_4)_2SO_4$ (w/v), 8 ml of 50% glucose, 1 ml of **200x Vitamins** and 171 ml of H_2O to a final volume of 200 ml.

SAP induction medium for *G. cingulata* growth contained 10 ml of **10x salts,** 8 ml of 4% BSA (w/v), 8 ml of 50% glucose, 1 ml of **200x Vitamins** and 173 ml of H_2O to a final volume of 200 ml.

Pectin lyase A induction medium for *G. cingulata* growth contained 10 ml of **10x salts,** 10 ml of 10% $(NH_4)_2SO_4$ (w/v), 50 ml of 20% pectin, 1 ml of **200x Vitamins,** and 173 ml of H_2O to a final volume of 200 ml.

Conidium induction medium or *G. cingulata* contained 10 ml of **10x salts,** 8 ml of 4% BSA (w/v), 50 ml of 50% glucose, 1 ml of **200x Vitamins** and 131 ml of H_2O to a final volume of 200 ml.

10x salts for minimum medium contain 1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$, 0.1% (w/v) NaCl, 0.1% (w/v) $CaCl_2$, and 1% (v/v) **1000x Trace Elements**.

1000x Trace Elements (per litre) contained 0.5g $Na_2B_4O_7.10H_2O$; 0.1 g $CuSO_4.5H_2O$; 0.1 g KI anhydrous; 0.2 g $FeCl_3$ anhydrous; 0.4 g $MnSO_4.4H_2O$; 0.2 g $Na_2MoO_4.2H_2O$; 0.4 g $ZnSO_4.7H_2O$.

200x Vitamins (per litre, filter sterilised): 20 mg Biotin; 400 mg Ca pantotherate; 2 mg Folic acid; 2 mg inositol; 400 mg Nicotinic acid; 200 mg p-aminobenzoic acid; 400 mg pyridoxine hydrochloride; 200 mg riboflavin; 400 mg thiamin

Appendix 1.2 Antibiotics and other supplement stocks

Antibiotics	Stock concentration	Final concentrations
Ampicillin	100 mg/ml	100 μg/ml
Hygromycin B	50 mg/ml	200 μg/ml, 100 μg/ml
Phleomycin	10 mg/ml	8 μg/ml

Appendix 1.3 Buffers and solutions for DNA isolation and detection

Alkaline lysis solution I contained 50 mM glucose; 25 mM Tris-HCl and 10 mM Na_2EDTA; pH 8.0.

Alkaline lysis solution II contained 0.2 M NaOH and 1 % (w/v) SDS.

Alkaline lysis solution III contained 29.44% (w/v) potassium acetate and 11.5% (w/v) glacial acetic acid per 100 ml.

Colony digestion buffer contained 0.1 M NaCl, 10mM Tris-HCl, pH8.0, 1mM EDTA, 0.5% TritonX-100, and 1x restriction digestion buffer.

Cell lysis buffer contained 40 mM Tris-acetate; 20 mM sodium acetate; 1 mM EDTA and 1% (w/v) SDS; pH 7.8.

RNaseA (DNase free) (Sigma) was prepared at 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. The solution was heated to 100°C for 15 min to inactivate DNase, cooled to room temperature and stored at -20 °C.

Exonuclease III buffer contained 660 mM Tris-Cl (pH 8.0), 66 mM $MgCl_2$ and 100 mM β-mercaptoethanol.

Nuclease S1 reaction mixture was made by mixing 172 μl of H_2O, 27 μl of 10x Nuclease S1 buffer and 60 units of Nuclease S1.

Nuclease S1 stop mixture contained 0.3 M Tris base and 50 mM EDTA (pH 8.0).

Klenow mixture was made by mixing 20 μl of H_2O, 6 μl of 1 M $MgCl_2$, 3 μl of 0.1 M Tris-Cl (pH 7.6) and 3 units Klenow fragment.

T4 bacteriophage ligase mixture was made by mixing 550 µl of H_2O, 100 µl of 10x bacteriophage T4 ligation buffer, 250 µl of PEG 8000 (30% w/v) and 5 Weiss units of bacteriophage T4 DNA ligase.

TE buffer contained 10 mM Tris-HCl and 1 mM Na_2EDTA, pH 8.0.

1 x TAE electrophoresis buffer contained 20 mM Tris; 10 mM glacial acetic acid and 1 mM Na_2EDTA. The pH was adjusted to 8.2.

SDS loading buffer contained 1% (w/v) sodium dodecyl sulphate (SDS); 0.02% (w/v) bromophenol blue; 20% (w/v) sucrose and 5 mM Na_2EDTA (pH 8.0).

Appendix 1.4 Buffers and solutions for RNA work

DNaseI buffer contained 100 mM sodium acetate and 5 mM $MgSO_4$. The pH was adjusted to 5.0.

DNase (RNase free) contained deoxyribonuclease I at a concentration of 2 mg/ml in 0.15 M NaCl and 50% (v/v) glycerol.

DNase stop mix contained 50 mM EDTA and 1% SDS.

5 x MOPS buffer contained 100 mM MOPS (3-[N-morpholino] propane-sulfonic acid); 15 mM sodium acetate and 5 mM EDTA. The pH was adjusted to 7.0.

RNA gel was prepared by adding 0.8 g agarose in 50 ml DEPC treated MQ, 16 ml 5x MOPS buffer and 14 ml 37% formaldehyde.

RNA denaturation buffer was prepared in a 14.5 µl volume by adding 9 µl of formamide, 4 µl 5x MOPS buffer, 1 µl of 37% formaldehyde and 0.5 µl of EtBr (10mg/ml).

RNA gel tank buffer contained 100 ml of 5 x MOPS buffer, 311 ml of DEPC treated MQ and 89 ml of 37% formaldehyde.

RNA loading buffer contained 50% glycerol; 1 mM EDTA pH 8.0; 0.25% bromophenol blue; and 0.25% xylene cyanol.

DEPC treated MQ was prepared by adding DEPC to H_2O at a final concentration of 0.01%, and the solution was stored at 37°C overnight before autoclaving twice.

Appendix 1.5 Buffers and solutions for Southern/northern blotting, hybridisation and detection

Denaturing solution contained 1.5 M NaCl and 0.5 M NaOH

Neutralising solution contained 1.5 M NaCl and 1 M Tris-HCl, pH7.4.

20 x SSC contained 3 M NaCl and 0.3 M sodium citrate.

2 x SSC contained 0.3 M NaCl and 0.03 M sodium citrate.

DIG Easy Hyb buffer contained 5 x SSC; 50% (v/v) formamide; 0.1% (w/v) sodium-lauroylsarcosine; 0.02% (w/v) SDS; 2% (w/v) blocking reagent (Roche).

Maleic acid buffer contained 100 mM maleic acid and 150 mM NaCl. The pH was adjusted to 7.5.

DIG washing buffer contained 100 mM maleic acid; 150 mM NaCl. The pH was adjusted to 7.5 and then Tween 20 was added to a final concentration of 0.3% (v/v).

Blocking solution contained 1% blocking reagent (Roche) in maleic acid buffer and heated to 60°C with stirring.

Detection solution contained 100 mM Tris-HCl and 100 mM NaCl. The pH was adjusted to 9.5.

Hybridization Solution for Northern Hybridization contained 0.5 M sodium phosphate (pH 7.2), 7% (w/v) SDS and 1 mM EDTA (pH 7.0).

Appendix 1.6 Buffers and solutions for *G. cingulata* protoplast preparation, transformation and characterisation

OM buffer contained 1.2 M of $MgSO_4.7H_2O$; 10 mM Na_2HPO_4 and 100 mM $NaH_2PO_4.2H_2O$ added until pH is 5.8.

ST buffer contained 0.6 M sorbitol and 100 mM Tris-HCl pH 8.0.

STC buffer contained 1 M sorbitol; 50 mM Tris-HCl (pH 8.0) and 50 mM $CaCl_2$.

Cell wall digesting enzyme solution contained Glucanex (NZ chemcolour Industries) in OM buffer in a final concentration of 5 mg/ml. The solution was sterilised by Millipore filtration.

PEG solution contained PEG 4000 dissolved in STC buffer to a final concentration of 40% (w/v).

Regeneration top agar contained 2.4% (w/v) dehydrated potato dextrose brothe, 0.8% agar and 27.38% (w/v) sucrose. The pH was adjusted to 6.5.

Regeneration base agar contained 2.4% (w/v) dehydrated potato dextrose brothe, 1.5% agar and 27.38% (w/v) sucrose. The pH was adjusted to 6.5.

Glycogen staining solution contained 60 mg of KI and 10 mg of I_2 per millimetre of MQ water.

Nile Red solution contained 50 mM Tris/maleate buffer, pH 7.5, with 20 mg/ml polyvinylpyrrolidone and 2.5 μg/ml Nile Red Oxazone (Sigma).

Appendix 2

The *G. cingulata SAP* gene sequence

```
   1  GAATTCGCGCGATATGCAATACAACAAACGAAACACCAGTGACGAAAGAAGCCGTGCTGT
      CTTAAGCGCGCTATACGTTATGTTGTTTGCTTTGTGGTCACTGCTTTCTTCGGCACGACA

  61  GTATGGTGTTGTGTGTGATACTGTGTGTGCCTGACCGACGATGGGATCAGTGAAGAGGAA
      CATACCACAACACACACTATGACACACACGGACTGGCTGCTACCCTAGTCACTTCTCCTT

 121  AGACGAACATGGTCGGACTGACTTCATGACTCTTCGGATACAAGTACCCCGAACGTCCAG
      TCTGCTTGTACCAGCCTGACTGAAGTACTGAGAAGCCTATGTTCATGGGGCTTGCAGGTC

 181  AGCTGGGGCCCTGATTCAAGAGCAATGACCTCAGACCTCGGGTACGGCGGTCGGGATTTG
      TCGACCCCGGGACTAAGTTCTCGTTACTGGAGTCTGGAGCCCATGCCGCCAGCCCTAAAC

 241  GGGAAGAAGCGCAGAGTCTCGTCGAGTTCAGATGACAGCCGCAACCACCACAATGATGAA
      CCCTTCTTCGCGTCTCAGAGCAGCTCAAGTCTACTGTCGGCGTTGGTGGTGTTACTACTT

 301  GAACATCAATAACCCAAAGATGGAAAGTCAACAAACAACACCACCAAACAGGCACGGGCA
      CTTGTAGTTATTGGGTTTCTACCTTTCAGTTGTTTGTTGTGGTGGTTTGTCCGTGCCCGT

 361  ACGACCCAAGGAAGGCCCACTCCGCTTTGCCGCGATGGGCAAACATCCCCAATGGTCTGC
      TGCTGGGTTCCTTCCGGGTGAGGCGAAACGGCGCTACCCGTTTGTAGGGGTTACCAGACG

 421  TCAGCGGTTGTGGGCCGCGATAATCAATCGACCAGGCAATTACTTAACGGCGTCTACGGT
      AGTCGCCAACACCCGGCGCTATTAGTTAGCTGGTCCGTTAATGAATTGCCGCAGATGCCA

 481  ACGTAATTGAATTTGAACCCCAAGGGTCAGGCACCTTACTGCGCCGTGAGGAAGCATTGG
      TGCATTAACTTAAACTTGGGGTTCCCAGTCCGTGGAATGACGCGGCACTCCTTCGTAACC

 541  ATCAGGGGAGACCCGTGTCGGGGGAGCTGAGAGAGCTCCGACGGTGCAATCTTAATTCAA
      TAGTCCCCTCTGGGCACAGCCCCCTCGACTCTCTCGAGGCTGCCACGTTAGAATTAAGTT

 601  TCATGCTTATTCGGGGGGTTTCCGCGCCCAACGGTTCTTATCGCGCGGTCGGCAGAGAGG
      AGTACGAATAAGCCCCCCAAAGGCGCGGGTTGCCAAGAATAGCGCGCCAGCCGTCTCTCC

 661  AGACTGACTGACTCTGACTGAGGACCCGACGAGAGAGTCTCTCGCCACTGTCTAGATTGC
      TCTGACTGACTGAGACTGACTCCTGGGCTGCTCTCTCAGAGAGCGGTGACAGATCTAACG

 721  GACATCCAATGGGACTGGCCTGACTGGACTGGACTGTACTTGTTGGCCTGCACCAGTTTG
      CTGTAGGTTACCCTGACCGGACTGACCTGACCTGACATGAACAACCGGACGTGGTCAAAC

 781  TGTGGCTGGCGCTTGTTCAACTGATGAGCACACGACTAGTACCTTCTTGAGCCTTATCTT
      ACACCGACCGCGAACAAGTTGACTACTCGTGTGCTGATCATGGAAGAACTCGGAATAGAA

 841  AAGAAAACATGGAGACCGGCTGACTCGGCAGTCTTCTCGAAGAAACGCCCAGCACGATTG
      TTCTTTTGTACCTCTGGCCGACTGAGCCGTCAGAAGAGCTTCTTTGCGGGTCGTGCTAAC


 901  AGGAAGCTCAAACAAATCACGATTCACCCCTCATCCGCAAAGTCAGTGAACCGCCGGAGC
      TCCTTCGAGTTTGTTTAGTGCTAAGTGGGGAGTAGGCGTTTCAGTCACTTGGCGGCCTCG

 961  GAAGAGATCAATCGGGAAAGAGAGAAGGTCACGAAGGTTTGTGAAATGTTCCGTCATGGA
      CTTCTCTAGTTAGCCCTTTCTCTCTTCCAGTGCTTCCAAACACTTTACAAGGCAGTACCT

1021  GTCGCGGGCAGAATATCAGCTCGTGCGAGCGATGGCACGACATGGACGAACTTTTGCAAA
      CAGCGCCCGTCTTATAGTCGAGCACGCTCGCTACCGTGCTGTACCTGCTTGAAAACGTTT
```

1081 GGCAACCCAGCCCAGCTTCCCGAATGATGAATCCCTCCTCCTCCAGCCCCAAATAATACG
CCGTTGGGTCGGGTCGAAGGGCTTACTACTTAGGGAGGAGGAGGTCGGGGTTTATTATGC

1141 CGCGAAGAAGGTCCTTGTGTTGAGTTGAATGCGACGGAGGGCAGGGTCCAGGCCGGTGCA
GCGCTTCTTCCAGGAACACAACTCAACTTACGCTGCCTCCCGTCCCAGGTCCGGCCACGT

1201 GCCGCCAGCACGTCATGAGAGCAGGGCAGCTCATGGCCAGACGTATCGCCAAGATGGCAA
CGGCGGTCGTGCAGTACTCTCGTCCCGTCGAGTACCGGTCTGCATAGCGGTTCTACCGTT

1261 CGACCGCCGAACAATTCCGCTTCTTTGGGATCCGGTGTTTGACGCAGAGAAGAGACGACG
GCTGGCGGCTTGTTAAGGCGAAGAAACCCTAGGCCACAAACTGCGTCTCTTCTCTGCTGC

1321 GAGACTTGGGTCCAGGCCCCCAGACATCTCATCAGCGCAGCGCATCGCATGCACGAGGGC
CTCTGAACCCAGGTCCGGGGGTCTGTAGAGTAGTCGCGTCGCGTAGCGTACGTGCTCCCG

1381 GGGCTCTGTCTGTGTCTGTCTGTGACTATGTCTCTCGCATGCAATCCATCCCTTCTCCCA
CCCGAGACAGACACAGACAGACACTGATACAGAGAGCGTACGTTAGGTAGGGAAGAGGGT

1441 CATCGCACGCATCTCGCCGTCTTCCATCACCTGCACCACCACCATCAGCAGAAGCACCCC
GTAGCGTGCGTAGAGCGGCAGAAGGTAGTGGACGTGGTGGTGGTAGTCGTCTTCGTGGGG

1501 AGTCCGCCTTGCCTTCCTTCCCGTCTTGGCCTCGATCTCGTGCCTGGGCGGCCCCAGACT
TCAGGCGGAACGGAAGGAAGGGCAGAACCGGAGCTAGAGCACGGACCCGCCGGGGTCTGA

1561 TGGCGGAGGGCTGGCGGAGGGTTGGAGTTGCAATTCCAAGTCTTTGCTCGCATACGGGAC
ACCGCCTCCCGACCGCCTCCCAACCTCAACGTTAAGGTTCAGAAACGAGCGTATGCCCTG

1621 TGTGGGTGGGGACTTGGCGGTAAAGCTTGTTTCCATGTGCCGAGGCGAATTGGCAAAGCG
ACACCCACCCCTGAACCGCCATTTCGAACAAAGGTACACGGCTCCGCTTAACCGTTTCGC

1681 AAGTGAACGGGGGATCTGCCATGCCAGTCTGCAGCTGTTGTGTTCAGTTATTGGCGATTA
TTCACTTGCCCCCTAGACGGTACGGTCAGACGTCGACAACACAAGTCAATAACCGCTAAT

1741 ACAATGGGAAACATGGTTGGGCCTGGTTCCAGCTCGGAGCCCCATGTGCGGTTTTGGGCT
TGTTACCCTTTGTACCAACCCGGACCAAGGTCGAGCCTCGGGGTACACGCCAAAACCCGA

1801 GGCGACTCCCGGAGCCATGTCCCGAGTAGGTAGGTGCTTTTATTTTCCTTCTTCTACGTA
CCGCTGAGGGCCTCGGTACAGGGCTCATCCATCCACGAAAATAAAAGGAAGAAGATGCAT

1861F
1861 CTGCGTATCCGTACGAGTACGTGTGAGCGAGCACTCAGACTTGTCTGTTCGACTCGAACA
GACGCATAGGCATGCTCATGCACACTCGCTCGTGAGTCTGAACAGACAAGCTGAGCTTGT

L2801
1921 CCGGTCCGGGGCTGTCGCGAGATGCGCTGGGAACATGGCAGAGACGATCAAGGATCTATC
GGCCAGGCCCCGACAGCGCTCTACGCGACCCTTGTACCGTCTCTGCTAGTTCCTAGATAG

2019F
1981 TGTCGAAGAGAGGAAAAGGGGTCCCGTCCGGCGTTGGCATTGGCTGGTTGCGAGATGCAT
ACAGCTTCTCTCCTTTTCCCCAGGGCAGGCCGCAACCGTAACCGACCAACGCTCTACGTA

2041 GGCTGAGTGAATCCATTCGTCTCCCCCGCCCTCGGCCCCATCATCAGCTGCTCCAGTCGT
CCGACTCACTTAGGTAAGCAGAGGGGGCGGGAGCCGGGGTAGTAGTCGACGAGGTCAGCA

2101 GCGCTGTGCTTTTTTTTTCATCTCATCTCCCGCCTTGGACGAACGGAGAGCCGCGGTTTG
CGCGACACGAAAAAAAAAGTAGAGTAGAGGGCGGAACCTGCTTGCCTCTCGGCGCCAAAC

2161 GTCTGGCTGGGGAATTCGACCTGCCTCTTTTTCATCTAGTTTCCCGCTGCTGGGGACTTG
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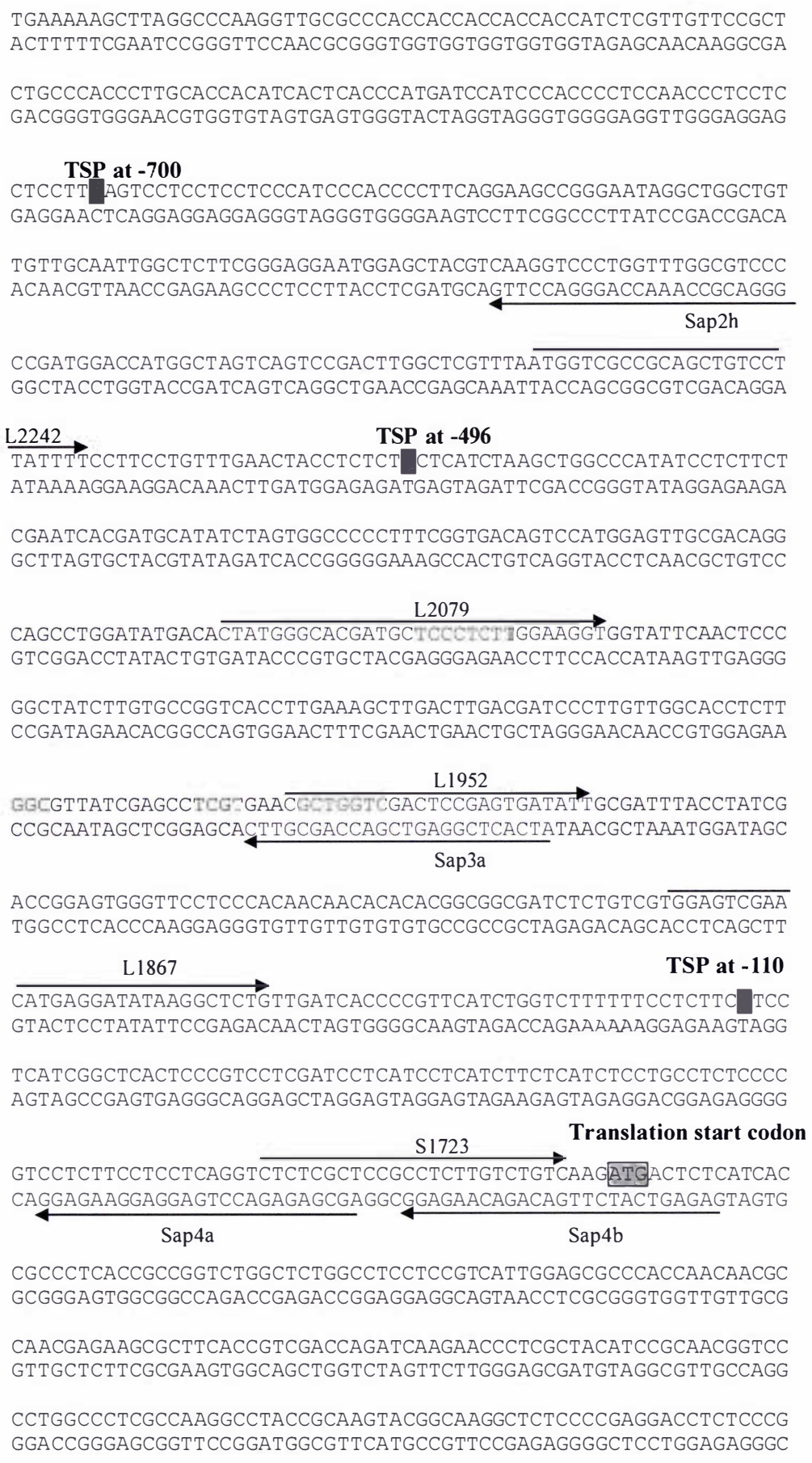
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Sap2h
L2242
TSP at -496
L2079
L1952
Sap3a
L1867
TSP at -110
Translation start codon
S1723
Sap4a
Sap4b

3241 CGTCGTCGCCAACATCACCTCCACCGGTGCCACCAAGCGCGCTACCGGCAGCGTCGCCGC
GCAGCAGCGGTTGTAGTGGAGGTGGCCACGGTGGTTCGCGCGATGGCCGTCGCAGCGGCG

3301 CACCCCCCAGGACTACGAYGTCGAGTACCTGAGCCCCGTCCAGATCGGCACCCCGGCCCA
GTGGGGGGTCCTGATGCTRCAGCTCATGGACTCGGGGCAGGTCTAGCCGTGGGGCCGGGT

3361 GACCCTCACCCTCGACTTCGACACCGGATCTTCCGATCTCTGGGTCTTCTCCACCTCCAC
CTGGGAGTGGGAGCTGAAGCTGTGGCCTAGAAGGCTAGAGACCCAGAAGAGGTGGAGGTG

3421 CCCGTCCAGCCAGCGCAACGGCCAGACCGTCTACGACCCCAGCAAGTCCAGCACCGCCTC
GGGCAGGTCGGTCGCGTTGCCGGTCTGGCAGATGCTGGGGTCGTTCAGGTCGTGGCGGAG

3481 CCGCCTCACCGGCGCCACCTGGTCCATCTCCTACGGCGACGGCTCCAGCTCCAGCGGTAT
GGCGGAGTGGCCGCGGTGGACCAGGTAGAGGATGCCGCTGCCGAGGTCGAGGTCGCCATA

3541 CGTCTACAAGGACACCGTCTCCGTCGGCRGCCTGTCCGTCACCGGCCAGGCTGTCGAGGC
GCAGATGTTCCTGTGGCAGAGGCAGCCGYCGGACAGGCAGTGGCCGGTCCGACAGCTCCG

3601 CGCCTCCAAGGTTTCCTCTTCCTTCTCCGAGGAGAGCGACCTGGACGGTCTCCTGGGTCT
GCGGAGGTTCCAAAGGAGAAGGAAGAGGCTCCTCTCGCTGGACCTGCCAGAGGACCCAGA

3661 CGGCTTCAGCTCCATCAACACCGTCTCCCCCACCCAGCAGAAGACCTTCTTCGAGACTGC
GCCGAAGTCGAGGTAGTTGTGGCAGAGGGGGTGGGTCGTCTTCTGGAAGAAGCTCTGACG

3721 CAAGTCCAAGTTGGATGCTTACCTCTTCACTGCTGACTTGAAACACAACACCCgtaagta
GTTCAGGTTCAACCTACGAATGGAGAAGTGACGACTGAACTTTGTGTTGTGGGcattcat

3781 tttcacttcccgtctcatatcacactcgcgagtgagacagtaaacatatactgacatcca
aaagtgaagggcagagtatagtgtgagcgctcactctgtcatttgtatatgactgtaggt

3841 caacagCCGGCAAGTACAACTTCGGCTACATCGACTCCTCTGCCTACACCGGCGCCATCA
gttgtcGGCCGTTCATGTTGAAGCCGATGTAGCTGAGGAGACGGATGTGGCCGCGGTAGT

C847

3901 CCTACGTCAGCATCGACAACTCCGACGGCTGGTGGCAGTTCACCTCCTCCGGCTACTCTG
GGATGCAGTCGTAGCTGTTGAGGCTGCCGACCACCGTCAAGTGGAGGAGGCCGATGAGAC

3961 TCGGCTCCGCCAGCTTCACCTCCACCTCCCTCAACGGCATCGCTGACACCGGAACCACCC
AGCCGAGGCGGTCGAAGTGGAGGTGGAGGGAGTTGCCGTAGCGACTGTGGCCTTGGTGGG

4021 TCCTCCTCCTCCCCCAGTCTGTCGTCACTGCCTACTACGCCAAGATCTCCGGCGCCAAGT
AGGAGGAGGAGGGGGTCAGACAGCAGTGACGGATGATGCGGTTCTAGAGGCCGCGGTTCA

4081 ACGACTCTTCCCAGGGAGGCTACACCTTCCCCTGCTCCGCCACCGTCCCCTCCTTCACCT
TGCTGAGAAGGGTCCCTCCGATGTGGAAGGGGACGAGGCGGTGGCAGGGGAGGAAGTGGA

4141 TCGGCGTCGGCTCCGCCCGCGTCACCATCCCCGCCAGCTACATGAACTACGCTCCCGTCA
AGCCGCAGCCGAGGCGGGCGCAGTGGTAGGGGCGGTCGATGTACTTGATGCGAGGGCAGT

4201 GCACCAGCACTTGCTTCGGTGGCCTGCAGAGCTCCTCCGGCATTGGCATCAACATCTTCG
CGTGGTCGTGAACGAAGCCACCGGACGTCTCGAGGAGGCCGTAACCGTAGTTGTAGAAGC

4261 GTGATGTCGCCCTCAAGGCTGCCTTCGTTGTCTTCGATGGTGCCTCCAACCGCCTTGGCT
CACTACAGCGGGAGTTCCGACGGAAGCAACAGAAGCTACCACGGAGGTTGGCGGAACCGA

4321 GGGCCGCCAAGACCCTCTCTTAAACACTCTTATAGAGACACAGAGAGAGARAGAACTTAG
CCCGGCGGTTCTGGGAGAGAATTTGTGAGAATATCTCTGTGTCTCTCTCTYTCTTGAATC

4381 AGAGGGATYTGCCCATTAGAGAGATGGCCTGAGGGATTTCGGAATGAGGACCTTGAGGAC
TCTCCCTARACGGGTAATCTCTCTACCGGACTCCCTAAAGCCTTACTCCTGGAACTCCTG

```
4441    GGAAGATYGAGGGCGCATCTGATGGGCAACTCCCCTGTTTATGGACAATCCATGTGATGA
        CCTTCTARCTCCCGCGTAGACTACCCGTTGAGGGGACAAATACCTGTTAGGTACACTACT

4501    GACGATCGAGTTTCACCAATCACTGCTTCTTATACATATTTCATATATCTTAGTATATTT
        CTGCTAGCTCAAAGTGGTTAGTGACGAAGAATATGTATAAAGTATATAGAATCATATAAA

4561    TGGTGGATTATGAGAGATAATGAGATCAAGATAAGTCTTTGAGATATAATGACTCAGAAT
        ACCACCTAATACTCTCTATTACTCTAGTTCTATTCAGAAACTCTATATTACTGAGTCTTA

4621    ATAAAAATCACGTTGAAGAAGTTTAATGTGCGTTTTCCGTGGTGACTTTTTTCCGTGCGG
        TATTTTTAGTGCAACTTCTTCAAATTACACGCAAAAGGCACCACTGAAAAAAGGCACGCC

4681    CACATTTATTCAAGTCTTTTTCCGAATTGCCAAGACGCCAGACTTAAGTTGCACTCACTC
        GTGTAAATAAGTTCAGAAAAAGGCTTAACGGTTCTGCGGTCTGAATTCAACGTGAGTGAG

4741    ACACCTCTTCCCGTCGCGCCGAATTC
        TGTGGAGAAGGGCAGCGCGGCTTAAG
```

Appendix 3

Oligonucleotide primers used in this study

Names	Sequences (5'-3')	Applications
5' RACE adapter	GCTGATGGCGATGAATGAACACTGCGTTTGCTG GCTTTGATGAAA	RACE
5RLM-RACE-Outer	GCTGATGGCGATGAATGAACACTG	RACE
5RLM-RACE-Inner	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGA TG	RACE
Oligo dT-anchor primer	GCGAGCACAGAATTAATACGACTCACTATAGGT $_{12\text{-}18}$VN	RACE
3' RACE Outer Primer	GCGAGCACAGAATTAATACGACT	RACE
3' RACE Inner Primer	CGCGGATCCGAATTAATACGACTCACTATAGG	RACE
C847	GTTGTCGATGCTGACGTAGGTGATGGC	PCR
S1723	CTCTCGCTCCGCCTCTTGTCTGTCA	PCR
L1867	GGAGTCGAACATGAGGATATAAGGCTCTG	PCR
L1952	CGCTGGTCGACTCCGAGTGATATTGCGATT	PCR
L2079	CTATGGGCACGATGCTCCCTCTTGGAAGGT	PCR
L2242	ATGGTCGCCGCAGCTGTCCTTATTT	PCR
L2801	GAGATGCGCTGGGAACATGGCAGAGACG	PCR
Sap1861F	TACGTACTGCGTATCCGTACG	PCR
Sap2019F	ATTGGCTGGTTGCGAGATGC	PCR
Sap 4a	AGCGAGAGACCTGAGGAGGAAGAGG	PCR
Sap 4b	GAGAGTCATCTTGACAGACAAGAGG	PCR
Sap 2h	GGGACGCCAAACCAGGGACCTTG	PCR
Sap 3a	ATCACTCGGAGTCGACCAGCGTTC	PCR
Sap 1a	CCTCTCTTCGACAGATAGATCC	PCR
Sod757F	CAAGGTCACCGGCTCCATCG	PCR
Sod1240R	CAAGCGGGACGGGGACCAG	PCR
T3 primer	AATTAACCCTCACTAAAGGG	PCR
T7 primer	GTAATACGACTCACTATAGGGC	PCR
Hyg308.26F	AGCGGATTCCTCAGTCTCG	PCR
Hyg1045.26R	AGGTCGCCAACATCTTCTTC	PCR
F 2019	ATTGGCTGGTTGCGAGATGC	PCR
GFP3132F	CAGCGGAGAGGGTGAAGGTGAT	PCR
GFP1	GAT GGT TCT CTC CTG CAC ATAG	PCR
DF1	GCCACDTYRTGGGARGAYGAG	PCR
DF2	CTVAAYGTYGCYGGHATG	PCR
DR1	TGVACRAAMARKGGGWARAG	PCR
DR2	TSKRRTTKGCDGGGTGGTA	PCR
iv_up	GCTTGGTGCCATTGATCATGTG	PCR
iv-down	CTGCCGAGTTCCAGCTAACG	PCR
1-24F	AGA TCT ATG AAT CAA CCC GCT GCC GAC	PCR
580up	AGATCTCTCAACTTACCAGACACCCTTC	PCR
1752R	GCG AAG TCG AGT GCC CTC TC	PCR
pAN662R	TCT ATG CTC CAA GCT AGA GTC	PCR

831R	CTC TCT CGC TGC TCA TGT ACC	PCR
420F	CCA CGA CCG TCC TGA TCT G	PCR
1054F	GTCGGTGCATACGATAGCTAC	PCR
PstT7-3rd-753	GCTGCTGGGCCATCAC	sequencing
XS20-T3R335	ACGGCAGGAGCAGAGTAG	sequencing
Pstl3T3-2nd	5-AAAGACTCGGCTGTTATTC-3	sequencing
PST13T7-2nd	5-CAATGTGGCTGTGAACTGG-3	sequencing
HE42T7-2nd658	ACCGGTGCTCGTCCCAGAT	sequencing
Sacl7T7-2nd614	CGCTCGACGCCTGGAAAGACA	sequencing
SMA20T3-2nd	AGGACGGTGACGGTAGGAAG	sequencing
SMA20T7-2nd	GGCTGCCACCATTACCAC	sequencing
PST13T3-3rd626	AAGTGTGCATTTTGTAGG	sequencing
P7399-240R	CTGCTATCTGGCTTCTGGAC	sequencing
SMA20T3-3rd	CAGAGTAAATACAAGAGATTGGG	sequencing
SMA20T3-4th	GCTTAGGTCATGTCCAAGGG	sequencing
He42T73rd	CATCTGAGTCGGAGTAGC	sequencing
xho43	GAG AGT CGA ATC CGA ATC CTC	sequencing
Hygl	GAA GTA CTC GCC GAT AGT GG	PCR
HYG3080F	GTGGTGGTGGTGTCACTTC	PCR
Xho63-160	GCA TGC CTA GTA TGG TGT GTC	PCR
act474F493	GACCGAGGCCCCCCATCAACC	PCR
act977R955	CCGCCGGACTCAAGACCAAG	PCR
Tub2-648F	CACGGCCTCGACAGCAATGG	PCR
Tub2-1210R	GGGAGGGAACGACGGAGAAG	PCR
gpd47F	CATCAAGGTCGGCATCAAC	PCR
gpd583R	GTGTAGGAGTGGACGGTGGT	PCR
pks2537R	GGAACCNGTNCCRTGCAT	PCR
pks558F	CCG CCA GCT CAA CGA ACG ATA	PCR
scd237F	CGT CCC CTG CTG GCA ACA TCAC	PCR
scd880R	ACG TCG GTC TTC TGG TCC TC	PCR
thr450F	GAT GCC TGG CGT TAC TTC TC	PCR
thrl234R	CGT TGA CGG TGA TCT TCT TGT	PCR
Pnl593F	TCACCGGTACCGAGGGAACTA	PCR
Pnl1379R	ACGGCCGGAGGTGTGGTGGAT	PCR

Appendix 4

Characterisation of the phenotypic changes for the complementation mutants

A qualitative analysis of the phenotypic changes for the wild type, the deletion mutants and the complementary mutants was carried out by examining the growth rate, conidia production, aerial hyphae and wetability as described in Section 4.5.1. The results were shown on the table below.

Strains	Growth rate	Conidia production	Aerial hyphae	Wetability
WT	+++	+++	+++	-
R532-4	+++	+	+	+
R532-6	+++	+	+	+
R532-8	+++	+	+	+
R532-2	+++	+++	+++	-
R532-9	+++	+++	+++	-
R532-10	+++	+++	+++	-
R532-11	+++	+++	+++	-

Appendix 5

The gene sequences used for northern probes

PCR products were sequenced from one strand using one of primers used in the amplification.

1. The *G. cingulata PKS1* gene sequence

```
   1   ATCGTACATGCAGCCCTCGCTTTGCATCACCCACCTGGCTCASTACATTGACCTCRCTGA
  61   GAAGGAGCCTCWAGAAGTTTGCCTTCRAGACCTGGATTCTTCTTGGGTCTCTGCWTGGGG
 121   STGTTTGCCGCCACTGCTATCGCCTCGACCCCCTTGKCCTCTACCTTGATACCCCTTGCC
 181   GTTCAGGTAGTCCTCATGGCCTTCARAACTGGCACTCTCCTCSGGTCCCTGGGTGAAAGA
 241   CTCMGCCCGGCSGTCGGCCGATCTGAACCTTGGACCCATATCCTTCCTGGCCTGAAGGAA
 301   CCCTGGTCCAAAGAGACTCTCTTTAATTTCCATGAATCTAATTACATCCCCGCCACACAG
 361   CCAAGCATACGTTAGCGCTGGGTCCGSMTCTGGTCTAGCRAGCTCCGGACCGGCWCCRAC
 421   TCTGAACGCCCTGGATGACCAGAACGTCTTCGGCGTCAAGACCACGGSMATCCACGTCTA
 481   CGGACATTACCATGGTATCTCACCTCCWGGGGCRCTGCCGATTTCRAGAAGATTCTTCGAC
 541   TTGACGACCCCRARGGCACCGAGGTCTTGGAGAAGAGCARACCCCGCTCTGCCATTATGT
 601   CTGGCACCAATGGTATGTGGCTCGCTGASACCMGYMCMMASTCTCTGCTGCATGCTGTMG
 661   STCWGGARTGCCTTGTCGACAKACTTGWTKACCARACTGGCATTGACGGRTGCRTCGAKA
 721   CTGCCCSTGACTTTGAGGGATCAACRTGCCTCGTTATCCCCTTCGGCCCTACTATCAWCG
 781   YCRAKACCCTCCGGAAGACGATCAAGGACCGCGACTCTTTGACRTTMTCGTCCGTCCTGS
 841   TGAMCTCTCCAGAKTGAGAGCTACTGCTCCAAKATCGGAAACCACSGTCCATMCCGGCAR
 901   TGCMAACTCKSCAYTGKCCGTATGGMGCGTCGCTWCCCMKAYKSTGCCWGCCSCTCTAMC
 961   CTTTGGGASTSCTGGCCGGGGGCCGGWAGTTCCMAGAGTCGTTCCTGCCTAGCKCTTCCT
1021   CKKCRCCSCTMCTACGAWATCRCTGGGACGATGTCRACAMCGAGTCMMTCTYAATACCGT
1081   ATGMTGCATTGACAGYCYCGATACCTYTGACCMKGACTYTTCGAATKTMTGCCGTGAGCA
1141   TGTCATACTGATCYCATGCATGCATCGGAGATKASGAGSKSTTCAGCATCGGAAATGYCT
1201   GTWTGCTMCCTTACCGTACTACCCTGACTACCTGATATCCYATAGTACGTTCACTGTTCG
1261   AACTCTCCCAAGMAAAATRGACCCTCTA
```

2. The *G. cingulata SCD1* gene sequence

```
   1   CRGKCRGGYGWCWSAYMRACSTACGAGAAAMTCGACGATAGTCTGCAGACTACCTCGGCT
  61   TGACGGCCTGCCTCTTCGAGTGGGCCGACTCCTACGACTCCAAGGACTGGGACCGTCTCC
 121   GCAAGTGCATCGCCCCAGAGCTCCGCGTACGTCCCCCCTCCACCCATCTCCCCCAATCCA
 181   TCACTACCTACCCAACTCCCGCCCTCGCGCCCCCCCTGCGCCCCTCCCACAACCCCAACC
 241   CCGTCTCCTCGCTCCTCAGCCGCCCGGTGGAGGGCGGCGACGATGACGACGCCCAAACCC
 301   ATCATCAAACTCACATCCCGCACCAGATCGACTACCGCTCCTTCCTCGACAAGATCTGGG
 361   AGGCCATGCCGGCCGAGGAGTTCATCGCCATGATCAGCGACAAGTCGGTCCTCGGCAACC
 421   CGCTGCTCAAGACGCAGCACTTCATCGGCGGCACGCGCTGGGAGAAGGTGTCGGACACGG
 481   AGGTCATCGGGCACCACCAGCTGCGCGTGCCGCACCAGAAGTACACGGACGCGAGCCGCA
 541   AGACGGTCGCCGTCAAGGGCCACGCGCACAGCTACAACATGCACTGGTACAAGAAGGTCA
 601   ACGGCGTGTGGAAGTTTGCCGGGCTCAACCCTGAGATCCGCTGGTCCGAGTACGACTTCG
 661   AGGCCGTGTTTGCCGATGGCCGCGACTCGTTTGGCGAGGACCAGAGAAMCCGACGW
```

3. The *G. cingulata THR1* gene sequence

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  1   TTCAAAYGCAAGTCCGGCTCCGACGCCGCTTCCATCAAGGCCAACGTCTCCGACGTCGAC
 61   CAGATCGTCAAGATGTTCGCCGAGGCCAAGAAGATCTTCGGCAAGCTCGACGTCGTCTGC
121   TCCAACTCCGGTGTCGTCTCTTTCGGCCACGTCAAGGACGTCACCCCCGAGGAGTTCGAC
181   CGCGTCTTCAGCATCAACACCCGTGGCCAGTTCTTCGTCGCCCGCGAGGCCTACAAGAAC
241   CTCGAGGTCGGTGGCCGCCTCATCCTGATGGGCTCCATCACTGGCCAGGCCAAGGGTGTC
301   CCCAAGCACGCCGTCTACTCCGGATCCAAGGGTACCATCGAGACCTTCGTCCGCTGCATG
361   GCCATCGACTTCGGTGACAAGAAGATCACCGTCAACGA
```

4. The *G. cingulata* Actin gene sequence

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  1   ATTGAKMKMKYRTRSMKCRSCCAGATCGTTTTCGAGACCTTCACGCCCCCGCGTTCTACG
 61   TCTCTATCCAGGCCGTYCTGTCTCTGTACGCCTCCGGTCGTACCACCGGTATCGTCCTCG
121   ACTCCGGTGACGGTGTCACCCACGTTGTCCCCATCTACGAGGGTTTCGCTCTTCCCCAGG
181   CCATTGCCCGTGTCGACATGGCTGGTCGTGACTTGACCGACTACCTCATGAAGATTCTCG
241   CTGAGCGCGGTTACTCTTTCTCCACCACTGCCGAGCGTGAAATCGTTCGTGACATCAAGG
301   AGAAGCTCTGCTACGTCGCCCTTGACTTCGAGCAGGAGCTCCAGACCGCTTCCCAGAGCT
361   CCAGCTTGGAGAAGTCCTACGAGCTTCCTGACGGTCAGGTCATCACCATTGGCAACGAGC
421   GTTTCCGTGCTCCTGAGGCTCTGTTCSCTYCTTTCCGTCCTTGGTCTTRGTCCCGSSGG
```

5. The *G. cingulata* β-tubulin gene sequence

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  1   GGCGTATCTTGGAGATCAGGAGGGTACCCATACCAGCACCGGTACCACCGCCGAGGGAGT
 61   GGGTGATCTGGAAACCCTGGAGGCAGTCGCAGCCCTCAGCCTCGCGGCGGACAACATCGA
121   GAACCTGGTCGACGAGCTCGGCACCCTCGGTGTAGTGACCCTTGGCCCAGTTGTTGCCGG
181   CACCAGACTGGCCGAAGACGAAGTTGTCGGGGCGGAAGAGCTGGCCGAAAGGACCGGCAC
241   GGACGGCGTCCATGGTACCGGGCTCCAAATCGACGAGGACGGSRCGGGGCACGTACTTGT
301   TGCCGGAAGCCTGGGTAGGAGCGAAGGTCAGTACTCGTCAATATGTTTATCTTGCACTCT
361   GGGGGCTATAAGGTAACAAACTTCGTTGAAGTAGACGCTCATGCGCTCGAGCTGGAGCTC
421   AGAGGTGCCGTTGTAGCTGTTCGAGTTTAGCGGTCAACCACCAATGTGGCCAGATAAGGG
481   GCATGACATACACTCCATTGCTGCGAAGGCCGTGA
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