Comparative Analysis of the Maize Smut Fungi *Ustilago maydis* and *Sporisorium reilianum*

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Erklärung

Ich versichere, dass ich meine Dissertation mit dem Titel ”Comparative analysis of the maize smut fungi *Ustilago maydis and Sporisorium reilianum*“ selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Ort, Datum	Bernadette Heinze

In memory of my fathers Jerry Goodman and Christian Heinze.

"Every day I remind myself that my inner and outer life are based on the labors of other men, living and dead, and that I must exert myself in order to give in the same measure as I have received and am still receiving. "

Albert Einstein (1879 - 1955)

Zusammenfassung

Die Maispflanze (*Zea mays*) ist Wirt für zwei engverwandte phytopathoge Pilze, *Ustilago maydis* und *Sporisorium reilianum*. Eine Infektion mit *U. maydis* führt zu mit Sporen gefüllten Gallen oder Tumoren an allen oberirdischen Teilen der Pflanze. *S. reilianum* infiziert junge Keimlinge, wächst systemisch und bildet Sporenlager in den Infloresenzen. Ziel dieser Arbeit ist die Identifizierung der Faktoren, welche die unterschiedlichen Infektionsspezifika zwischen beiden Pilzen erklären. Da *S. reilianum* dimorphisch ist, war es wichtig, die Paarungstyploci, die für die morphologischen Veränderungen grundlegend sind, zu identifizieren. *S. reilianum* hat wie *U. maydis* ein tetrapolares Paarungssystem. Interessanterweise zeigte eine molekulare Charakterisierung der Paarungstyploci, dass *S. reilianum* drei unterschiedliche *a*-loci bebesitzt, *a1*, *a2* und *a3*. Jedes der *a*-loci kodiert für ein Rezeptor Gen und zwei Pheromon Gene. Eine funktionale Analyse ergab, dass ein Rezeptor nur durch ein Pheromon stimuliert werden kann, das auf einem anderen *a* Allel kodiert wird. Es wurden fünf *b*-Paarungstyploci von *S. reilianum* identifiziert und charakterisiert. Diese enthalten zwei divergent transkribierte offene Leserahmen, die für Untereinheiten eines Transkriptionsfaktors kodieren.

In einem weiteren Versuch wurden anhand der amplifizierten Fragmentlängen Polymorphismen von cDNA (cDNA-AFLP) die Gen expressionsprofile von *U. maydis* und *S. reilianum* infizierten Maispflanzen verglichen. Dabei wurde ein Gen aus *U. maydis*, das für eine Nitrilase kodiert, als differentiell exprimiert identifiziert. Da eine Nitrilase den letzten Schritt der Auxin biosynthese katalysieren könnte, wurden *U. maydis* Deletionsmutanten, denen neben der Nitrilase auch die Acetaldehyd dehydrogenasen *Iad1* und *Iad2* fehlen, auf ihre Fähigkeit zur Auxinproduktion und Tumorbildung untersucht. *In vitro* Auxinmessungen zeigten eine drastische Reduktion der Auxinproduktion der dreifach Deletionsmutanten. Pflanzeninfektionsexperimente zeigten, dass die Fähigkeit zur Tumorinduktion der Dreifachmutanten nicht beeinträchtigt war. Die Fähigkeit von *U. maydis* Auxin zu produzieren, scheint nicht mit seiner Fähigkeit Tumore zu induzieren verbunden zu sein.

Summary

The maize plant (*Zea mays*) is host to two closely related phytopathogenic fungi *Ustilago maydis* and *Sporisorium reilianum*. *U. maydis* infects all aerial parts of the plant, rapidly forming galls or tumours filled with spores. *S. reilianum* infects young seedlings, remains asymptomatic and grows systemically until it replaces the inflorescence with a mass of sooty spores. The identification of the factors responsible for the difference in infection specificity was the main aim of this work. As *S. reilianum* is known to be dimorphic it was important to characterise the mating type loci that are fundamental for the morphological changes. *S. reilianum*, like *U. maydis*, has a tetrapolar mating system. Molecular characterisation revealed that *S. reilianum* is an exceptional smut fungus that has three *a* loci: these were termed *a1*, *a2*, and *a3*. The *a* loci were found to each contain one receptor gene but two pheromone genes. Functional analysis proved that the native receptor does not recognise the two native pheromones. Instead it can only be stimulated by one pheromone, which is carried by each of the alternate *a* alleles. *S. reilianum*, similar to *U. maydis*, has multiple *b* mating type loci of which five were isolated and characterised. The *b* loci of *S. reilianum* contain two divergently transcribed open reading frames that each contains a homeobox motif. This is as is found in the *b* loci of *U. maydis,* which encode proteins that function as a transcription factor.

Microscopic comparison of the early stages of infection caused by *U. maydis* and *S. reilianum* in maize demonstrated that the two fungi differ remarkably during the colonisation of the host tissue. *U. maydis*' proliferative capacity is initiated as early as three dasy post infection (dpi) while *S. reilianum* shows only sustained hyphal growth at the same time point. Thus, the gene expression profiles produced by *U. maydis* and by *S. reilianum* infected maize using amplified fragment length polymorphism on cDNA (cDNA-AFLP) were compared. Interestingly, a nitrilase gene was identified as being differentially expressed in *U. maydis* tissue three dpi. As a nitrilase may be involved in the final stages of auxin biosynthesis, the auxin generating capability of the *U. maydis* triple deletion strains missing the nitrilase and the two indole acetaldehyde dehydrogenase genes *iad1* and *iad2* was investigated. Although auxin production was reduced in the mutants, plant infection assays revealed that tumour formation was unaffected. Consequently, the ability of *U. maydis* to produce auxin may not be directly related with its ability to produce tumours.

Abbreviations

AFLP	Amplified fragment length polymorphism
AM	Ammonium minimal medium
Amp	Ampicillin
Ara	Arabinose
bp	Base pair(s)
C-terminal	carboxyterminal
Cbx	Carboxin
cDNA	complementary DNA
CLP	Chromosomal length polymorphism
CM	Complete medium
cpm	Counts per minute
DAPI	4',6'-Diamidino-2-Phenylindol
DIC	Differential interference contrast
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
dpi	Days post infection
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EST	Expressed sequence tag
GFP	Green fluorescent protein
Glc	Glucose
$H_20_{bid.}$	Bi-distilled water
HMG	High mobility group
Hyg	Hygromycin
kb	Kilo base pair
MAPK	Mitogen activated protein kinase"
MAPKK	MAPK-Kinase
MAPKKK	MAPKK-Kinase
MOPS	3-(N-Morpholino)propane-sulphonate
N-terminal	aminoterminal
Nat	Nourseothricin
NLS	Nuclear localisation sequence
NM	Nitrate minimal medium
OD_{600}	Optical Density at 600 nm
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PC	Phenol/Chloroform
PCR	Polymerase chain reaction
PD	Potato dextrose
PEG	Polyethylene glycol
Phleo	Phleomycin
PIPES	Piperazine N-N'-bis (2-Ethane-sulphonate)
PKA	Protein kinase A
PRE	Pheromone response element
QRT-PCR	Quantitative real time RT-PCR
RACE	Rapid amplification of cDNA ends
RT	Reverse transcription or Room temperature
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SGFP	Synthetic green fluorescent protein
TAE	Tris-Acetate + Na_2-EDTA
TBE	Tris-Borate + Na_2-EDTA
TE	Tris-Cl + Na_2-EDTA
TEMED	N,N,N',N'-Tetramethylen-diamine
Tris	Trishydroxymethylamino-methane
U	Unit (Enzyme activity)
UARS	*U. maydis* autonomously replicating sequence
UAS	Upstream activating sequence
UTR	Untranslated region
UV	Ultraviolet light
wt	Wildtype

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General Introduction

The world population, consisting of over six billion people, derives most of its caloric intake from three plants: maize, rice and wheat. Approximately 650 million hectares of agricultural land is under the cultivation of cereal plants, with maize being the highest yielding (in terms of tonnage) followed by rice and wheat (Anon., 2006). Therefore, the economic importance of cereal crops cannot be over-emphasised especially when yields are reduced due to disease. Cereal plants are plagued by a plethora of diseases caused by several organisms such as insects, nematodes, fungi, bacteria and viruses. Estimates of annual financial losses caused by fungal diseases are difficult to calculate as they affect both pre- and post-harvest yields, but they range from 8 to 25% (Weber, 1993; Börmer, 1997). The most important group of fungal pathogens that attack cereal plants are known as the smuts.

1.1 The Smut Fungi

The term smut fungi is used to describe a group of more than 1000 species of fungal pathogens that infect flowering plants resulting in characteristic symptoms, namely, the replacement of plant organs by black masses of teliospores resembling soot or smut. These Basidiomycete fungi belong to the order Ustilaginales, which contains over 50 genera that are capable of infecting more than 4000 plant species. Most notable among these plant species is the grass family (Gramineae), to which the cereal crops belong (Deacon, 2005). All cereals are attacked by smut fungi but each fungus has a very narrow host range. One species seldom infects more than three different host plants (Bauer *et al*., 2001). The most distinct general feature among smut fungi is that they live in an intimate balance with their host plants until the plant flowers; thereafter usually the inflorescence is replaced by a mass of teliospores thereby rendering the crops unusable. These fungi are therefore biotrophic pathogens as they are dependent on their host plants to complete their life cycle but do not kill them. Several different smut diseases in cereal crops are known and these are mostly caused by species of the genus *Ustilago*, but also by other genera such as *Tilletia* and *Sporisorium*. The most prevalent smut diseases of cereals are briefly described below.

Common smut is found in maize (corn) and is caused by *Ustilago maydis*. The disease is characterised by the presence of tumours of varying sizes on any or all parts of the plant (leaves, stalks, ears, kernels, husks and tassels) (Fig. 1A). Tumours are covered by a thick, white membrane while the inside contains a mass of powdery black spores. Interestingly, certain regions in Mexico cultivate maize infected with common smut as it is considered a delicacy known as 'huitlacoche' or maize truffle.

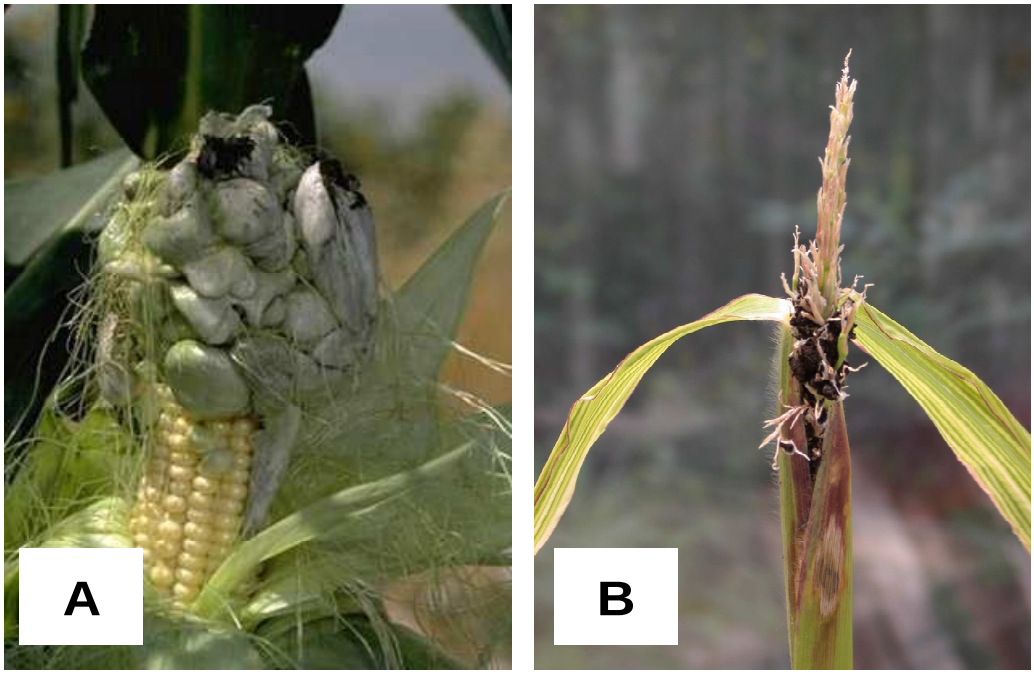

Figure 1. Symptoms of the distinct smut diseases of maize characterised by the presence of black teliospores in the inflorescence. **A**: common smut of maize characterized by the large galls (tumours) filled with teliospores. **B**: head smut of maize characterized by compact spores.
Pictures courtesy of Hannaford (Chemtura Australia) (A) and J. Schirawski (B).

Covered smut of barley and oats is caused by *Ustilago hordei* and *Ustilago kolleri (hordei),* respectively. The infection is visible in the crop as a greyish-black head instead of normal grain.

Head smut caused by *Sporisorium reilianum* is found in both maize and sorghum. Infection by this soil-borne fungus causes the inflorescence partly or completely to develop into smut sori (compact mass of black spores) (Fig. 1B).

False loose smut of barley is caused by *Ustilago nigra* and infection with this fungus causes the formation of smutted grain heads containing loosely packed spore masses.

Kernel smut of rice is caused by *Tilletia barclayana* and *Tilletia horrida*. Infected rice kernels are easily recognised by their black colour as these are filled with the spores.

Barley, oats and wheat are all susceptible to loose smut disease caused by *Ustilago tritici*, *Ustilago avenae*, and *Ustilago nuda* respectively. Infected plants appear normal until heading time; thereafter the disease is easily recognised by the characteristic dusty black appearance of diseased heads instead of normal flowering heads.

Stinking smut (common bunt) is a distinctive disease that affects both wheat and triticale and is caused by *Tilletia caries* and *Tilletia foetida*. The kernels of infected plants are replaced with smut balls filled with dark spores. When these balls rupture, the loose black powdery spores have a distinctive fishy odour. Dwarf bunt of wheat caused by the soil-borne *Tilletia controversa* has the same symptomatology as common bunt described above but in addition causes severe stunting of plants.

The whip smut disease of sugarcane is caused by *Sporisorium scitamineum*. Infection is characterised by the development of a curled, whip-like, spore covered rachis at the top of the shoot or stalk instead of the normal flower (Agrios, 2005).

An integral part of the life cycle of smut fungi is a dimorphic transition: the saprotrophic haploid phase is yeast-like (termed sporidia) and the parasitic dikaryotic phase is mycelial. The transition between these phases involves mating. Mating between haploid sporidia only occurs if two strains with different mating type interact. This process is termed heterothallism. A unifactorial mating system with two alleles, *Mat*1 and *Mat*2 is more common in the Ustilaginales than is the bifactorial system consisting of two unlinked mating loci (*a* and *b*). A bifactorial mating type system is present in *Ustilago maydis*. As a result, a germinating teliospore of *U. maydis* can produce sporidia of four different mating types and is therefore termed tetrapolar (Carlile *et al*., 2001).

1.2 The Smut Fungus *U. maydis*

U. maydis (DeCandole) Corda, the causal agent of common smut of maize, has been under intensive investigation over the past two decades and has thus etched its status as a model organism among phytopathogenic fungi. Several factors have raised it to the status of model organism namely, infection of seedlings results in tumour formation in approximately seven days, therefore allowing rapid assessment of disease symptoms; the relatively short period required (three weeks in a controlled environment) to complete its sexual life cycle; the plethora of molecular techniques available for genetic manipulation to analyse gene function and finally, the availability of the complete genome sequence for rapid identification of unique genes that may

be associated with pathogenicity (Kahmann *et al*., 2000; Banuett, 2002; Kämper *et al*., 2006).

Life Cycle

The dimorphic life cycle of this fungus has been well characterised (Snetselaar & Mims, 1992; Snetselaar, 1993) and is schematically represented in Fig. 2. Under favourable conditions the diploid teliospores that are produced in tumour tissue germinate, undergo meiosis and produce haploid sporidia of four different mating types. Such a tetrapolar mating system is uncommon amongst the *Ustilago spp*. Strains of opposite mating type sense each other and grow towards each other, forming long tubes (so called conjugation tubes) at one end of the cell. When these cell tips meet, they fuse and form a dikaryotic filament (Snetselaar & Mims, 1992).

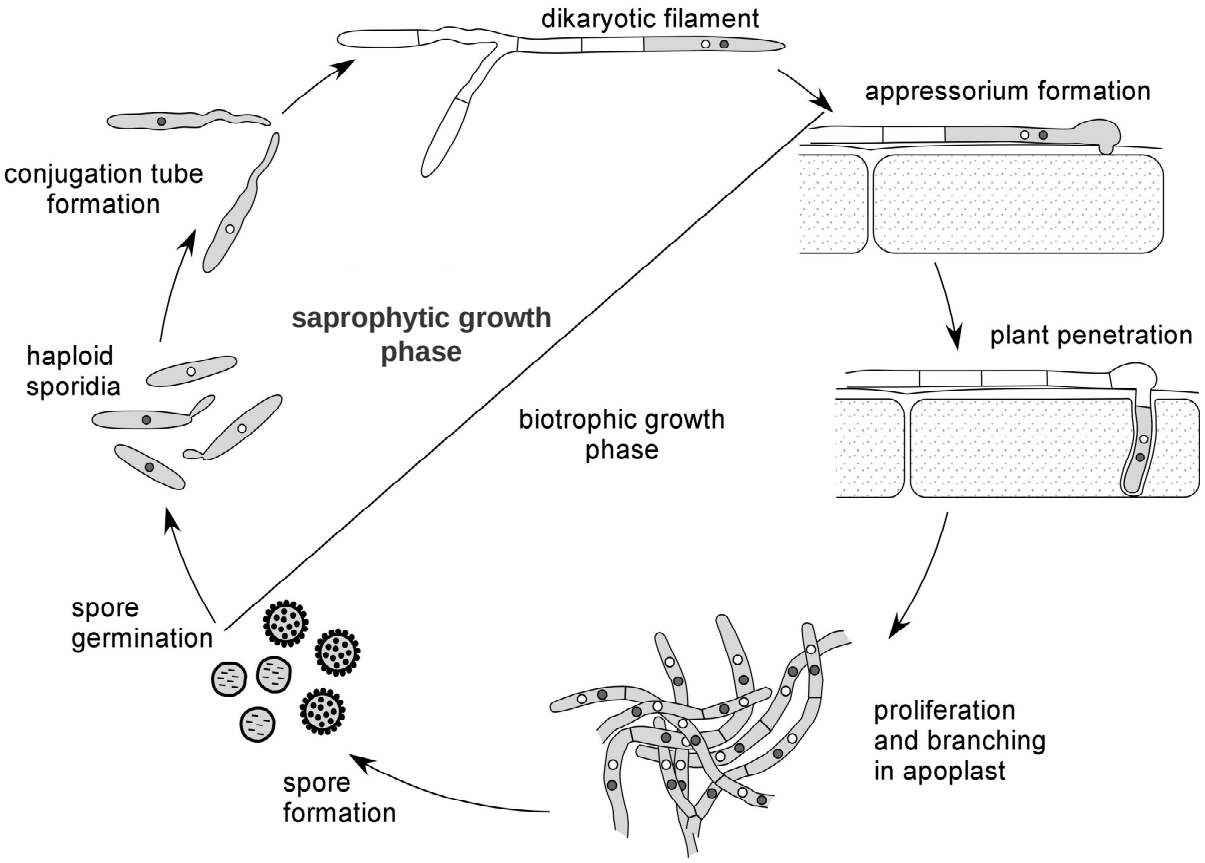

Figure 2. Diagrammatic representation of the dimorphic life cycle of *Ustilago maydis*. Teliospores germinate and undergo meiosis to produce haploid sporidia of four different mating types (different coloured nuclei). Cells of opposite mating type grow towards each other and fuse at their tips forming a dikaryotic filament. This dikaryon requires certain plant signals to continue growth and initiates infection by penetrating the plant surface through the formation of an infection structure known as an appressorium. Once inside the plant, the fungus grows as dikaryotic mycelia inter- and intracellularly by branching. After a few days the fungus induces tumour formation and the mycelia contract their cytoplasm, fragment and undergo karyogamy to produce teliospores, which mature. The mature teliospores are released into the environment when the tumours rupture. Under favourable conditions the cycle is then repeated when the teliospores germinate once more. Illustration courtesy of J. Schirawski, altered.

These morphological transitions are controlled by the *a* and *b* mating genes. Initial sensing of cells of opposite mating type and formation of conjugation tubes is controlled by the bi-allelic *a* mating genes that encode a pheromone / pheromone receptor system (Bölker *et al*., 1992). Maintenance of the dikaryotic filament and subsequent plant penetration are controlled by the *b*-mating genes, which encode homeodomain proteins that are active as a heterodimer and function as a transcription factor (Schulz *et al*., 1990; Kronstad and Leong, 1990; Gillissen *et al*., 1992; Schlesinger *et al*., 1997).

For the fungus to enter the biotrophic phase, compatible haploid cells need to fuse to form a filamentous dikaryon. Entry into the plant is achieved by the formation of appressorium-like structures that are formed at the hyphal tip (Snetselaar and Mims, 1992). The invading hyphae gain entry into the plant by direct penetration or through natural openings such as stomata and floral organs (Snetselaar and Mims, 1992). Once plant penetration is achieved the fungus grows apparently unimpeded by the plant as no obvious defence reactions are initiated (Snetselaar and Mims, 1993) even though the expression level of several defence related genes is dramatically increased (Döhlemann et al., 2008). Although the invading hyphae rupture the host cell walls, the host plasma membrane remains intact around the intracellular growing hyphae (Snetselaar & Mims, 1992). However, after several days of fungal infection limited necrosis and anthocyanin formation at the site of penetration are observed (Banuett and Herskowitz, 1996). The fungus' mode of growth two days post infection (dpi) entails rapid, unbranched hyphal tip growth, in which older compartments become devoid of cytoplasm, are sealed off and collapse as the tip cell passes through several host cells (Snetselaar and Mims, 1992; Banuett and Herskowitz, 1996). Growth at this stage is mostly intracellular. Between 3 and 4 dpi hyphae start branching and are filled with cytoplasm. Approximately 5 dpi hyphal branching is profuse and occurs at frequent intervals. Growth is mostly intercellular and the formation of clamp-like structures is observed, which has not been observed in axenic culture (Snetselaar & Mims, 1992; 1993; 1994; Banuett & Herskowitz, 1994). The formation of tumours is induced that consist of hypertrophied plant tissue and fungal hyphae (Snetselaar and Mims, 1994). This massive proliferation of plant tissue is associated with the presence of elevated levels of the phytohormone auxin (indole-3-acetic acid) (Klee and Estelle, 1991). In tumour tissue, 5 – 20 fold higher levels of indole-3-acetic acid (IAA) have been measured as compared to healthy tissue (Moulton, 1942; Wolf, 1952; Turian and Hamilton, 1960). Concomitantly, *U. maydis* has been shown to produce IAA in axenic culture in the presence of tryptophan

(Wolf, 1952). It is therefore possible that the IAA produced by *U. maydis* is responsible for the induction of tumour formation. Other phytohormones associated with cell elongation such as the cytokinins and gibberellins have also been shown to be produced by *U. maydis* (Mills and van Staden, 1978; Sokolovskaya and Kuznetsov, 1984) and may therefore also be able to induce tumour formation. However, the biosynthetic pathways for these hormones have not yet been described in *U. maydis* (Kahmann *et al*., 2001). About 7 dpi hyphae proliferate in the intercellular space or within host cells of the plant tumour (Snetselaar and Mims, 1992; Banuett and Herskowitz, 1996). The onset of spore formation in the tumour material is evident and is characterised by the presence of uni-nucleated hyphae, indicating that nuclear fusion has taken place (Snetselaar and Mims, 1994). Maturation of the teliospores within the tumour material takes place through the fragmentation of the sporogenous hyphae followed by the development of a thick pigmented cell wall (Banuett and Herskowitz, 1996; Fisher and Holten, 1957). The mature teliospores are then released in the environment when the tumour ruptures and the spores are spread by wind and rain (Christensen, 1963).

The a-mating type locus of U. maydis and the mating response

Cellular recognition between haploid cells is mediated by the gene products of the bi-allelic *a*-locus through a pheromone and cognate receptor system. Sequence lengths of the *a1* and *a2* loci are 4.5 kb and 8 kb, respectively, and the loci share little sequence homology (Froeliger and Leong, 1991; Bölker *et al*., 1992). The pheromone precursors are encoded by the mating factor *a1* (*mfa1*) and *a2* (*mfa2*) genes and pheromone receptors by *pra1* and *pra2* genes (Fig. 2A). Pheromones from one mating type are recognised by the cognitive receptor of the opposite mating type hence, pheromone from an *a1* strain (*mfa1*) is recognised by the receptor of an *a2* strain (*pra2*) and vice versa. The *a1* pheromone binds to the receptor on *a2* cells and activates the formation of conjugation tubes. Likewise *a2* pheromone activates a similar response in *a1* cells (Bölker *et al*., 1992). To test the functionality of the *a1* pheromone or *a1* receptor, each gene is separately transformed into a strain, in which the entire *a*-locus has been deleted. The resultant mutants are then co-cultured with a compatible wild-type strain and assayed for conjugation tube formation. No conjugation tubes are formed when the mutant containing the entire *a*-locus deletion is grown with a compatible wild type strain. In the case of the mutant containing only the pheromone gene (*i.e*. no receptor present), only the wild type cells respond by conjugation tube formation. While only the mutant cells form

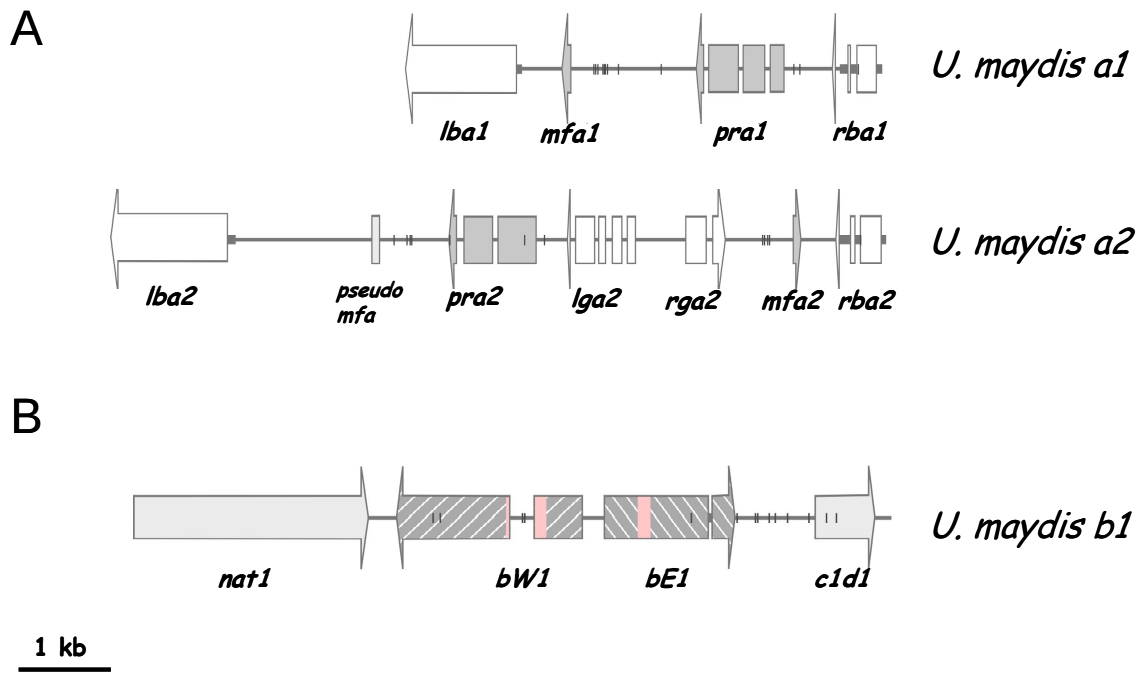

Figure 2. Schematic diagram of the organization of the *a*- and *b*-mating type loci of *U. maydis*. **A:** The *a*-locus is bi-allelic and contains genes for pheromones (*mfa1* and *mfa2*) and pheromone receptors (*pra1* and *pra2*). The *a2* allele has additional genes *lga2* and *rga2*, which are involved in mitochondrial inheritance and a pseudo pheromone gene. Both alleles are flanked by identical sequences designated *lba* and *rba.* **B:** The multi-allelic *b*-locus contains two divergently transcribed genes termed *bE* and *bW*. These genes encode proteins that function as a heterodimeric transcription factor when the gene products are derived from different alleles. Each b-locus is flanked by *nat1,* which encodes a putative N-terminal acetyl transferase and *c1d1,* which encodes a putative nuclear regulator. Short perpendicular lines indicate PREs (pheromone response elements).

conjugation tubes in the case of a mixture of a compatible wild type strain and cells containing only the receptor gene (*i.e*. no pheromone present). Active pheromones are only 13 and nine amino acids respectively, and are post-translationally modified at their C-terminal cysteine by farnesylation and carboxyl methyl esterification. Receptor genes are 200 and 220 amino acids for *a1* and *a2* strains respectively and belong to the family of seven trans-membrane class that are coupled to heterotrimeric G proteins (Bölker *et al*., 1992; Spellig *et al*., 1994; Urban *et al*., 1996). The *a2*-locus contains two additional genes, *lga2* and *rga2* (Fig. 2A), which have a function in uniparental mitochondrial DNA (mtDNA) inheritance during sexual development (Bortfeld *et al*., 2004; Felder *et al*., 2009). A pseudo-pheromone gene is also present in the *a2* locus. Identical flanking sequences are found to the left and right of each *a* allele. These are designated *lba* for left border of *a* and *rba* for right border of *a* (Fig. 2A) (Bölker *et al.*, 1992). Gene products of the *a*-locus in addition to *b*-locus gene products are required for the induction of filamentous growth during the mating process (Spellig *et al*., 1994).

When pheromone perception takes place, the *a*- and *b*-mating type genes are transcriptionally activated by the action of a transcriptional activator termed Prf1 (pheromone response factor 1) (Hartmann *et al*., 1996). The regulatory regions of the *a*- and *b*-mating type genes contain pheromone response elements (PREs) that are recognized by the DNA-binding domain of the HMG box type (high mobility group) present in *prf1* (Urban *et al*., 1996). The activity of Prf1 is regulated transcriptionally in response to different carbon sources and cyclic AMP (cAMP) levels as well as post-transcriptionally in response to cAMP and pheromone levels (Hartmann *et al*., 1996; 1999). Transcriptional regulation of *prf1* involves auto-regulation via two PREs within its own promoter. Post-transcriptional regulation of Prf1 is achieved by phosphorylation through a conserved cAMP dependent protein kinase A (PKA) signaling pathway (Gold *et al*., 1994; Regenfelder *et al*., 1997; Dürrenberger *et al*., 1998) and by mitogen activated protein kinase (MAPK) cascade signaling (Banuett and Herskowitz, 1994; Mayorga and Gold, 1999; Müller *et al*., 1999; Andrews *et al*., 2000; Müller *et al*., 2003). Prf1 has six MAPK phosphorylation sites and five PKA sites. While PKA phosphorylation sites are essential for induced expression of both *a*- and *b*-mating type genes, the MAPK phosphorylation sites are required for *b* gene expression only. Thus one transcription factor is capable of integrating signals from two different pathways and initiates different transcriptional responses by an alteration in its phosphorylation status (Kaffarnik *et al*., 2003). Pheromone-regulated target genes respond differentially to MAPK phosphorylation of transcription factor Prf1 (Zarnack *et al*., 2008).

The b-mating type locus of U. maydis

The *b*-locus in *U. maydis* is multi-allelic containing at least 25 alternate sequences of two divergently transcribed genes termed *bEast* (*bE*) and *bWest* (*bW*) (Fig. 2B). Their predicted polypeptides comprise 473 and 644 amino acids respectively, with an intergenic spacer region of 200 base pairs (Gillissen *et al*., 1992). Both genes have a similar organization with a homeodomain motif in the C-terminal region that is conserved among the different alleles, while the N-terminal region exhibits the most allelic variability. With the exception of the homeodomain motif, *bE* and *bW* are not related by amino acid sequence. The polypeptides encoded by *bE* and *bW* function as a heterodimeric transcription factor that is active only when either gene is derived from a different allele (Kämper *et al*., 1995). Hence, to form a functional heterodimer, the gene product of *bE* from strain *b1* has to dimerise with the gene product of *bW* from strain *b2* or any other allelic variant to be functional (Schulz *et al*., 1990;

Gillissen *et al*., 1992; Kämper *et al*., 1995). Homeodomain motifs present in both *bE* and *bW* have been shown to be responsible for the functionality of the heterodimer as a transcriptional regulator as deletions or substitutions in this motif rendered the protein inactive (Schlesinger *et al*., 1997). The variable domain present in *bE* and *bW* allows the discrimination of self and non-self and therefore maintains the non-infectious state in haploid cells (Bölker *et al*., 1995; Kämper *et al*., 1995). The *bE* / *bW* transcription factor has been determined to be the central factor in triggering pathogenic development through a regulatory cascade that affects a large number of genes involved in the regulation of the cell cycle, mitosis and DNA replication (Feldbrügge *et al*., 2004). These genes are grouped into two classes: class one genes are regulated directly through the binding to a conserved DNA motif termed *b*-binding sequence (bbs) in the upstream region of *b*-responsive genes; class two genes are indirectly regulated by a *b*-dependent signal cascade (Romeis *et al*., 2000; Brachmann *et al*., 2001). Of the class one genes, *lga2* was the first direct target to be identified (Romeis *et al.*, 2000). This gene is present only in the *a2* mating type locus and has been shown to be involved in mitochondrial inheritance (Bortfeld *et al*., 2004). Another gene identified is *clp1* (Scherer *et al*., 2006), related to the *C. cinerea clp1,* which is responsible for clamp formation and subsequent nuclear distribution (Inada et al., 2001). Clamp formation in *U. maydis* has not been observed in axenic culture, although clamp-like structures have been observed in *U. maydis*-infected plant tissue (Snetselaar & Mims, 1994). However, the nuclear distribution associated with clamp formation is impaired in strains lacking *clp1* thereby rendering them nonpathogenic (Scherer *et al*., 2006). Several class two genes have been identified and these include *dik1* and *dik6* that encode hypothetical proteins of unknown function (Bohlmann *et al*, 1994); *egl1* that encodes an endoglucanase (Schauwecker *et al*., 1995); *rep1* that encodes a repellent (Wösten *et al*., 1996; Teertstra *et al*., 2009); *hum2* that encodes a hydrophobin (Bohlmann, 1996); *kpp6* that encodes a MAP kinase (Brachmann *et al*., 2003) and *biz1* that encodes a zinc finger protein (Flor-Parra *et al*., 2006). All the above-mentioned genes with the exception of *kpp6* and *biz1* have no effect on pathogenic development when individually deleted (Grandel *et al*., 2000). However, *kpp6* is required for plant penetration after appressoria are formed (Brachmann *et al*., 2003) and *biz1* mutants show a reduction in appressorium development and arrested growth after plant penetration (Flor-Parra *et al*., 2006).

Since the *bE* / *bW* heterodimer is considered the master regulator of pathogenic development, manipulation of the *b*-locus would facilitate the construction of a

solopathogenic strain. Such strains contain compatible alleles of the *b*-locus and are thus able to cause infection in the plant without the need for a mating partner (Bölker *et al*., 1995). Bakkeren and Kronstad (1996) revealed that the *a*-locus controls compatibility between species of smut fungi and that the process of dikaryon establishment and maintenance controlled by the *b*-locus is conserved between species, but other genetic factors that control virulence and host specificity are not. A solopathogenic strain can thus be used in various ways to investigate the factors involved in symptom development.

1.3 Mating type loci in other basidiomycete fungi

The basidiomycete fungi are mostly heterothallic, that is they require a mate of a different sex or mating type to enter the sexual phase. The mating type genes ensure that only genetically different nuclei will fuse and undergo meiosis prior to the formation of sexual spores. In addition to tetrapolar mating (two or more specificities at each of two unlinked mating type loci), heterothallic basidiomycete fungi exhibit two other mating patterns, a bipolar system (two alternate specificities at a single *MAT* locus) and a system with a single *MAT* locus with multiple alternative specificities (Kronstad and Staben, 1997). In the following, the bipolar mating system of the smut fungus *U. hordei* and the tetrapolar mating system found in the two mushroom species *Schizophyllum commune* and *Coprinopsis cinerea* are described.

In *U. hordei* mating is controlled by a single genetic locus (*MAT*). The *MAT* locus consists of two alleles designated *MAT-1* and *MAT-2*. The *MAT* locus is located on the largest chromosome of *U. hordei* (Lee *et al.*, 1999) and is unusually large, 526 kb for *MAT-1* and approximately 430 kb for *MAT-2* (Bakkeren & Kronstad, 1994; Lee *et al.*, 1999; Bakkeren *et al.*, 2006). Each *MAT* allele has genes equivalent to those of both the *a*- and *b*-loci of *U. maydis* (Bakkeren & Kronstad, 1993; Bakkeren *et al.*, 1993). Sequence analysis of the *U. hordei a*-locus showed the presence of one pheromone gene (*Uhmfa*) and one pheromone receptor gene (*Uhpra*) specific for each mating type and these are involved in cell – cell recognition (Bakkeren & Kronstad, 1996; Sherwood *et al.*, 1998; Anderson *et al.*, 1999). Interspecies hybrids of *Ustilago hordei* containing the *a*-locus of *U. maydis* revealed that these genes are also responsible for intercompatibility among different *Ustilago* species (Bakkeren and Kronstad, 1996). The *U. hordei bE* and *bW* genes from *MAT-1* and *MAT-2* are similar to their counterparts in *U. maydis* and also control filamentous growth and pathogenicity (Bakkeren & Kronstad, 1993; Bakkeren *et al.*, 1993). Linkage of the *a*-

and *b*-loci within the *MAT* locus in *U. hordei* and the separation of the loci on different chromosomes in *U. maydis* is the reason for the difference between bipolar and tetrapolar mating systems for these fungi (Bakkeren *et al.*, 2006; 2008).

The tetrapolar species *C. cinerea* and *S. commune* each have two unlinked mating type factors designated *A* and *B*. The *A* mating type factors in these fungi are equivalent to the *b* mating type genes in *U. maydis* and the *B* factors are equivalent to the *a* mating type genes. Each of the *A* and *B* factors have subloci designated α and β. These two loci are functionally redundant since alleles at either α or β need to vary between different strains for a compatible interaction. In *C. cinerea* there are an estimated 160 specificities at *A* and 79 at *B* but the actual numbers of the α and β alleles are unknown (Raper, 1966). In *S. commune* the *A* factor has 288 specificities (9 for α and 32 for β) and *B* has 81 specificities (9 for α and 9 for β) (Casselton and Kües, 1994). The *A*-regulated events include nuclear pairing, hook cell formation, conjugate division of the nuclei in the tip cell and hook septation. The *B* factors regulate nuclear migration and fusion of the hook cell with the sub apical cell. The mating type proteins of the *A* / *b* loci fall into two distinct subgroups on the basis of the homeodomain sequence, and these have been termed HD1 and HD2 (Kües and Casselton, 1992). In *S. commune* the homeodomain sequence-containing genes have been designated *Y* and *Z* and encode homeodomain proteins (Specht *et al*., 1992; Stankis *et al*., 1992). The *A* factors of *C. cinerea* contain three pairs of homeodomain genes (Kües *et al*., 1992; Pardo *et al.*, 1996). *HD1* is similar to *S. commune Z* genes and *U. maydis bE* genes, while *HD2* is similar to *Y* and *bW* genes of *S. commune* and *U. maydis,* respectively.

The *B* factors of *C. cinerea* contain three pheromone receptor genes and six pheromone genes. The *B* mating type genes of *C. cinerea* are sequestered into a single locus. This locus derives its numerous specificities from three functionally independent genes. Each set of genes belongs to an independent subfamily and each consists of a cassette of one receptor and two pheromone genes. A pheromone from a single subfamily can stimulate only all the receptors within that subfamily excluding its own (Olesnicky *et al.*, 2000). The *B* factors of *S. commune* encode one receptor gene and three pheromone genes. In *S. commune* the *B* mating type genes are separated into two discrete loci *B*α and *B*β (Parag and Koltin, 1971). Based on the sequence of one locus of each type (*B*α*1* and *B*β*1*) each contains a receptor and three pheromone genes (Wendland *et al*., 1995; Vaillancourt *et al*., 1997). Previously it was shown that pheromones with *B*α specificity can stimulate *B*α receptors from other cassettes but cannot stimulate their own resident receptor or any *B*β receptors

(Wendland *et al*., 1995). Likewise, it was shown that a *Bβ* pheromone can stimulate other *Bβ* receptors except its own and cannot activate *Bα* receptors (Vaillancourt *et al*., 1997). However, it was recently reported that *S. commune* pheromones can be classified into five groups based on certain amino acids critical for recognition by compatible receptors that have close sequence relationships within each group but not between groups. Therefore, based on sequence similarity, pheromones with Bα specificity can stimulate receptors of both Bα and Bβ specificity (Fowler *et al*., 2001; 2004). Pheromones appear to have no role in cell fusion in mushroom species; this process is mating type independent. Pheromone signalling is activated after cell fusion and is necessary to initiate and maintain the dikaryon (Casselton, 2002).

1.4 *Sporisorium reilianum*, the close relative of *U. maydis*

Sporisorium reilianum (Kühn) Langdon & Fullerton causes head smut on both maize (Fig. 1B) and sorghum. *S. reilianum* and *U. maydis* share maize as a common host and the similarity of these two distinct fungi extends to a molecular level. Phylogenetic analysis of the internal transcribed spacer (ITS) and large subunit of ribosomal DNA revealed that *U. maydis* is more related to *S. reilianum* than to other *Ustilago* species (Bergerow *et al*., 1997; Bakkeren *et al*., 2000; Piepenbring *et al*., 2002; Stoll *et al*., 2005). It was previously shown that the *U. maydis a*- and *b*-loci can hybridise to DNA from other smut fungi, demonstrating sequence conservation in these loci (Bakkeren *et al*., 1992). Although *S. reilianum* and *U. maydis* are both capable of infecting maize, they differ remarkably from each other in their aetiology. Infection with *S. reilianum* is via soil-borne teliospores that are capable of surviving in the soil for several years (Potter, 1914) and infect the plants as the kernels germinate. Low soil moisture content as well as a temperature of 28℃ is optimal for infection (Christensen, 1926). The fungus initially invades the apical meristem (Stromberg *et al*., 1984) and grows systemically without causing symptoms. It then invades the undifferentiated floral tissue and the emerging inflorescence is partially or totally replaced by the smut sorus (Wilson and Frederiksen, 1970). When no sori are present, phyllody (the process by which floral organs turn into leaves) is observed in the tassels (Halisky, 1963). In some seedlings chlorotic spots containing hyphae occur along the midrib and the leaf blade of the fourth and fifth emerged leaf (Matyac and Kommendahl, 1985).

Although *S. reilianum* has been prevalent on both maize and sorghum for centuries, not much research has been done on its molecular characterisation. Hanna (1929)

illustrated the stages of spore germination and subsequent budding growth of the haploid sporidia. Later Maytac (1985) described the distribution of fungal mycelia during the development of sori of maize. Recently Martinez *et al*. (1999) showed evidence of the presence of intracellular fungal hyphae in the shoot apex. Llike *U. maydis*, *S. reilianum* was shown to be heterothallic and have a tetrapolar mating system (Hanna, 1929). Its dimorphic life cycle was shown to be similar to that of *U. maydis* described above, with differences associated with biotrophic phase (Martinez *et al*., 2002). *S. reilianum* has been shown to penetrate the maize shoot apex epidermis directly (Wilson and Frederiksen, 1970) or has been suggested to locally dissolve the epidermal cell wall to penetrate the root without appressoria formation (Martinez *et al*., 2000). Prolific fungal growth is restricted until floral initiation and no hypertrophy is induced by the fungus during sporogenesis (Martinez *et al*., 2002).

Elucidation of the factors responsible for the differences in the specificity of the infection process of *U. maydis* and *S. reilianum* will provide valuable insights into the mechanisms of host-pathogen interactions.

1.5 Comparative Transcript Profiling of the Early Plant Infection Stages in *U. maydis* and *S. reilianum*

One of the aims of this study was to identify genes in *U. maydis* associated with tumour induction and formation and / or genes responsible for the absence of this phenotype in *S. reilianum* by comparative expression analysis. Several methods have been developed and refined since a little more than a decade ago, which allow the analysis of genome-wide gene expression patterns. The most widely used gene profiling techniques include differential display after reverse transcription and polymerase chain reaction (DD-RT-PCR) (Liang & Pardee, 1992;1995;1998; Welsh *et al*., 1992; McClelland *et al*., 1995;1997); serial analysis of gene expression (SAGE) (Velculescu *et al*., 1995); DNA microarrays (Schena *et al*., 1995); amplified fragment length polymorphism based on cDNA (cDNA-AFLP) (Bachem *et al*., 1996; 1998); and suppression subtractive hybridisation (SSH) (Diatchenko *et al*., 1996). The nature of the expression analysis provided by these techniques is the simultaneous examination of up to tens of thousands of genes, thus providing an invaluable resource for the large-scale identification of gene functions (Breyne & Zabeau, 2001). Microarrays offer the best solution for genome-wide expression profiling. Two different kinds of microarrays are common: cDNA microarrays and oligonucleotide microarrays. cDNA microarrays are prepared by high-density printing

of complementary DNAs on glass (Schena *et al*., 1995) while oligonucleotide microarrays are high-density microarrays containing several thousands of synthetic oligonucleotides (Lochart *et al*., 1996). The latter were used by Affymetrix™ in the design of the customised *U. maydis* GeneChip. Advantages of oligonucleotide microarrays are that the probes are present at higher densities and that probes are designed to represent unique gene sequences. This design feature minimises the common occurrence of cross-hybridisation between related gene sequences (gene families or shared functional domains) associated with cDNA microarrays. In addition, the probes are uniform in length, concentration and melting temperature, consequently very little experimental variation is encountered (Alba *et al*., 2004). Unfortunately, the use of microarrays for full genome-wide expression is limited to model organisms for which the complete genome sequence is determined or the availability of large databases of well characterised expressed sequence tags (ESTs) exists (Breyne & Zabeau, 2001). Fortunately, other profiling techniques such as SAGE, SSH and cDNA-AFLP offer an invaluable advantage compared to microarrays in that they allow genome-wide expression analysis in any organism without prior knowledge of the genome sequence and have the inherent advantage of identifying and assessing new genes (Vos *et al*., 1995, Bachem *et al*., 1996). In addition, the cDNA-AFLP technique allows the investigation of a wide variety of tissue types, developmental stages, or time points to be compared concurrently (Alba *et al*., 2004).

1.6 Objectives of the study

The focus of this study was the investigation of the differences in infection specificity of the two closely related phytopathogenic fungi *U. maydis* and *S. reilianum*. *U. maydis* on the one hand infects all aerial parts of the plant, rapidly proliferates within the infected tissue and leads to the formation of spore-filled tumours. On the other hand, *S. reilianum* is reported to infect via the roots, grow systemically within the plant without causing symptoms and only upon flowering replace the inflorescence with spores. Although these fungi have a common host plant *Zea mays* (maize) and both cause smut disease, the aetiology of the disease resulting from infection by the two fungi differs substantially. A cornucopia of techniques has been established for the study and manipulation of *U. maydis* at a molecular level, however, a dearth of molecular analysis of *S. reilianum* exists. The aims of the study were therefore to 1) establish molecular techniques for the

characterisation of *S. reilianum* to facilitate its manipulation at a molecular level; 2) identify the mating type loci of *S. reilianum* and characterise the associated genes; 3) determine the factors responsible for the difference in modes of infection by testing which traits specific to each organism are dominant, 4) microscopically follow the infection process of *U. maydis* and *S. reilianum* to define the differences during biotrophic development and lastly, 5) to detect genes whose presence or difference in expression level can be correlated with the differences observed during biotrophic development using comparative expression analysis.

Results I

Characterisation of mating type loci

Mating type genes play a key role in formation and maintenance of the infectious cell type and hence pathogenicity. It is therefore of importance to understand the structure and function of the responsible genes. As no molecular characterisation of *S. reilianum* was ever published, it was necessary to first characterise the mating type loci.

2.1 Screening of mating type in *S. reilianum* isolates

To determine the mating types present in *S. reilianum,* two assays were developed to distinguish between strains containing different *a*- and *b*-mating type genes. Haploid cells of different mating type are morphologically indistinguishable and can only be differentiated when mixed together and grown on nutrient media (Rowell, 1955; Puhalla, 1970). Since cells can differ at both the *a*- and the *b*-locus, it is necessary to determine the exact genotype using mating assays. When cells contain different *a* alleles and are co-cultivated, they respond to the pheromone secreted by the compatible cell and grow towards each other by producing conjugation hyphae. In the assay to determine if cells differ at the *a*-locus, cells are mixed together and grown in liquid medium and checked microscopically to determine if they produce conjugation hyphae or not (Fig. 4 upper left panel). The absence of conjugation hyphae is indicative of cells having the same *a* alleles. This assay is not capable of differentiating between *b* alleles.

Plate mating assays are used to determine if cells differ at both the *a*-locus and the *b*-locus and thus form stable dikaryotic filaments (Fig. 4 upper right panel). In this assay cells are mixed together, dropped on water agar plates and left overnight. The plates are checked for the formation of aerial hyphae which give the colony a white, fuzzy appearance. Cell mixtures that have the same *a*- and *b*-locus result in colonies with a smooth appearance, while mixtures of cells that have different *a*-loci but the same *b*-loci result in colonies that have a rough appearance (Fig. 4 lower right panel).

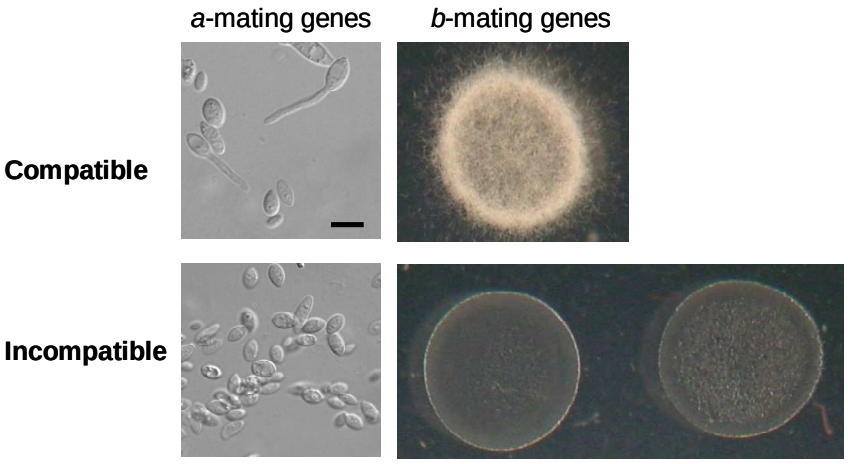

Figure 4. Assays for determining *a*- and *b*-mating type genes of *Sporisorium reilianum*.
Two sporidial cultures of comparable cell densities were mixed and assayed in liquid cultures for the development of conjugation hyphae, indicative of compatible *a*-mating genes (upper left panel). Incompatible *a*-mating genes do not develop conjugation hyphae (lower left panel). A mixture of the sporidial cultures were dropped on water agar plates and assayed for the development of aerial hyphae resulting in fuzzy white colonies, indicative of compatible *b*-mating genes (upper right panel). Incompatible *b*-mating genes result in smooth colonies on plate (lower right panel). Compatible *a*-mating genes but incompatible *b*-mating genes result in rough colonies on plate (lower right panel). Bar = 10µm.

Spore samples obtained from infected field-grown maize from Germany and France were germinated and the haploid sporidia obtained were tested for their *a*-mating genotype using the above-mentioned assays. Once strains had been identified to contain different *a*-mating genes it was then possible to determine whether they carried compatible *b*-mating genes by using the plate mating assay.

Four different mating types were identified in the samples from both Germany and France designated *a*1*b*1, *a*1*b*2, *a*2*b*1 and *a*2*b*2 (Table 1) and were subsequently used as tester strains. Results of the mating assays confirmed previous findings that *S. reilianum* has a tetrapolar mating system. However, the finding of only two alleles of the *b*-mating type was in contrast to that of the close relative *U. maydis* which has at least 23 different *b* alleles (Puhalla, 1970; Silva, 1972). To determine whether more than two *b* alleles existed in *S. reilianum,* spore samples were obtained from widely differing geographic regions namely China, USA and South Africa. These spores were germinated and the genotypes of the resultant sporidia were determined using the above-mentioned mating assays. Several strains from China and USA were found to form fuzzy filaments on plates with strains carrying the *a*1 allele regardless of the *b1* or *b2* allele. Similarly, isolates from South Africa were found to form fuzzy filaments on plates with strains carrying the *a*2 allele regardless of the *b1* or *b2* allele. These sporidia from China and USA were therefore classified *b3.* In sporidia from South Africa an additional two different *b* alleles, designated *b4* and *b5* were identified. Hence a total of at least five *b* alleles must exist in *S. reilianum*

(Table 1). These additional *b* alleles therefore confirmed the multi-allelic *b*-mating type nature of *S. reilianum* to be similar to its relative *U. maydis*.

Table 1. Geographic distribution of *Sporisorium reilianum* genotypes

Genotype	Germany	France	China	USA	South Africa
a1b1	47	25	0	15	0
a1b2	12	20	3	3	0
a1b4	0	0	0	0	19
a1b5	0	0	0	0	14
a2b1	2	26	22	7	0
a2b2	59	36	26	10	0
a2b3	0	0	22	4	0
a3b1	0	0	25	0	0
a3b2	0	0	31	8	0
a3b3	0	0	7	3	0
a3b4	0	0	0	0	6
a3b5	0	0	0	0	28
Total number of sporidia tested[1]	120 (2)	107 (7)	136 (4)	50 (4)	67 (3)

[1]Sporidia were obtained from germinated spores from field samples that were streaked out to single colonies. Spores were obtained from several field samples of infected plants for each country - respective numbers of field samples tested are given in brackets.

Surprisingly, in addition to the various different *b* alleles, a third *a* allele was also identified in the samples obtained from China, USA and South Africa (Table 1). These strains were able to form conjugation hyphae when combined with strains containing both *a*1 and *a*2 alleles, thus indicating the presence of a different *a* allele.

Geographic distribution of the *S. reilianum* genotypes identified is listed in Table 1. Isolates from Germany and France had the genotypes *a1b1*; *a1b2*; *a2b1;* and *a2b2* with *a2b2* being the most prevalent. Sporidia from South Africa, like those of Germany and France, contained two *a* and two *b* alleles. However, differences were observed at both *a and b* alleles (*a1b4*; *a1b5*; *a3b4*; *a3b5*). The greatest genotypic variation was observed in the isolates obtained from China and USA as three *a* alleles as well as three *b* alleles were identified in the sporidia tested. Genotypes found in China were *a1b2*; *a2b1*; *a2b2*; *a2b3*; *a3b1*; *a3b2*; and *a3b3* with *a3b2* being most prevalent. Finally, genotypes found in the USA were *a1b1*; *a1b2*; *a2b1*; *a2b2*; *a2b3*; *a3b2*; and *a3b3* with *a1b1* being the most prevalent.

The presence of the third *a* allele in sporidia from three different geographic locations confirmed its authenticity and revealed a difference to the bi-allelic *a*-mating genes identified in other members of the smut fungi characterised to date.

2.2 Isolation and sequence characterization of *S. reilianum* mating type loci

2.2.1 Isolation of *S. reilianum a*-mating type genes

To identify the *a*-mating type locus of *S. reilianum,* an 8 kb *U. maydis* DNA fragment containing the *a*1 locus was used as a probe in Southern hybridisation analysis using *BamHI* I digested genomic DNA of *S. reilianum*, *S. scitamineum* and *U. maydis* of genotypes specifying different *a*-alleles. A band of approximately 4 kb only observed in *S. reilianum a*1 mating type strains hybridised relatively strongly to the *U. maydis* probe as well as the expected 8 kb band in the *U. maydis* strain of *a*1 mating type, which served as a positive control (Fig. 5A). Several weaker bands were observed in

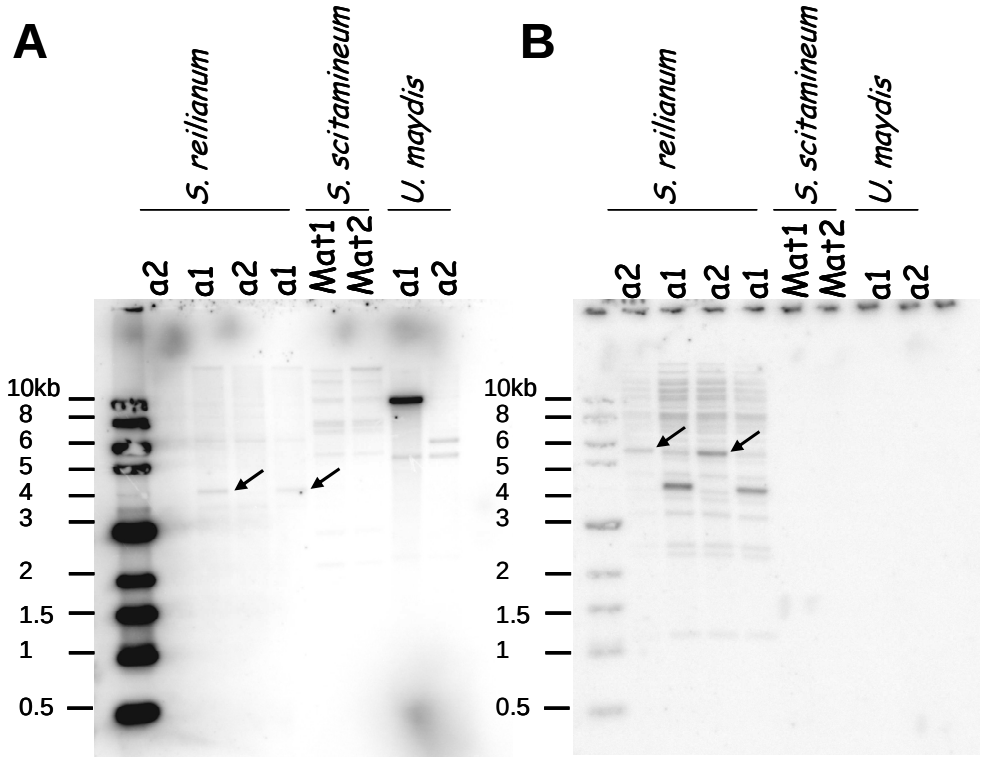

Figure 5. Southern hybridisation analysis of genomic DNA for the identification of *Sporisorium reilianum a*-mating type loci. Genomic DNA of *S. reilianum*, *U. maydis* and *S. scitamineum* of different mating types were digested with *BamH* I endonuclease, separated on a 0.8% agarose gel and transferred to nylon membranes. **A.** An 8 kb fragment of *U.maydis a*1 locus used as a heterologous probe hybridized strongly to an approximately 4 kb fragment in *S. reilianum* strains of *a*1 mating type indicated by the arrows. The strong signal in *U.maydis a*1 mating type served as a positive hybridization control. **B.** The 4 kb *S. relianum a*1 fragment was cloned and used as a probe to identify an approximately 5.5 kb fragment in *S. relianum* strains of *a*2 mating type indicated by the arrows.

the *S. reilianum a*2 mating type strains, both mating types of *S. scitamineum* as well as two weak bands in *U. maydis a*2 mating type (Fig. 5A). As no specific hybridisation was observed in *S. reilianum a*2 mating type strains, the approximately 4 kb region was isolated from *a*1 strains, cloned in pCR-Topo vector and used as a more specific probe in Southern analysis in order to identify *a*2-specific fragments. Using this probe a strong hybridisation signal was observed in the *S. reilianum a*2 mating type at approximately 5.5 kb as well as the expected 4 kb band in the *a*1 mating type of *S. reilianum* (Fig. 5B). Several other bands also hybridised to the 4 kb probe but were present in both *a1* and *a2 S. reilianum* mating types and might be indicative of repetitive sequences present in the probe fragment. No hybridisation to this probe was observed in either of the *U. maydis* or the *S. scitamineum* strains (Fig. 5B). The *S. reilianum* 4 kb *a*1 fragment and the 5.5 kb *a*2 fragment were cloned into a plasmid vector for sequence characterisation, which revealed incomplete regions of the *a*-loci. In order to identify the remainder sequences of the *a*-loci, a genomic library was constructed from two compatible strains (*a1b1* and *a*2*b*2). Genomic DNA was partially digested using *Sau*3AI, 8 - 9 kb fragments isolated and cloned in pCR4-Topo vector. The library consisted of approximately 24000 colonies. The average insert size was determined by restriction analysis to be approximately 5kb. Border sequences of the *a*-locus were used as probes in colony hybridisations. Sequences of the *a*1 locus were represented on two overlapping plasmids designated p5211 and p527. The 5.5 kb *a*2 fragment was identified in a single plasmid designated pG10 but only contained a portion of the *a*-locus. A second overlapping 3.5 kb *Sac* I fragment was identified from a genomic library and subcloned in pG10 to yield pG11. All intervening sequences and the right border were obtained by PCR amplification.

None of the *U. maydis* or the *S. reilianum a*-mating type fragments that were used as probes in Southern analysis on genomic DNA of the *S. reilianum a*3 strains resulted in a hybridization signal (not shown). Therefore, a PCR approach was used to identify the locus containing *a3* information. This work was part of a Diploma thesis by Martin Wagenknecht (2005) and all the specifics have been described by him. In brief, an approximately 9.5 kb fragment was amplified by long range PCR and cloned into pCR4Blunt-Topo generating pMW3 to facilitate sequence determination.

2.2.2 Sequence characterisation of the *S. reilianum a*-mating genes

Sequence characterisation of the three *a*-loci *a1*, *a2*, and *a3* of *S. reilianum* determined them to be 6.2 kb, 11.0 kb and 9.1 kb in length respectively between the border genes. Each allele contains three open reading frames (ORFs), which were

found by comparison to public sequence databases to encode a receptor gene and two pheromone genes. The pheromone receptor genes were designated *pra*1, *pra*2 and *pra*3 for the respective alleles. Pheromone genes were designated *mfa1.2* and *mfa1.3* for the a1 allele, *mfa2.1* and *mfa2.3* for the *a*2 allele and *mfa3.1* and *mfa3.2* for the a3 allele (Fig. 6A). In addition, the *S. reilianum a*2 allele contains two ORFs which are equivalent to the *U. maydis a*2-specific genes *lga* and *rga.* Similar to *U. maydis*, the *S. reilianum a*-loci are flanked by conserved ORFs termed *lba* (left border *a*) for the left ORF and *rba* (right border *a*) for the ORF on the right. Sequences obtained for the *S. reilianum a*-loci were aligned with the *U. maydis a*-loci to determine the degree of similarity between the two organisms. The schematic diagram of the sequence alignment revealed that the gene order in both organisms is highly similar (Fig. 6A). Except the presence of a second pheromone gene, the *S. reilianum a*1 allele is analogous to the *U. maydis a*1 allele. Similarly, the *S. reilianum a2* allele is analogous to the *U. maydis a2* allele as *S. reilianum* also contains the *a2*-specific genes *lga* and *rga.* Interestingly, the *U. maydis a2* allele contains a pseudo-pheromone gene which is located at a comparable genic region to the second pheromone gene in the *S. reilianum a*2 allele. The *S. reilianum a*3 allele is least similar to the *a1* and *a2* loci of *U. maydis* (Fig. 6A).

Functionality of the pheromone and receptor genes was established by transformation of the specific genic region of the *a* loci into strains of different mating type and the mating response determined. Altered strains are assayed for conjugation hyphae formation which is indicative of the recognition of the receptor by the nascent pheromones. However, a double mater phenotype is indicative of successful mating where pheromone stimulation of each mating partner is required. To test the function of the pheromone genes, a fragment containing the *mfa1.2* and *mfa1.3* pheromone precursor genes was introduced via a self-replicating plasmid pSr-a1 into *S. reilianum* SRZ2 (*a2b2*). In the absence of a mating partner, the altered strain formed conjugation hyphae in liquid medium. Plate mating assays of the altered SRZ2 revealed that it formed aerial filaments with both an *a1b1* strain (SRZ1) and an *a2b1* strain (SRZ3), characteristic of a strain expressing two pheromone genes that can stimulate both *a1* and *a2* mating partners. The double mater phenotype was attributed to the introduced *mfa1.2* gene, as the nascent *mfa2.3* pheromone precursor gene in the altered *a2* strain is identical to the introduced *mfa1.3* gene. Introduction of the *mfa1.2* gene in *a3* strain produced no morphological response, thereby illustrating the functionality of the *mfa1.2* gene required for mating with *a2* strains. To confirm that *mfa2.3* is specifically recognised by Pra3 a genomic

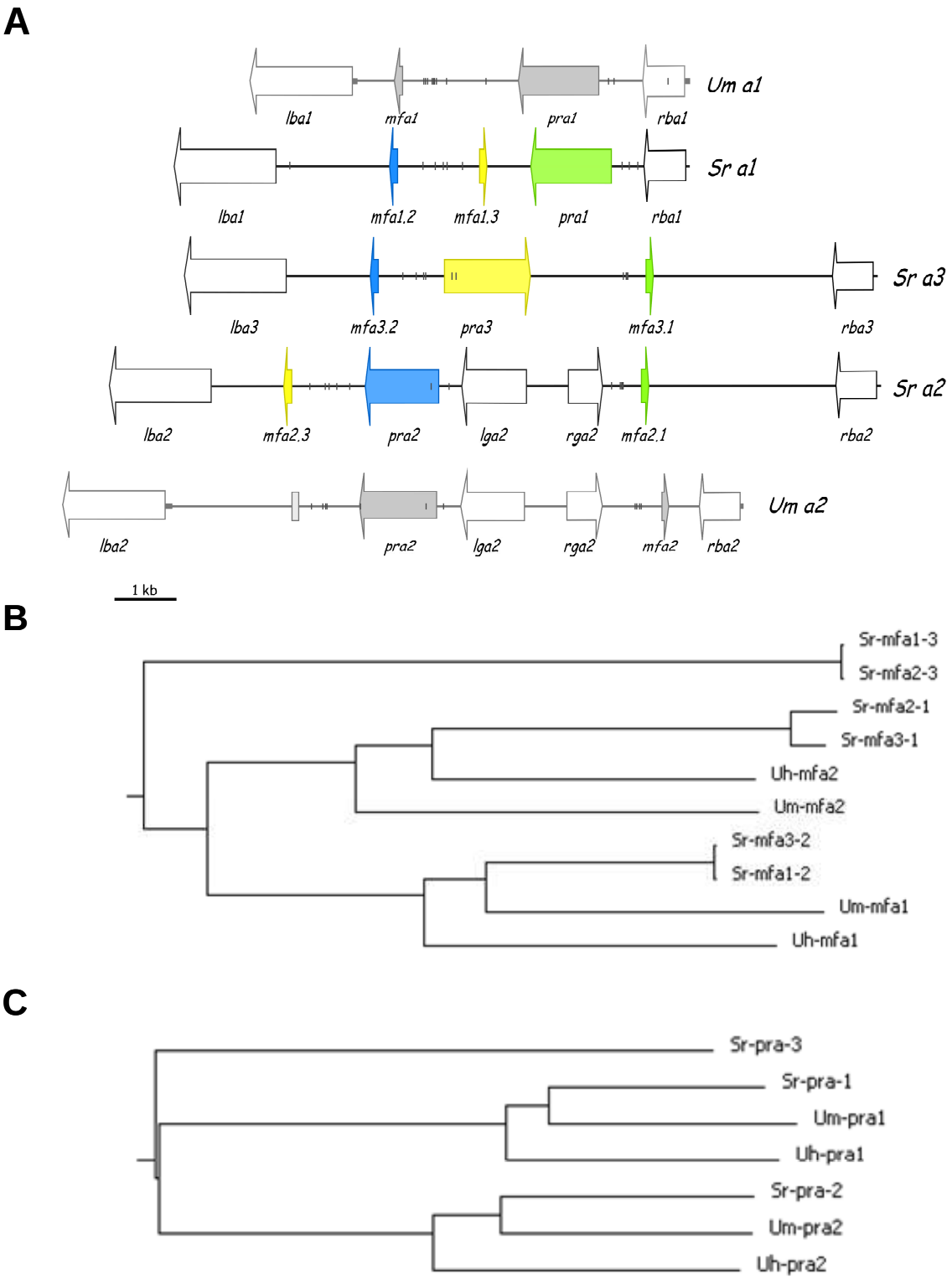

Figure 6. Alignment of the *Sporisorium reilianum a*-loci with the *a*-loci of *Ustilago maydis* and comparison with mating type genes of *Ustilago hordei*. **A.** Graphic representation of the alignment of the three *a*-loci of *S. reilianum Sr a1*, *Sr a2*, *and Sr a3* with the two *a*-loci of *U. maydis Um a1* and *Um a2*. *Sr a1* and *Sr a2* are most similar to *Um a1* and *Um a2* respectively. Gene order of the pheromone receptors (*pra1* and *pra2*) and the pheromone genes (*mfa1.2* and *mfa2.1*) of *Sr* a1 and Sra2 shows perfect synteny with *Um a1 pra1 and mfa1* and *Um a2 pra2* and *mfa2*. The exception being the presence of a second pheromone gene in *Sr a1* (*mfa1.3*) and *Sr a2* (*mfa2.3*). *Um a2* contains a pseudo-pheromone gene (open vertical bar) at a similar genic position to the *Sr a2* second pheromone gene *mfa2.3*. *Sr a2* also contains the *Um a2*-specifc genes *lga2* and *rga2*. The third *S. reilianum a*-locus *Sr a3* is distinct from both *a*-loci in *S. reilianum* and *U. maydis*, however the pheromone genes *mfa3.1* and *mfa3.2* are identical to *mfa2.1* and *mfa1.2* respectively. Similar to *U. maydis*, the *a*-loci of *S. reilianum* are flanked by two conserved open reading frames termed *lba* (left border *a*) and *rba* (right border *a*). **B and C.** Phylogenetic analysis of the inferred amino acid sequences of the pheromone genes and pheromone receptor genes of *S. reilianum* (*Sr*), *U. maydis* (*Um*) and *U. hordei* (*Uh*). The phylogenetic tree was generated using CloneManager multiway analysis and the PAM250 residue weight table with default parameters.

fragment containing *mfa2.3* was introduced into *a1* and *a3* strains. Only the altered *a3* strain formed conjugation hyphae without a mating partner and displayed a double mater phenotype. This displayed that Mfa2.3 can specifically be recognized by Pra3 but not by Pra1 or Pra2. Pheromone genes of the *a3* (*mfa3.1* and *mfa3.2*) strain were similarly assayed.

To test the function of the *a2* receptor gene, a genomic fragment containing the putative *pra2* gene was introduced into strain SRZ1. Conjugation hyphae formation without a mating partner was observed, but the strain did not show a double mater phenotype. The same was observed when the putative *pra*2 gene was introduced into an *a3* strain, thereby confirming that the *pra*2 gene of *S. reilianum* encodes a functional pheromone receptor. Similarly, *pra1* and *pra3* were assayed and their functionality confirmed. In summary, the identical pheromones Mfa1.2 and Mfa3.2 are exclusively recognised by Pra2. Similarly, Mfa2.1 and Mfa3.1 stimulate Pra1 and Mfa1.3 and Mfa2.3 stimulate Pra3. The functional analysis of the mating genes of the *a1* and *a*2 strains was conducted by J. Schirawski (Schirawski *et al*., 2005) and that of the *a3* strain was conducted by M. Wagenknecht (Wagenknecht, pers comm.). It is also interesting to note that *S. reilianum* and *U. maydis* do not pheromone-stimulate each other (Schirawski pers comm.).

To determine the phylogenetic relatedness of the *a*-loci of *S. reilianum* to *U. maydis*, predicted amino acid sequences were compared *in silico*. A phylogenetic tree was generated using the software programme CloneManager multiway analysis and the PAM250 residue weight table with default parameters. Phylogenetic analysis of the pheromone genes of *S. reilianum* revealed that *mfa*1.3 and *mfa*2.3 are identical and are quite distinct from the other pheromone genes (Fig. 6B). *mfa*2.1 and *mfa*3.1 are identical and most similar to *U. maydis mfa*2 and *U. hordei mfa*2 (48.7% and 53.8% amino acid identity, respectively), while *mfa*1.2 and *mfa*3.2 are also identical and most similar to *U. maydis mfa*1 and *U. hordei mfa*1 (52.4% and 58.5% amino acid identity, respectively). Comparison of the pheromone receptor genes revealed that the *S. reilianum pra*1 is most similar to *U. maydis pra*1 and *U. hordei pra*1 while *S. reilianum pra*2 gene is most similar to *U. maydis pra*2 and *U. hordei pra*2. The *pra*3 gene of *S. reilianum* is quite distinct from both *pra*1 and *pra*2 as well as the orthologues of *U. maydis* and *U. hordei*. (Fig. 6C). These analyses revealed that the overall structure of the *a*-loci of *S. reilianum* is very similar to *U. maydis*, with the exception of the presence of a third *a*-allele and two active pheromone genes per allele.

2.2.3 Isolation of *S. reilianum b*-mating type genes

Albert and Schenk (1996) were able to amplify a 450 bp PCR fragment from the *Ustilago scitaminea* (*Sporisorium scitamineum*) *b*E gene using primers based on the *U. maydis b*E mating type gene. The 450 bp product showed 70% nucleotide sequence homology to *U. maydis* and *U. hordei* and specifically hybridised to single *BamHI* I fragments in the *Mat1* and *Mat2* mating types of *S. scitamineum* (Albert and Schenk, 1996). We therefore used the 450 bp PCR fragment as a heterologous probe in Southern hybridisation analysis to identify the *b*-loci of *S. reilianum*. Using *BamHI* I-digested genomic DNA of different mating types, an approximately 9 kb fragment was observed to hybridise specifically in all the *S. reilianum* strains containing *b1* and *b2* mating type genes (Fig. 7). Strong hybridisation was observed at approximately 7 kb and greater than 10 kb in the *S. scitamineum Mat1* and *Mat2* strains respectively, thus confirming the specificity of the hybridisation. Surprisingly, no hybridisation was observed in either of the *U. maydis b* alleles (Fig. 7). The 9 kb *BamHI* fragments from *S. reilianum b1* and *b2* strains were isolated, cloned into

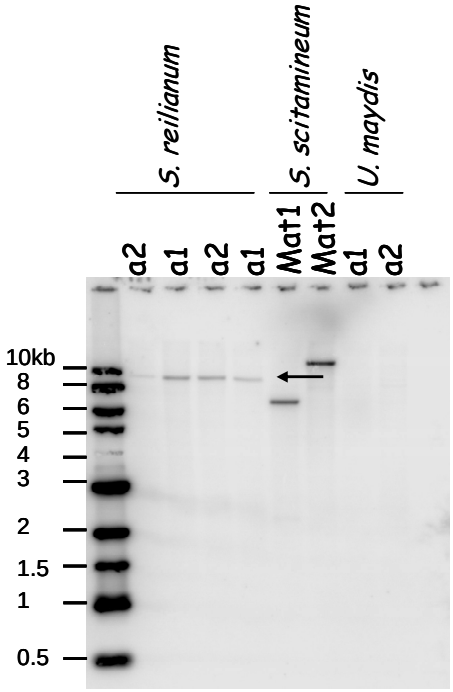

Figure 7. Southern hybridisation analysis of genomic DNA for the identification of *S. reilianum b*-mating type loci. Genomic DNA of *S. reilianum*, *S. scitamineum* and *U. maydis* of different mating types were digested with *BamH* I endonuclease, separated on a 0.8% agarose gel and transferred to a nylon membrane. A 0.45kb PCR fragment of *S. scitamineum b*-locus used as a probe hybridized specifically to an approximately 9 kb fragment in *S. reilianum* strains of both *b*1 and *b*2 mating type (indicated by the arrow). A strong specific band in both *S. scitamineum* mating types served as a hybridization positive control.

pCR4 BamHI and screened by colony hybridisation using the original 450 bp *S. scitamineum* b*E* PCR fragment as probe. The screen identified two clones containing the *b*1 and *b*2 9 kb fragments inserted in the plasmids p17-1 and p114-1 respectively. The cloned *b*-loci were sequence analysed and their identity confirmed by comparison to public sequence databases. For the isolation of *b3, b4* and *b5* PCR was used to amplify two fragments of 1642 bp and 4587 bp from the respective genomic DNAs using the primer pairs oJS31/oJS33 and oJS57/oJS69. The 1642 bp fragment covered the *bW* gene and part of the potential N-terminal acetyltransferase gene *nat1*, while the 4587 bp included the *bE* gene and part of the proposed nuclear regulator *c1d1* gene, related to human C1D. Amplified gene fragments were directly sequenced.

2.2.4 Sequence characterisation of the *S. reilianum b*-mating genes

The cloned fragments containing the *S. reilianum b1* and *b2* loci were determined to be 9.0kb in length, while the PCR-amplified *b*-genes of the *b3*, *b4* and *b5* loci were 5.5kb in length. All five *b*-loci contain two ORFs which are divergently transcribed and sequence comparison with public sequence databases revealed the highest degree of similarity with the *bE* and *bW* genes of *U. maydis* and *U. hordei*. Partial sequences of the borders of the *b*-loci showed that similar to *U. maydis* the *bW* gene is flanked by the *nat*1 gene which encodes a potential N-terminal acetyltransferase, while the *bE* gene is flanked by the *c1d1* gene which encodes a proposed nuclear regulator related to the human C1D. Interestingly, the *S. reilianum b1* and *b2* loci contain a 2.4 kb transposon of the Fot I family situated between the *b*E and *c1d1* genes (Fig. 8A). Both the *bE* and *bW* genes contain one intron each as determined by sequence analysis of RT-PCR products from RNA obtained from dikaryotic filaments. Introns are situated at similar positions to those observed in the *b* genes of *U. maydis* (Fig. 8A). Both *U. maydis bE* and *bW* genes have been shown to contain a conserved C-terminal domain, a variable N-terminal domain as well as a homeodomain motif (Kämper *et al*., 1995). Analysis of the inferred protein sequence of the *S. reilianum b* genes revealed a similar organisation, with all five *bE* genes having conserved C-terminal domains but N-terminal domains that are more variable. The same applies to the *bW* genes (not shown).

To determine the phylogenetic relatedness of the *b*-loci of *S. reilianum* to other smut fungi (*U. maydis* and *U. hordei*), predicted amino acid sequences were compared *in silico*. A phylogenetic tree was generated using the software programme CloneManager multiway analysis and the PAM250 residue weight table with default

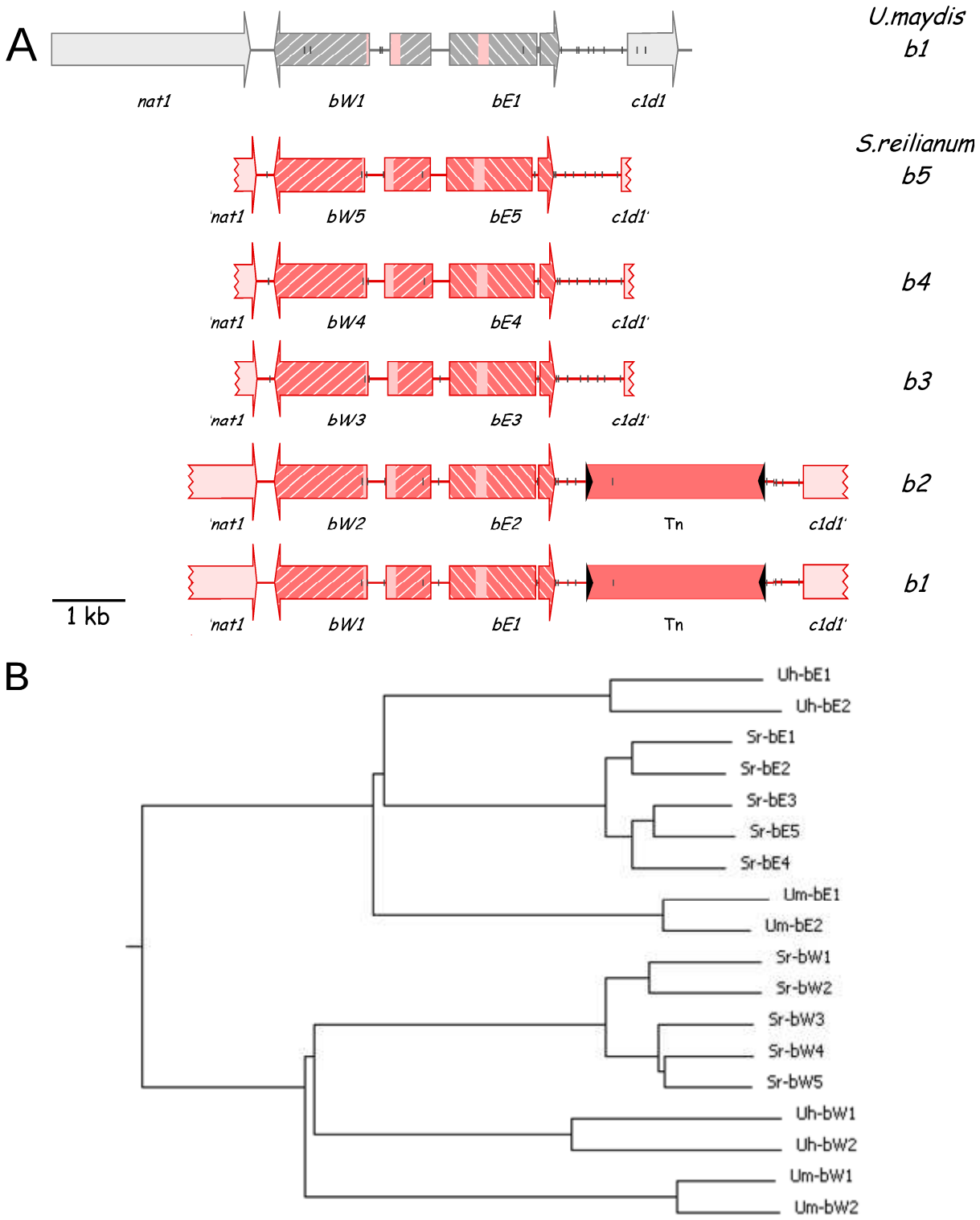

Figure 8. Alignment of the *b*-loci of *Sporisorium reilianum* with the *b*-locus of *Ustilago maydis* and *Ustilago hordei*. **A.** Graphic representation of the alignment of the five *b*-loci of *S. reilianum b1*, *b2*, *b3, b4 and b5* with the *b1* locus of *U. maydis*. Similar to *U. maydis*, *S. reilianum b*-loci contain two divertly transcribed open reading frames (ORFs) that are flanked by two conserved ORFs. Partial sequences obtained revealed these ORFs to be similar to a potential N-terminal acetyltransferase encoded by *nat1* and a proposed nuclear regulator related to the human C1D encoded by *c1d1*. A transposon of the FotI family situated between the *bE* and *c1d1* is unique to the *b1* and *b2* alleles. The *bE* and *bW* genes contain one intron each and are situated at similar positions to those observed in the *b* genes of *U. maydis* (shown by an interruption in the arrow). As in *U. maydis*, *S. reilianum* b*E* and b*W* genes contain a conserved C-terminal domain, a variable N-terminal domain as well as a homeodomain motif (light shaded box). **B.** Phylogenetic analysis of the inferred amino acid sequences of the b*E* and b*W* genes of *S. reilianum* (*Sr*), *U. maydis* (*Um*) and *U. hordei* (*Uh*). The phylogenetic tree was generated using CloneManager multiway analysis and the PAM250 residue weight table with default parameters.

parameters. The phylogenetic tree revealed that the five *S. reilianum* bE proteins are more closely related to each other than to those of either *U. maydis* or *U. hordei* (Fig. 8B). The same holds true for the bW proteins. In comparison to the close relatives *U. maydis* and *U. hordei*, the bE proteins of *S. reilianum* share 48% and 47% amino acid identity, with their respective orthologues. While the bW proteins share 36% and 33% amino acid identity with their respective *U. maydis* and *U. hordei* orthologues. These relationships are revealed in the phylogenetic tree (Fig. 8B) and displays that the overall structure of the bE and bW proteins is conserved among the smut fungi.

Results II

Hybrids of *Ustilago maydis* and *Sporisorium reilianum*

An infection of maize plants with either *U. maydis* or *S. reilianum* leads to very different symptoms. Evidently, *U. maydis* contains genes that allow the induction of tumours on leaves, while *S. reilianum* contains genes that restrict spore development to the floral tissue of the maize plant. To know which of these genes would be dominant and be identified using a BAC-transformation approach, the creation of hybrids was attempted by protoplast fusion. Protoplast fusion permits the generation of hybrids that naturally would be inhibited by sexual, somatic and / or nuclear incompatibility (Peberdy, 1979) as shown by the cross between *Candida tropicalis* and *Yarrowia fibuligera* (Provost *et al*., 1978). Diploid strains of *U. maydis* have been created and are viable and stable (Banuett & Herskowitz, 1994b). The following strains were chosen to create hybrids: the *U. maydis b*-null strains AB1 and AB2, in which the *b*-locus is replaced by a phleomycin resistance ($Phleo^R$) cassette (Brachmann, 2001); and the *S. reilianum* JS333 solopathogenic strain, in which the native *bE1* allele was replaced by a heterologous *bE2* allele and a mutated version of the *U. maydis* succinate dehydrogenase gene, which confers resistance to the antibiotic carboxin (Cbx^R) (J. Schirawski, pers. comm.). The *U. maydis* strains lacking the *b*-mating type genes were selected as it was previously shown that the *b*-locus is the central regulator for pathogenicity. The solopathogenic *S. reilianum* strain was chosen to alleviate the need for a mating partner thereby reducing the genetic complexity.

3.1 Hybrid Formation and Symptom Development Assessment

Protoplasts of *U. maydis b*-null strains AB1 ($Phleo^R$) or AB2 ($Phleo^R$) were mixed with protoplasts of the *S. reilianum* solopathogenic strain JS333 (Cbx^R) in the presence of polyethylene glycol (PEG) and subjected to antibiotic selection pressure. DNA was then isolated from single Cbx and phleomycin resistant colonies. Six potential hybrids were obtained from the fusion of AB1 and JS333 (JA1-1 to JA1-6) and five hybrids from the fusion of AB2 and JS333 (JA2-1 to JA2-5). The presence of genes of both parent strains in the hybrids was tested by PCR using specific primer pairs. Marker

genes of *U. maydis* were identified by specific fragments of the *a1* and *a2* mating type loci. Specific fragments of the *a1* mating type locus, the b*E*2 gene and the succinate dehydrogenase gene (Sdh) served to identify marker genes of *S. reilianum*. PCR results of the putative hybrids and the progenitor strains for the various marker genes are listed in Table 2.

Table 2. Results of specific PCR amplification of marker genes for hybrid status verification

Transformant	Sdh (Sr) *oJS124 / oJS140	*bE2* (Sr) *oJS216 / oJS219	*a1* (Um) *oBH1 / oBH2	*a2* (Um) *oJS6 / oJS7	*a1* (Sr) *oJS105 / oJS195
JA1-1	+	-	+	-	+
JA1-2	+	+	+	-	+
JA1-3	+	+	+	-	+
JA1-4	(+)	-	+	-	(+)
JA1-5	(+)	-	+	-	(+)
JA1-6	+	-	+	-	(+)
JA2-1	+	-	(+)	+	-
JA2-2	-	-	-	+	-
JA2-3	-	-	-	+	-
JA2-4	-	-	-	+	-
JA2-5	-	-	+	+	-
Parent - JS333	+	+	-	-	+
Parent - AB1	-	-	+	-	(+)
Parent - AB2	-	-	(+)	+	-

* specific primer pair used to amplify the marker gene fragment, (+) indicates a very weak band, (Um) *Ustilago maydis* (Sr) *Sporisorium reilianum*

Only two putative hybrids, JA1-2 and JA1-3, gave positive PCR results for all the marker genes tested. The PCR result for the marker gene *a*2 of *U. maydis* is negative since these hybrids were obtained from a fusion between JS333 and AB1, which is an *a*1 strain. Both hybrids produced aerial filaments when grown on water agar as well as the progenitor strain JS333, while both AB1 and AB2 only formed smooth colonies (not shown). Although the hybrid JA1-1 gave a negative PCR result for the *bE2* gene of *S. reilianum*, it also produced aerial filaments when spotted on water agar (not shown). This suggested that the hybrid JA1-1 indeed had the *bE2* gene from *S. reilianum* as it was able to grow filamentously on its own, which would not be possible in the absence of a functional *b*-complex. An explanation for the negative PCR result might be due to an alteration in the primer recognition sequence during the fusion event in JA1-1 resulting in the primer not binding during PCR. Alternatively, the PCR reaction may have failed in that instance.

The cell and colonial morphology of the three hybrids were different from the progenitor strain AB1 (Fig. 9A) and JS333 (Fig. 9B). Hybrid JA1-1 had a cigar cell shape typical of *U. maydis* but shorter in length and had a lighter colony colour on PD plates than AB1 or JS333 (Fig. 9C). JA1-2 had a mixture of mostly shorter cigar-shaped cells and some having a more lemon shape. This was also reflected in the colonial morphology on PD plates, which appeared to be an intermediate of AB1 and JS333 (Fig. 9D). Hybrid JA1-3 had a lemon cell shape resembling JS333 but had a lighter colony colour on PD plates (Fig. 9E).

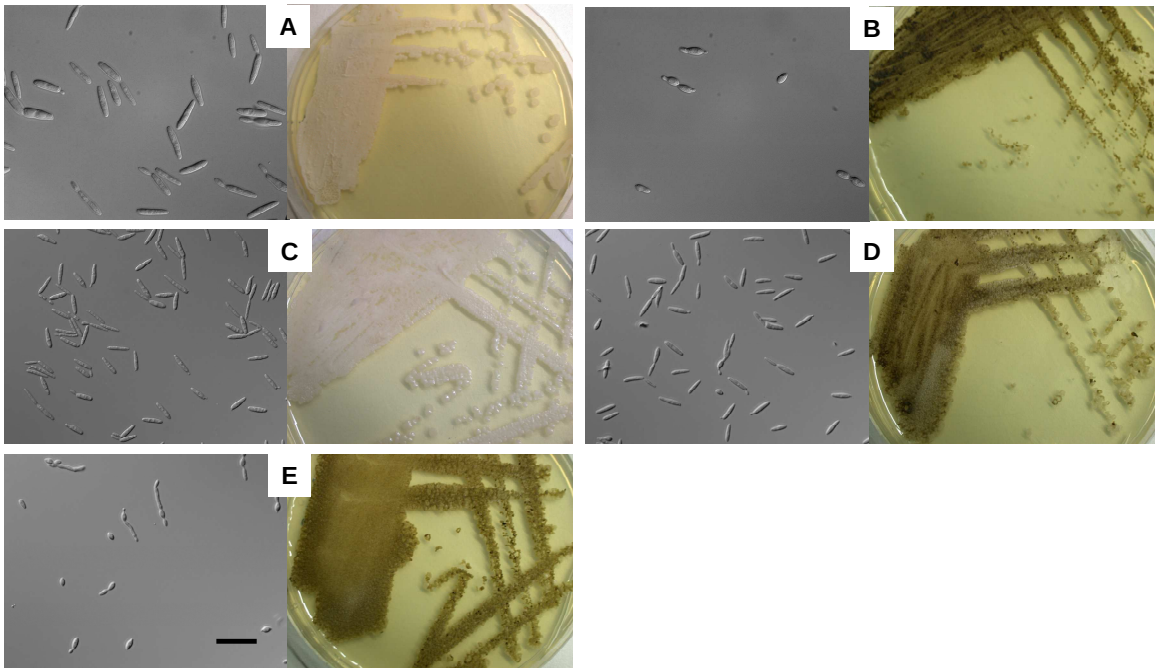

Figure 9. Cell and colony morphology of *U. maydis* and *S. reilianum* progenitor strains and the putative hybrids obtained by protoplast fusion. Each image represents cells grown in liquid medium on the left and the respective strains after growth on PD solid medium for 3 days at 28^{o}C on the right. **A**. *U. maydis* strain AB1 **B.** *S. reilianum* strain JS333 **C.** putative hybrid JA1-1 **D**. putative hybrid JA1-2 **E.** putative hybrid JA1-3. Bar = 10 µm applies to images on the left.

Since the three putative hybrids were able to grow filamentously on water agar, it was of interest to test their ability to infect maize plants and observe the symptoms, if any, that were produced. The dwarf maize variety Gaspe Flint was used to assess symptom development as the mature plants are not very big and flower development is observed within two months. It was essential to maintain the plants until they flowered, since *S. reilianum* only produces symptoms in the inflorescence. Approximately 50 plants were infected with each of the hybrids along with the progenitor strains and the *U. maydis* solopathogenic strain SG200, which served as a

positive control. However, only chlorosis was observed in most of the plants infected with the putative hybrids and their progenitor strains while 82% of the plants infected with SG200 formed tumours (not shown). It was then decided to increase the number of plants to approximately 300 and thereby increase the possibility of observing symptoms. With the larger number of plants infected with the hybrids, tumour development was observed in one plant each of those infected with hybrids JA1-1 and JA1-3, while no symptoms besides chlorosis was observed with hybrid JA1-2 (not shown). One tumour was formed by JA1-1, while two tumours developed from JA1-3 infection. In both infections tumour formation was restricted to the cob (Fig. 10A, B). The tumour produced by JA1-1 was smaller than the one produced by JA1-3 since part of the cob was still visible in JA1-1 while the tumour in JA1-3 completely replaced the cob. Tumours produced by JA1-1 and JA1-3 infections were excised from extraneous plant material and dried for two days to destroy any vegetative cells still present. Spores were allowed to germinate on PD plates containing ampicillin, tetracyclin and chloramphenicol to reduce bacterial contamination. Germinated

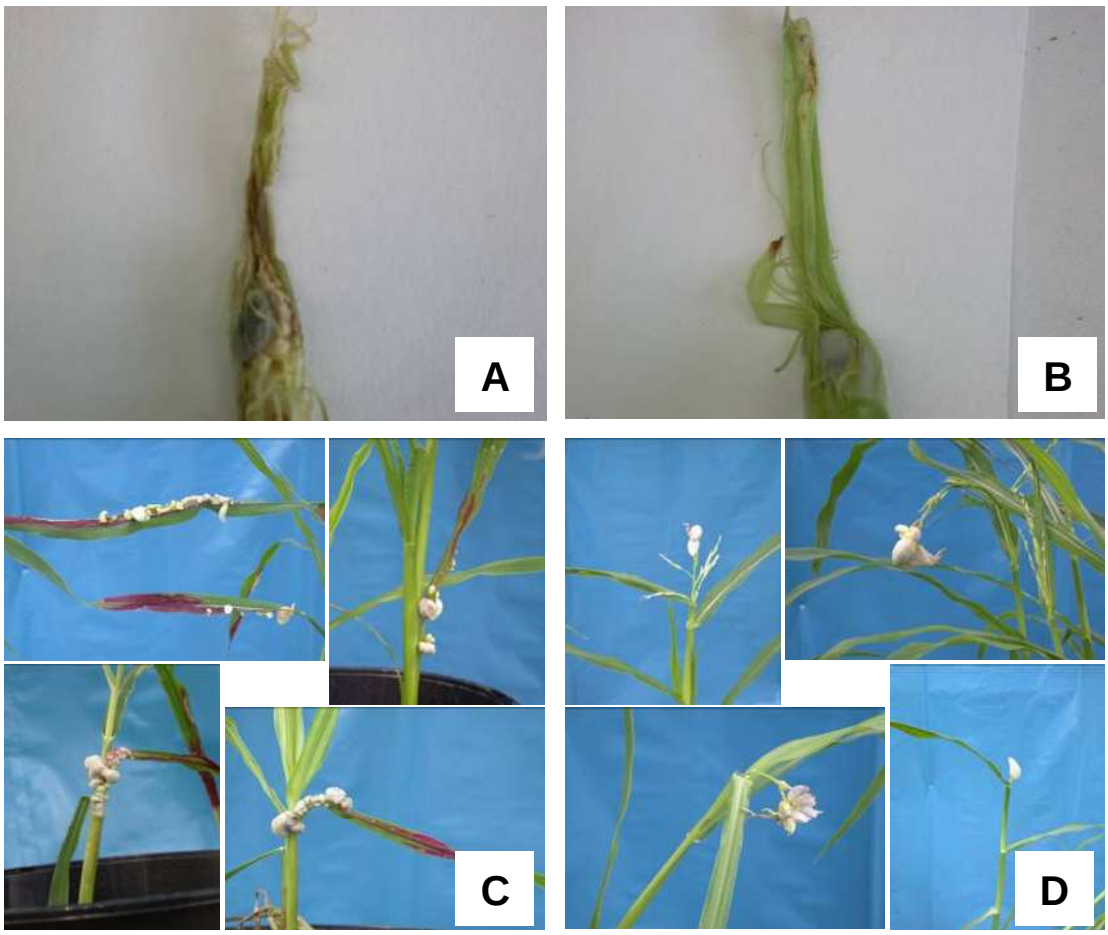

Figure 10. Symptoms of infection on the dwarf maize Gaspe Flint produced by the two intergeneric hybrids JA1-1 and JA1-3 and their progeny B7 and T2-22 respectively. **A.** Tumour formed on the cob by hybrid JA1-1. **B.** Tumour formed by hybrid JA1-3 completely replacing the cob with some leaf tumour at the tip. **C.** Several examples of the abnormally large leaf tumours and anthocyanin (red pigment on leaf) produced by infection with the progeny of hybrid JA1-1, B7. **D.** Several examples of the tumours produced predominantly in the flowers in infections with the progeny of hybrid JA1-3, T2-22.

colonies were transferred to plates containing either phleomycin or carboxin to determine the segregation of the marker genes. Twenty-four colonies from each tumour were tested for their resistance to the antibiotics as determined by the ability to grow on solid medium containing standard concentrations of the respective antibiotic. As expected, JS333 was resistant to carboxin and sensitive to phleomycin, while AB1 was sensitive to carboxin and resistant to phleomycin. A large proportion (17/23) of the progeny from hybrid JA1-1 was resistant to both antibiotics. Similarly, progeny from hybrid JA1-3 tumour 1 and tumour 2 had 21/24 and 20/24 colonies, respectively, resistant to both antibiotics.

The progeny were screened by PCR for the presence of the marker genes as determined for the hybrid parent strains i.e. *a1* for *U. maydis* and *bE2*, *a1* and Sdh genes for *S. reilianum*. A mosaic of presence and or absence of the marker genes was obtained for the progeny of both hybrid JA1-1 and hybrid JA1-3 (not shown). Unfortunately, almost all the progeny were non-viable after transferring to fresh medium. Only one strain from putative hybrid JA1-1, B7, and two strains from putative hybrid JA1-3, T2-21 and T2-22, were still viable. Interestingly, all three strains gave positive PCR results for all the above-mentioned marker genes tested, with the exception that strains B7 and T2-21 were both sensitive to phleomycin. Since the viable progeny contained most of the markers present in the parent strains the three strains were tested in plant pathogenicity assays for their ability to induce symptoms. Interestingly, two of the hybrid progeny B7 and T2-22 produced symptoms in the plant while T2-21 was completely apathogenic. Plant infections with the strain B7 resulted in unusually large tumours on the leaf and significant anthocyanin production (Fig. 10C). Symptoms of T2-22 infection were the formation of tumours predominantly in the male flower (Fig. 10D), although some were also observed on leaves (not shown). Similar symptoms were observed in a replicate experiment. These results suggest that the putative hybrids exhibit traits characteristic of both progenitor strains, but appear to encounter problems during plant infection as the rate of tumour formation is low. However, the hybrid progeny appear to be more stable and are able to lead to higher rate of tumour formation.

3.2 Molecular Characterisation of Hybrids

PCR amplification of the various marker genes available indicated that the three strains obtained from the protoplast fusion were *U. maydis* / *S. reilianum* hybrids. To analyse whether these strain were diploid and thus contain the complete genomes of

both the *U. maydis* and *S. reilianum* progenitor strains, the DNA content of each putative hybrid strain and the progenitor strains was analysed by fluorescence assisted cell sorting (FACS). This method employs nuclear-specific fluorescence labelling of cells. A measurement of the amount of fluorescence emitted by each cell in a population is made and then displayed in a histogram. Histograms of DNA fluorescence of the progenitor strains AB1 and JS333 and their fusion products JA1-1, JA1-2 and JA1-3 are shown in Fig. 11. The established *U. maydis* diploid strain FBD11 was used as a control to display the contrasting histogram to that of the haploid progenitor strains. The haploid *U. maydis* progenitor strain AB1 produced a distinct histogram from that of the diploid FBD11. Fusion product JA1-1 had a histogram that was very similar to that of AB1, indicating that hybrid JA1-1 is a haploid strain. The histograms of the hybrids JA1-2 and JA1-3 more closely resembled that of the diploid FBD11 than that of AB1, indicating that they are indeed

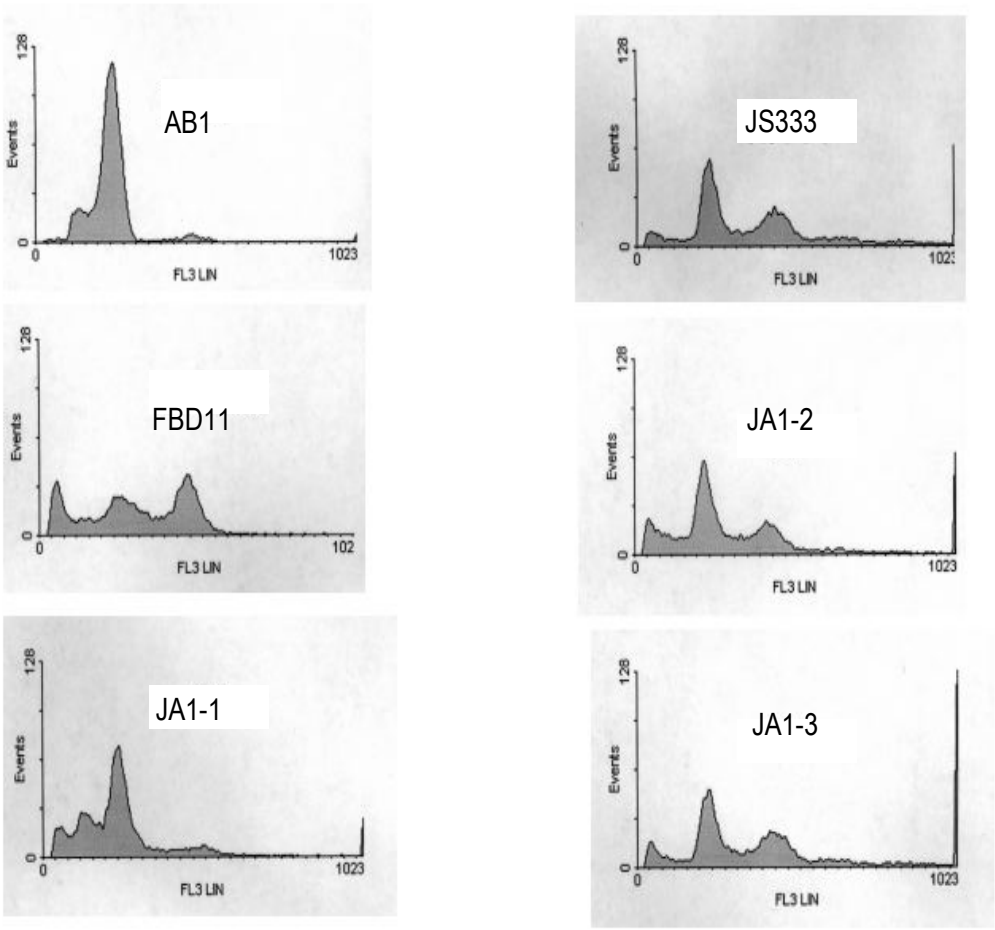

Figure 11. Histograms of fluorescence intensity determined by fluorescence assisted cell sorting (FACS) analysis to ascertain the DNA content of the *U. maydis* / *S. reilianum* putative hybrids and their progenitor strains. *U. maydis* AB1 and *S. reilianum* JS333 were used as progenitor strains in protoplast fusion to generate putative hybrids JA1-1, JA1-2 and JA1-3. *U. maydis* diploid strain FBD11 served as a control in DNA content determination. (FACS analysis was kindly performed by I. Flor-Parra)

diploid strains. Surprisingly, the histogram of the *S. reilianum* progenitor strain JS333 looked similar to that of the diploid FBD11, suggesting that the strain used to create the fusion products was already diploid. Therefore the diploid nature of the fusion products JA1-2 and JA1-3 does not necessarily imply that they carry the full genomic complements of both *U. maydis* and *S. reilianum*.

To elucidate whether complete chromosomes were inherited from the progenitor strains by the hybrids, their chromosomal DNA was analysed by pulse field gel electrophoresis using a contour-clamped homogenous electric field (CHEF) system. Since the hybrid JA1-2 was unable to produce symptoms when infected into maize plants, it was excluded from this analysis. Samples included in the analysis were the two parent strains AB1 and JS333, the hybrids JA1-1 and JA1-3, their respective progeny B7 and T2-21 & T2-22, *U. maydis* strains AB2 and FB1 and *S. reilianum* strain SRZ2. By including the strains FB1, AB2 and SRZ2 we would be able to determine if there are any chromosomal length polymorphisms (CLP) present between any of the *U. maydis* strains and similarly between any of the *S. reilianum* strains. CLPs are not a rare phenomenon as they are seen frequently in natural isolates as well as in laboratory strains and the occurrence of CLPs is increased by treatments such as transformation (Fierro and Martin, 1999). As FB1 and SRZ2 were the progenitors of AB1 and JS333 respectively, they would therefore serve as good controls for this purpose. Whole cells were imbedded in the agarose plugs before treatment with proteinase to remove the cell wall and thus ensure minimal damage to the chromosomal DNA. None of the *U. maydis* strains showed any CLP and all investigated *U. maydis* strains produced very similar banding patterns (Fig. 12). One CLP was evident between the *S. reilianum* strains JS333 and SRZ2 as seen in the size difference present in the smallest chromosomal band (Fig. 12). Hybrid JA1-1 had the same chromosomal banding pattern as that of the *U. maydis* progenitor strain AB1, while hybrid JA1-3 had the same chromosomal banding pattern as that of the *S. reilianum* progenitor strain JS333 (Fig. 12). However, the banding patterns of the hybrid JA1-1 and its progeny B7 revealed several differences indicative of an increase in size of some of the chromosomes (arrows, Fig. 12). No obvious differences were observed between the banding pattern of hybrid JA1-3 and its progeny T2-21. Most striking were the differences observed between the banding patterns of the hybrid JA1-3 and its progeny T2-22 (Fig. 12). The chromosomal banding pattern of the hybrid progeny T2-22 suggests that chromosomal

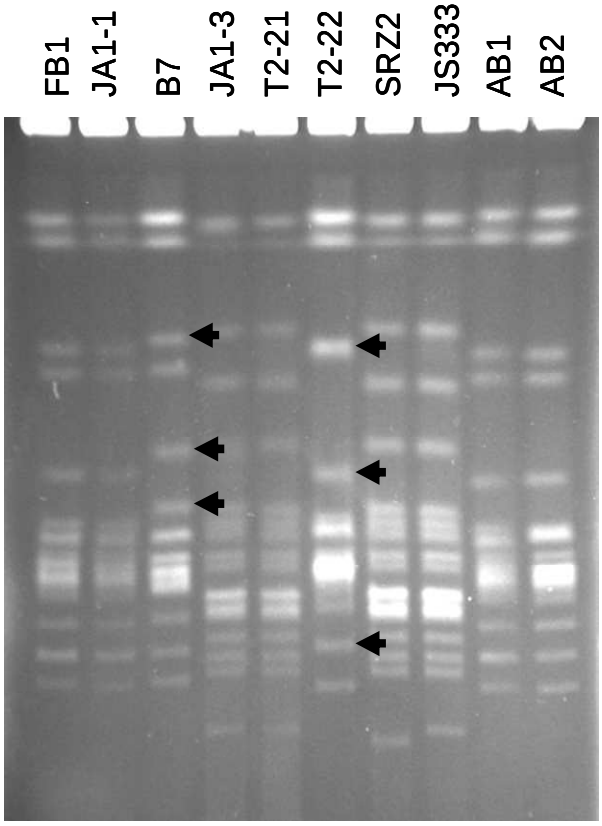

Figure 12. Pulse field gel electrophoresis using a contour-clamped homogenous electric field (CHEF) system for the separation of chromosomal DNA of *U. maydis* and *S. reilianum* progenitor strains, putative hybrids and hybrid progeny.
DNA plugs were separated in 1% TBE agarose (chromosomal grade) using 6 V/cm for 27 h with switch times of 46 sec for 10 h followed by 56 sec for 17 h and an angle of 120°. Gels were stained with ethidium bromide for visualization. The progenitor strains: *U. maydis* AB1 & AB2 and *S. reilianum* JS333. The putative hybrids: JA1-1 and JA1-3, and their progeny: B7 and T2-21 & T2-22 respectively. The *U. maydis* strain FB1 and *S. reilianum* SRZ2 were included as controls as they were the progenitor strains of the parent strains AB1 and JS333, respectively. Arrows indicate the chromosomal rearrangments observed in the progeny of the putative hybrids.

rearrangement had taken place. It most closely resembled that of *U. maydis* with loss of some chromosomes and the appearance of new ones (arrows, Fig. 12).

For complete analysis of the identity of the chromosomal bands present in each hybrid and the progeny, one would need to perform hybridisation analysis using marker genes specific to each chromosome. However, as the complete sequence of *S. reilianum* was not available at the time of this study, we employed the use of DNA microarray analysis using the customised high-density oligonucleotide *Ustilago* chips designed by Affymetrix™. Highly pure DNA obtained from the progenitor strains AB1 and JS333 and the progeny B7 and T2-22 of the respective fusion hybrids JA1-1 and JA1-3 was DNAse-treated to digest the DNA into fragments of 50 - 200 nucleotides (nt) in length. The DNA fragments were then biotinylated and hybridised to the *U. maydis* microarray chips containing 33 pairs of 25-mer oligonucleotides for each of the ca 6500 *U. maydis* genes represented, and their hybridisation signals were compared. The percentage of DNA hybridisation to the microarray for AB1, B7 and T2-22 were all 99%, while only 22% was achieved for JS333. At hybridisation levels of 80% or less, the accuracy of the data is markedly reduced (Flynn & Carr, 2007). Hybridisation signal intensities of all the genes present on the microarrays were used to make comparisons between the two progenitor strains AB1 and JS333, and the

hybrid progeny B7 and T2-22 to identify the genes that might be present or absent in the hybrid progeny as compared to either of the progenitor strains. These comparisons were displayed in the form of a scatter plot graph, in which each gene is represented by a dot. Red dots represent genes whose hybridisation signal intensities are higher in one microarray than another, while blue dots represent genes whose hybridisation signal intensities are lower. In the comparison of AB1 with B7, only one gene um11789 encoding a putative senescence associated protein was absent in B7 otherwise there was very little difference observed in the hybridisation signal intensities. All the dots cluster tightly with a few random dots falling outside the cluster but with no great significance as they represented control genes (Fig. 13A). However, in the comparison of JS333 with B7 there is a very big difference observed in the hybridisation signal intensities as they spread widely along the horizontal with most of the dots being significantly different *i.e.* either having lower intensities (blue dots) or higher intensities (red dots) (Fig. 13B). This result confirmed both the FACS analysis (of the hybrid parent JA1-1) and the CHEF gel patterns which revealed the high degree of similarity of B7 to AB1. The very weak hybridisation of JS333 in the microarray might obscure any possible existing similarities with B7. The comparison of AB1 and T2-22 revealed a similar scatter plot as that of B7 with very little significantly different signal intensities (Fig. 13C). Similarly, the comparison of JS333 with T2-22 revealed very little similarity in signal intensities as shown by the wide horizontal spread in Fig.13D. Results of this comparison are surprising as this hybrid progeny appeared to have chromosomal bands that were similar to those of JS333. The use of the *Ustilago* oligonucleotide microarrays was not able to identify any significant differences between the *U. maydis* progenitor strain and the hybrid progeny but did reveal the presence of 99% of *U. maydis* genome in both hybrid progeny. Due to the design of the microarray that relies on perfect hybridisation, it was not possible to obtain reliable information on the *S. reilianum* genomic contribution to the hybrid progeny. A conclusive result of the origin of the chromosomes would be supplied with the hybridisation of chromosomal markers specific to each of the progenitor strains

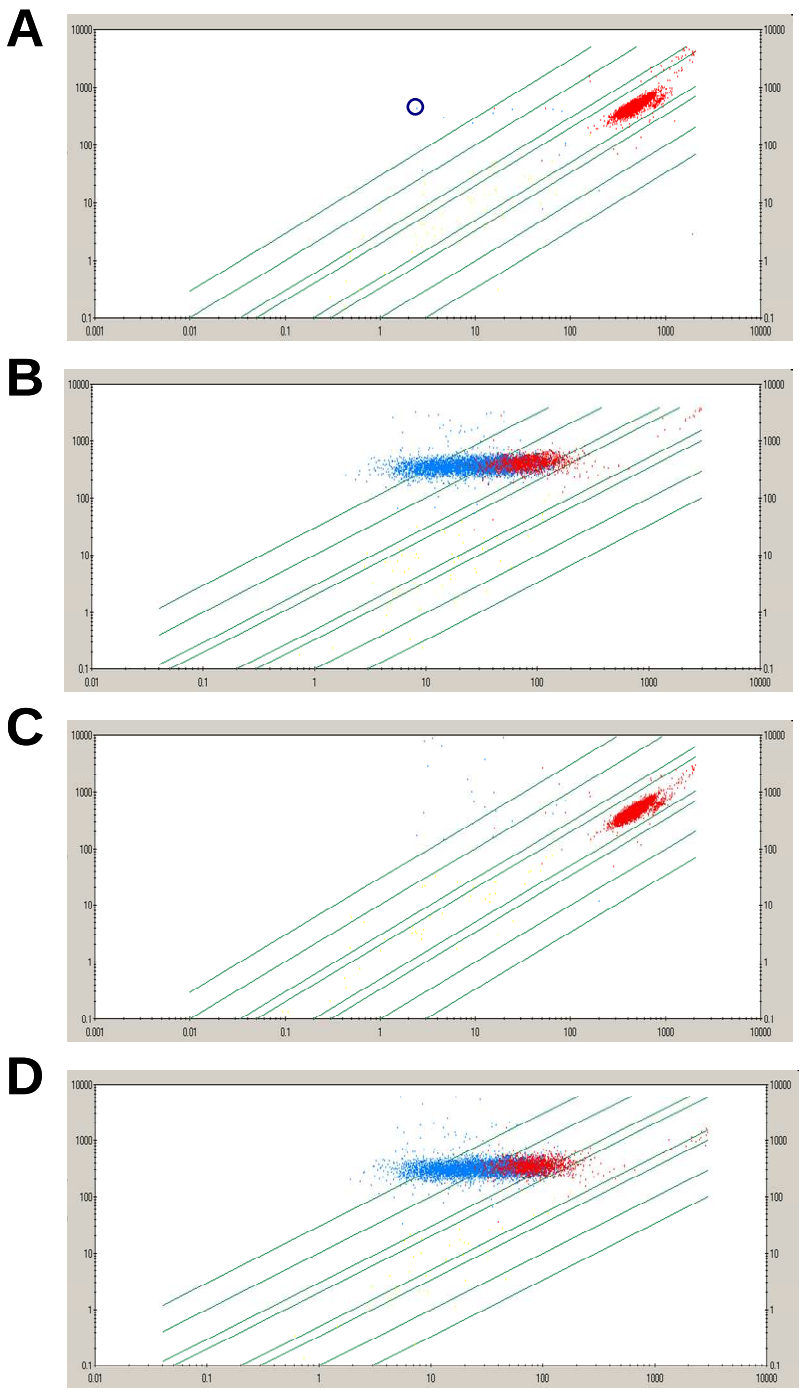

Figure 13. Scatter plot graph of the DNA microarray analysis of the progenitor strains AB1 and JS333 and the progeny B7 and T2-22 of the respective fusion hybrids JA1-1 and JA1-3. DNA obtained from each strain was digested (DNAse-treated) and hybridised to the *U. maydis* custom-made Affymetrix™ chips containing approximately 6500 genes represented by 33 pairs of 25 oligonucleotides and their hybridisation signals compared. Each gene present on the chip is represented by a dot. Comparison of hybridisation signals of: **A.** AB1 with hybrid progeny B7 (um11789 a putative senescence gene absent in B7 is circled). **B.** JS333 with hybrid progeny B7. **C.** AB1 with hybrid progeny T2-22. **D.** JS333 with hybrid progeny T2-22.

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Results III

4.1 Comparative Microscopic Analysis of the Early Infection Process in *U. maydis* and *S. reilianum* infected maize leaves

In order to identify genes related to tumour induction we chose to compare the expression profiles of *U. maydis* infected plants with those of *S. reilianum* infected plants. Therefore, it was essential to determine the time point at which a distinct alteration in fungal growth between the two pathosystems takes place. The concurrent observation of the infection process in maize infected with *U. maydis* and *S. reilianum* has never been performed at a microscopic level. As it is known that *U. maydis* infects maize seedlings and within a few days of infection induces the formation of tumours, while *S. reilianum* grows systemically within the plant, the early stages of infection were compared to identify distinct differences between the two fungi. Seven day old maize seedlings were infected with a mixture of compatible strains for both *U. maydis* (FB1xFB2) infections as well as *S. reilianum* (SRZ1xSRZ2) infections. Plants were subsequently observed and samples taken at 24 h intervals after the initial infection during a five day period. Monitoring of fungal growth within the plant microscopically was made possible by selective staining of fungal hyphae. One day post infection (dpi) both organisms show similar growth patterns and are capable of attaching to and penetrating the maize leaf surface (Fig. 14). Hyphae started growing uni-directionally weaving through the upper epidermal cell layers of the leaf. Similarly at two dpi both organisms showed no difference in hyphal growth, in both cases long hyphae were detected that branched and started to develop hyphal complexes as they grew across the expanse of the leaf and in the direction of the growing leaf (holes created by hypodermic needle served as orientation point). However, at three dpi the difference in hyphal growth between the two organisms became apparent as the hyphae of *U. maydis* appeared to be more prolific than that of *S. reilianum*. Hyphae of *U. maydis* are much shorter than those of *S. reilianum* with more branching apparent. The depth of hyphal growth had moved to the mesophyll cell layer while that of *S. reilianum* appeared to still be within the epidermal layer. *S. reilianum* hyphae were still long and continued forming hyphal complexes by branching occasionally. Four dpi *U. maydis* continued to grow within the deep

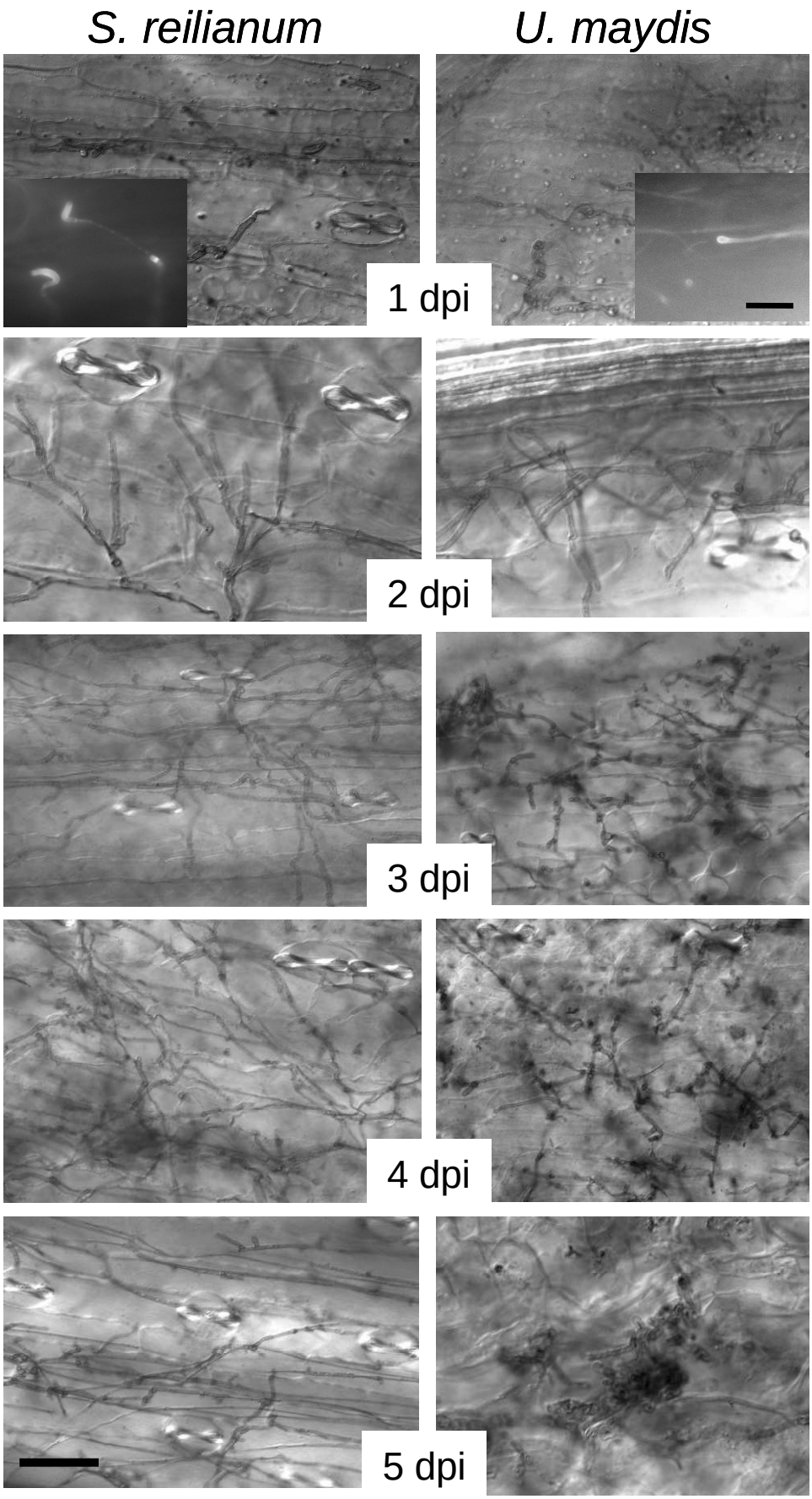


Figure 14. Microscopic images of the infection process in maize caused by *S. relianum* and *U. maydis* during a five day period. Third-leaf samples were collected at 24h intervals after initial infection. Inset at 1 dpi shows the calcofluor-stained fungal appresoria on leaf surface under UV illumination and DAPI filter. Fungal hyphae in maize leaves were selectively stained with Chlorazole Black E and visualised using differential interference contrast (DIC) microscopy. Representative samples of *S. reilianum*-infected maize leaves are on the left while *U. maydis*-infected maize leaves are on the right. Bar = 30µm

mesophyll cell layer with hyphae that were short and branched very often. No distinct difference was observed in *S. reilianum* hyphal growth from three dpi. On the fifth dpi *U. maydis* hyphae appeared to have undergone another alteration in growth as the hyphae appeared to be thicker, had condensed dramatically and were highly segmented. An alteration in the appearance of the plant tissue in the form of enlarged plant cells was also evident. Again no distinct difference in hyphal growth compared to three dpi was observed in *S. reilianum*. This comparative analysis revealed that distinct differences in mode of fungal growth are most evident at three and four dpi between the two pathosystems and would serve as suitable time points to identify genes responsible for these differences.

4.2 cDNA-AFLP Transcript profiling of the *U. maydis*-maize and *S. reilianum*-maize pathosystems

We employed the cDNA-AFLP transcript profiling technique to concurrently compare the expression profiles of *U. maydis* infected maize and *S. reilianum* infected maize. Since the transcript profiling was to be of infected maize tissue, extraneous fungal material on the leaf surface first had to be removed from leaf samples. Several methods were tested to determine an efficient method for the removal of extraneous fungal material from maize leaves. These included sonication, treatment with solutions of SDS or fungal lytic enzyme (Novozyme234) or liquid latex. Evidence of the removal of fungal material from the leaves was confirmed by staining the leaves with Calcofluor and visualisation of any residual fungal material by microscopy. Stripping of leaves after the application of liquid latex was the most efficient treatment for the removal of fungal material from maize leaves while treatment with Novozyme234 and sonication were the most inefficient (Fig. 15).

To identify genes that are differentially regulated between the two pathosystems, two time points were selected at which significant differences in fungal growth were evident between the two fungi, namely three and four dpi. Seven day-old maize plants of Early Golden Bantam variety (Old Seeds) were inoculated by injecting a sporidial suspension of compatible strains of either *U. maydis* (FB1 x FB2) or *S. reilianum* (SRZ1 x SRZ2) into the leaf whorl. A single sporidial culture of *U. maydis* (FB1) or *S. reilianum* (SRZ1) inoculated into plants served as negative controls to subtract any wounding induced genes. Uninfected plants were also kept as a

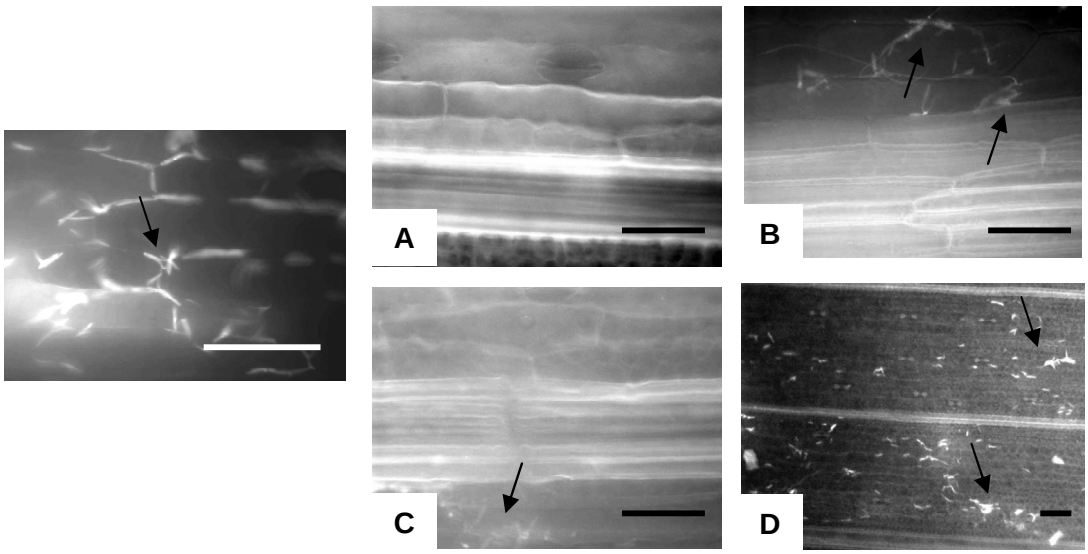


Figure 15. Microscopic images of maize leaves after different leaf pre-treatments applied to remove extraneous fungal material from leaf surfaces. Leaf samples were first treated to remove fungi then stained with Calcofluor (Sigma^{TM}Fluorescent Brightner) and viewed by microscope using a fluorescent filter. Image on the left was taken as a before treatment control to confirm the presence of fungi on the leaf surface. **A**. Liquid latex; **B**. Novozyme234; **C**. SDS; **D**. Sonication. Residual fungal material remaining after treatment is indicated by arrows. Bar = 30µm

control. Plants were harvested three and four days post infection. The third leaf of 16-20 plants per infection was excised and latex-treated to remove fungal material on the surface. Stripped leaves were flash-frozen in liquid nitrogen, pooled and used for cDNA preparation. cDNA-AFLP reactions were performed with 64 primer combinations for selective amplification, with three selective bases per primer (*EcoR* I-NNN / *Mse* I-NNN). An example of a typical cDNA-AFLP autoradiogram generated with the primer combinations indicated is shown in Fig. 16. AFLP fragments ranged from approximately 40 – 800 bp in size, with an average number of 40 bands per lane generated for each primer combination. Thus, it is estimated that approximately 2560 different messenger RNAs have been visualised using a set of 64 primer combinations. Selection of the transcript derived fragments (TDFs) was on the basis of the fragment being present only in the sample of interest and absent in all other samples and controls. Use of this criterion was based on the premise that the genes of interest would be expressed exclusively in one pathosystem and at one time point during plant infection. This is illustrated in Fig. 16 as indicated by the arrows. Resultantly, 322 bands ranging in length from 31 bp to 820 bp were excised from gels. All the excised bands were reamplified, cloned and sequenced at least in duplicate for each TDF. Sequence analysis of the 322 TDFs revealed a total number of 218 unique sequences where 197 were singletons and a low redundancy value (10%) where sequences were duplicated. A complete list of the 322 TDFs and their

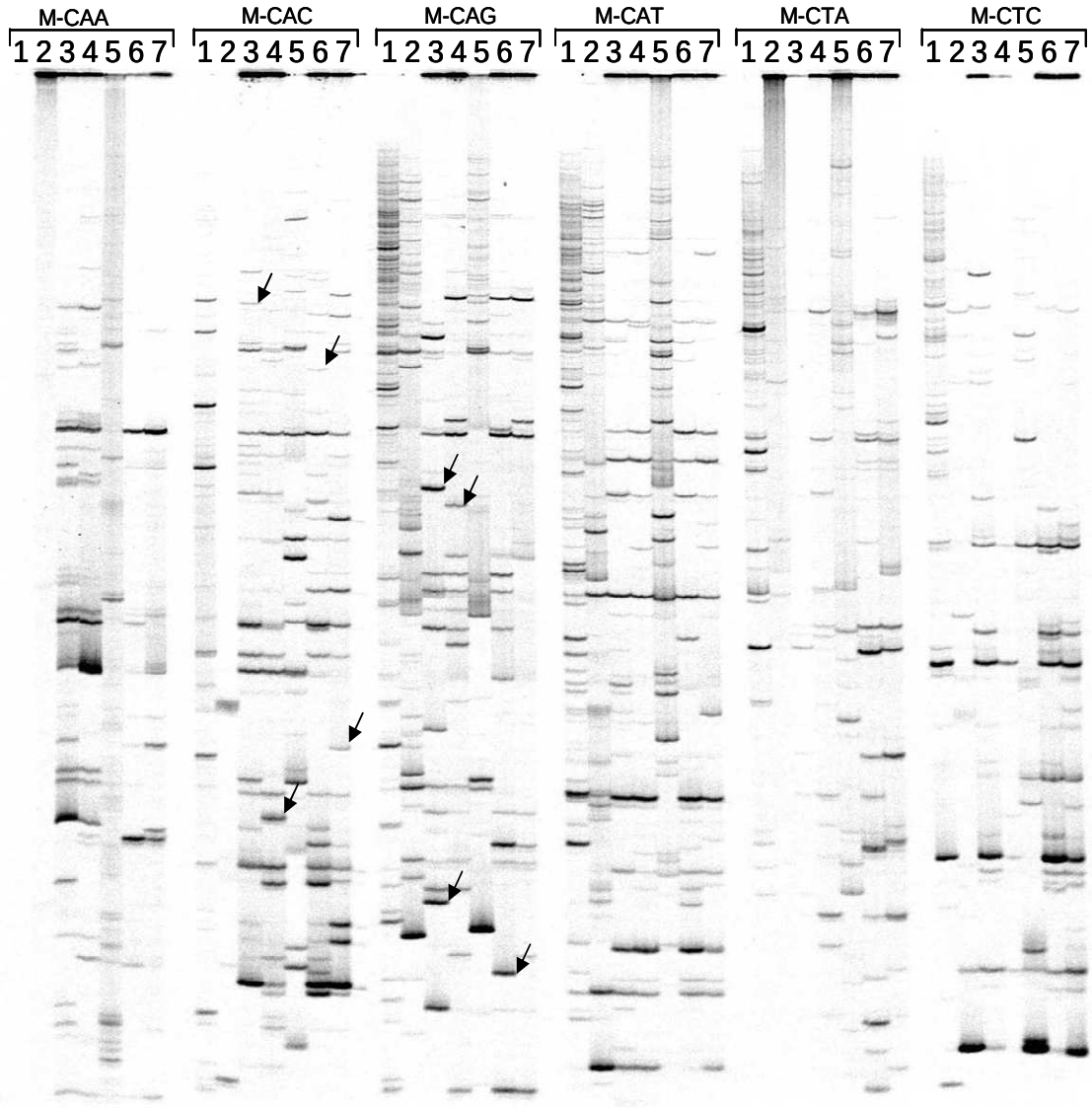


Figure 16. An autoradiogram of the cDNA-AFLP transcript profiles of samples from *U. maydis* infected tissue, *S. reilianum* infected tissue and necessary controls. Amplicons were generated using the *EcoR* I primer E-ACC and the different *Mse* I primers labeled above each set of lanes. 1 = Uninfected maize, 2 = *U. maydis* FB2 infected maize, 3 = *U. maydis* FB1xFB2 infected maize 3dpi, 4 = *U. maydis* FB1xFB2 infected maize 4dpi, 5 = *S. reilianum* SRZ1 infected maize, 6 = *S. reilianum* SRZ2xSRZ1 infected maize 3dpi, 7 = *S. reilianum* SRZ2xSRZ1 infected maize 4dpi. Amplicons resulting from specific primer combinations were separated by PAGE (5%). Amplicons were selected as being present only in *U. maydis* 3dpi and absent in the rest and likewise for 4dpi and *S. reilianum* 3dpi and 4dpi (indicated by arrows). Differential fragments were excised from the gel, reamplified with the same selective primers, cloned into pCR-Topo vector and sequenced. Sequences were analysed by comparison to public databases (BLAST-N).

corresponding nucleotide similarities to annotated genes is given in Table 3. The largest percentage of genes identified was of plant origin (60%) while 22% were of fungal origin and the remaining 18% could not be assigned. This is quite encouraging as the total amount of fungal biomass in the plant during early stages of infection has been shown to be very low (Basse *et al*., 2000; 2002). Of the fungal genes identified more than 70% (34/48) were specific to the pathosystem and time point, i.e. were solely identified in tissue isolated from *U. maydis* infection 3 dpi for example. Table 4

contains a list of the 34 differentially identified TDFs of fungal origin. Most of the sequences were of genes coding for hypothetical proteins of unknown function (40%), while those of known function were mostly those involved in basic metabolism or transport (Table 4). Interestingly, approximately 30% of the TDF sequences were identical to fungal intergenic or spacer DNA. There are several possible explanations for this occurrence: it might be due to the presence of long 5' or 3' UTRs in fungal mRNAs, indicate the presence of small non-coding RNAs, or be a result of the incorrect assignment of ORFs in the *U. maydis* genome. In addition several of the TDFs (TDF 10; 26; 158; 178; 202), derived from *S. reilianum* 3 dpi and 4 dpi infected material, have no sequence similarity with the *S. reilianum* genome database. This suggests that these TDFs are probably *U. maydis* contaminants since they all have sequence similarity to the *U. maydis* genome database.

Table 3. List of differentially occurring fragments as identified by cDNA-AFLP transcript profiling of the *U. maydis*-maize and *S. reilianum*-maize pathosystems.

Source*	TDF & Clone	Annotation	Blast N identities	Accession number
Um 3dpi	1#7	*Zea mays* PCO073595 mRNA sequence	266/267	AY103693
Um 3dpi	2#5	*Zea mays* PCO068796 mRNA sequence	114/114	AY108004
Um 3dpi	3#2	*Homo sapiens* DNA sequence from clone RP11-492O8 on chromosome X Contains a H2A histone family B pseudogene	21/77	AL592156
Um 3dpi	4#5	*Zea mays* PCO063180 mRNA sequence	48/56	AY103778
Um 3dpi	5#4	*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 7, BAC clone:OJ1354_H07	19/55	AP003755
Um 3dpi	11#1	*Lotus corniculatus* var. japonicus genomic DNA	25/450	AP004973
Um 3dpi	12#5	*Ustilago maydis* conserved hypothetical protein	401/402	XM_754006
Um 3dpi	13#1	*Oryza sativa* (japonica cultivar-group) chromosome 3	36/251	DP000009
Um 3dpi	14#5	*Zea mays* clone Contig220 mRNA sequence	230/232	BT016387
Um 3dpi	15#4	*Ustilago maydis* between related to SFT2/YBL102W & probable CCT6	154/155	(um02349 & um02350)
Um 3dpi	16#1	*Zea mays* clone EL01N0447F12.c mRNA sequence	99/107	BT017726
Um 3dpi	17#1	*Zea mays* clone Contig250 mRNA sequence	138/175	BT016417
Um 3dpi	32#1	*Oryza sativa* (japonica cultivar-group) chromosome 5 clone P0486C01	64/372	AC135924
Um 3dpi	33#4	*Zea mays* clone EL01T0403A05.c mRNA sequence	356/358	BT018338
Um 3dpi	34#6	*Zea mays* CL9605_1 mRNA sequence	319/337	AY109619
Um 3dpi	35#1	*Zea mays* chloroplast rbcL gene for ribulose bisphosphate carboxylase	221/221	CHZMRBCLG
Um 3dpi	36#8	*Zea mays* CL9999_1 mRNA sequence	119/160	AY109995
Um 3dpi	52#1	*Oryza sativa* (japonica cultivar-group)	32/254	XM_450624
Um 3dpi	65#3	*Lotus corniculatus* var. japonicus genomic DNA, chromosome 3	25/452	AP004973
Um 3dpi	66#2	*Ustilago maydis* probable RLI1 - Protein promoting pre-initiation complex assembly	214/317	(um03351)
Um 3dpi	67#4	*Ustilago maydis* probable RLI1 - Protein promoting pre-initiation complex assembly	213/317	(um03351)
Um 3dpi	77#4	*Zea mays* single myb histone 6 (Smh6) mRNA, complete cds	275/279	AY280632
Um 3dpi	78#3	*Zea mays* chloroplast rbcL gene for ribulose bisphosphate carboxylase	227/229	CHZMRBCLG
Um 3dpi	83#1	*Zea mays* PCO144739 mRNA sequence	96/150	AY107571
Um 3dpi	87#4	*Oryza sativa* (japonica cultivar-group) cDNA clone:J033074G02	309/365	AK099385
Um 3dpi	88#4	*Zea mays* ZmRR10 mRNA for response regulator 10	281/284	AB071695
Um 3dpi	89#2	*Oryza sativa* (japonica cultivar-group), predicted mRNA sequence	555/781	XM_473901
Um 3dpi	97#3	*Zea mays* strain NB mitochondrion	223/288	AY506529
Um 3dpi	98#2	*Zea mays* CL7689_-2 mRNA sequence	218/263	AY111537
Um 3dpi	99#4	*Oryza sativa* (japonica cultivar-group) cDNA clone:J033030G12	133/182	AK073324
Um 3dpi	107#1	*Zea mays* clone Contig77 mRNA sequence	119/213	BT016244
Um 3dpi	108#1	*Zea mays* clone Contig77 mRNA sequence	119/167	BT016244
Um 3dpi	111#1	*Zea mays* clone EL01N0422F01 mRNA sequence	178/256	BT024030
Um 3dpi	112#1	*Homo sapiens* PAC clone RP4-745K6 from 7	22/247	AC004875
Um 3dpi	113#1	*Zea mays* PCO156137 mRNA sequence	95/148	AY103541
Um 3dpi	114#1	*Oryza sativa* (japonica cultivar-group)	105/129	XM_463434
Um 3dpi	116#2	*Zea mays* CL7689_-2 mRNA sequence	218/263	AY111537
Um 3dpi	126#1	*Zea mays* beta-D-glucosidase precursor (glu2) mRNA sequence	107/107	ZMU44087
Um 3dpi	131#1	*Ustilago maydis* hypothetical protein	166/258	(um05920)
Um 3dpi	144#2	*Ustilago maydis* related to nitrilase	152/152	(um10444 / um03453)
Um 3dpi	146#1	*Ustilago maydis* hypothetical protein	165/273	(um05920 / umd216-90)
Um 3dpi	147#2	*Ustilago maydis* related to nitrilase	390/392	(um10444 / um03453)
Um 3dpi	150#5	*Zea mays* PCO069828 mRNA sequence	290/305	AY108770
Um 3dpi	151#6	*Oryza sativa* (japonica cultivar-group) cDNA clone:J033049G04	98/247	AK121627

Table 3. (Cont.)

Source*	TDF & Clone	Annotation	Blast N identities	Accession number
Um 3dpi	167#2	*Oryza sativa* (japonica cultivar-group) cDNA clone:J013116G03	397/431	AK072081
Um 3dpi	168#1	*Ustilago maydis* between two conserved hypothetical proteins	130/130	(um01136 & um01137)
Um 3dpi	169#1	*Sporisorium reilianum* between rba2 gene & mfa2.1 gene	104/104	AJ884589
Um 3dpi	179#1	*Oryza sativa* (japonica cultivar-group)	104/181	NM_191100
Um 3dpi	180#1	*Oryza sativa* (japonica cultivar-group)	105/181	NM_191100
Um 3dpi	181#3	*Oryza sativa* (japonica cultivar-group)	105/181	NM_191100
Um 3dpi	182#4	*Sporisorium reilianum* between rba2 gene & mfa2.1 gene	101/104	AJ884589
Um 3dpi	183#4	*Sporisorium reilianum* between rba2 gene & mfa2.1 gene	103/104	AJ884589
Um 3dpi	184#6	*Yarrowia lipolytica* chromosome C of strain CLIB122	19/53	CR382129
Um 3dpi	185#4	*Ustilago maydis* hypothetical protein	20/36	(um04147)
Um 3dpi	194#4	*Ustilago maydis* between conserved hypothetical & hypothetical protein	129/130	(um01136 & um11641)
Um 3dpi	195#5	*Ustilago maydis* between conserved hypothetical & hypothetical protein	128/128	(um01136 & um11641)
Um 3dpi	196#4	*Zea mays* clone EL01N0551A05.d mRNA sequence	105/113	BT018825
Um 3dpi	203#5	Zea may BAC clone c573L14	129/166	AY555143
Um 3dpi	206#1	*Zea mays* PCO131995 mRNA sequence	100/102	AY105969
Um 3dpi	214#3	*Mus musculus* BAC clone RP23-492P8	19/60	AC126686
Um 3dpi	218#1	*Zea mays* strain NB mitochondrion	57/72	AY506529
Um 3dpi	221#2	*Zea mays* 18S small subunit ribosomal RNA gene	327/614	AF168884
Um 3dpi	225#1	*Oryza sativa* (japonica cultivar-group)	275/339	XM_480608
Um 3dpi	229#1	*Oryza sativa* (japonica cultivar-group)	136/251	DP000009
Um 3dpi	230#1	*Oryza sativa* (japonica cultivar-group)	136/251	DP000009
Um 3dpi	231#1	*Oryza sativa* (japonica cultivar-group)	56/107	DP000009
Um 3dpi	232#1	*Zea mays* PCO139174 mRNA sequence	85/85	AY108191
Um 3dpi	233#4	*Ustilago maydis* gene for small subunit ribosomal RNA	42/55	X62396
Um 3dpi	248#1	*Oryza sativa* (japonica cultivar-group) chromosome 5 clone P0486C01	207/371	AC135924
Um 3dpi	249#2	*Zea mays* PCO120439 mRNA sequence	162/220	AY108271
Um 3dpi	250#4	*Zea mays* clone EL01N0406D12.c mRNA	65/76	BT017449
Um 3dpi	260#1	*Mus musculus* chromosome 3, clone RP223-387I7	25/152	AC113278.15
Um 3dpi	261#1	*Ustilago maydis* conserved hypothetical protein	86/111	(um04464)
Um 3dpi	262#1	*Penaeus monodon* clone 050 AFLP marker	25/96	DQ132927
Um 3dpi	270#1	*Ustilago maydis* related to nitrilase	152/152	(um10444 / um03453)
Um 3dpi	274#1	*Ustilago maydis* related to WD40 repeat protein CreC	704/707	(um02846)
Um 3dpi	275#1	*Lotus corniculatus* var. japonicus gDNA	25/450	AP004973
Um 3dpi	276#1	*Oryza sativa* clone:J033091J01	286/324	AK102362
Um 3dpi	277#1	*Oryza sativa* clone:J033091J01	286/317	AK102362
Um 3dpi	284#2	*Oryza sativa* (japonica cultivar-group)	313/397	XM_476365
Um 3dpi	285#4	*Zea mays* PCO107820 mRNA sequence	116/116	AY105079
Um 3dpi	286#4	*Oryza sativa* (japonica cultivar-group) clone OSJNBb0063D09 map S1559	66/107	AC120539.4
Um 3dpi	292#2	*Zea mays* CL598_2 mRNA sequence	403/685	AY109415
Um 3dpi	293#1	*Homo sapiens* DNA sequence from clone RP11-987D21	22/494	BX119919.5
Um 3dpi	294#4	*Oryza sativa* (japonica cultivar-group) cDNA clone:J023025N13	388/466	AK069442.1
Um 3dpi	295#3	*Zea mays* clone EL01N0316B09.c mRNA sequence	244/273	BT017299.1
Um 3dpi	300#4	*Zea mays* PCO110957 mRNA sequence	274/276	AY108546
Um 3dpi	305#1	*Ustilago maydis* related to Acetoacetyl-CoA synthetase	100/383	(um10088)
Um 3dpi	306#3	*Zea mays* CL7400_1 mRNA sequence	128/196	AY110160
Um 3dpi	307#1	*Triticum aestivum* clone wlmk4.pk0003.f5:fis	167/189	BT009434.1
Um 3dpi	310#4	*Zea mays* DnaJ-related protein ZMDJ1 (mdJ1) gene	485/486	AF053468
Um 3dpi	311#1	*Oryza sativa* (japonica cultivar-group) cDNA clone:J033147K10	397/483	AK122164
Um 3dpi	314#1	*Ustilago maydis* related to Na-K-Cl cotransporter	99/175	(um10987)

Table 3. (Cont.)

Source*	TDF & Clone	Annotation	Blast N identities	Accession number
Um 3dpi	318#5	*Ustilago maydis* related to Acetoacetyl-CoA synthetase	168/383	(um10088)
Um 3dpi	319#4	*Cintractia sorghi-vulgaris* isolate AFTOL-ID 867 25S large subunit ribosomal RNA gene	109/169	AY745726
Um 3dpi	321#2	*Zea mays* CL64795_1 mRNA sequence *Zea mays* CL64795_1 mRNA sequence	59/125	AY109924
Um 4dpi	6#3	*Mus musculus* BAC clone RP24-211B22 from chromosome 14	19/31	AC126437.3
Um 4dpi	7#2	*Zea mays* PCO133340 mRNA sequence	451/454	AY109265
Um 4dpi	8#1	*M. capricolum* DNA for CONTIG MC434	21/67	MC434
Um 4dpi	18#3	*Zea mays* CL7689_-2 mRNA sequence	214/329	AY111537
Um 4dpi	19#2	*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 7	21/193	AP008213
Um 4dpi	20#6	*Zea mays* PCO137834 mRNA sequence	98/102	AY103926
Um 4dpi	21#1	*Zea mays* PCO139174 mRNA sequence	85/85	AY108191
Um 4dpi	22#1	*Zea mays* pyruvate dehydrogenase kinase isoform 2	63/94	AF038586
Um 4dpi	37#4	*Oryza sativa* (japonica cultivar-group) chromosome 3	36/250	DP000009
Um 4dpi	38#5	*Zea mays* PCO140120 mRNA sequence	67/217	AY107993
Um 4dpi	39#4	*Ustilago maydis* hypothetical protein	181/187	XM_756707
Um 4dpi	40#7	*Zea mays* CL9999_1 mRNA sequence	126/157	AY109995
Um 4dpi	47#1	*Ustilago maydis* between conserved hypothetical protein & probable GTPase Rab7 protein	367/368	(um05510 & um05511)
Um 4dpi	53#4	*Zea mays* CL9999_1 mRNA sequence	126/160	AY109995
Um 4dpi	54#3	*Oryza sativa* (japonica cultivar-group) chromosome 3	36/254	DP000009
Um 4dpi	55#8	*Triticum aestivum* partial mRNA for IAA1 protein	54/68	AJ575098
Um 4dpi	68#1	*Zea mays* CL337_1 mRNA sequence	27/95	AY109445
Um 4dpi	79#3	*Zea mays* PCO074909 mRNA sequence	88/88	AY104770
Um 4dpi	80#3	*Ustilago maydis* between related to microsomal glutathione s-transferase 3 & related to stress response protein rds1p	69/69	(um05561 / um05562)
Um 4dpi	84#2	*Zea mays* PCO144739 mRNA sequence	97/150	AY107571
Um 4dpi	90#1	*Zea mays* clone Contig745 mRNA sequence	622/624	BT016912
Um 4dpi	91#1	*Zea mays* PCO154872 mRNA sequence	233/238	AY103621
Um 4dpi	92#4	*Zea mays* PCO154872 mRNA sequence	232/238	AY103621
Um 4dpi	93#1	*Zea mays* PCO071008 mRNA sequence	53/53	AY107260
Um 4dpi	100#5	Asimina triloba 26S ribosomal RNA gene	109/109	AY095451
Um 4dpi	109#3	*Ustilago maydis* between hypothetical protein & conserved hypothetical protein	250/251	(um05573 & um05574)
Um 4dpi	115#5	*Zea mays* clone Contig584 mRNA sequence	363/363	BT016751
Um 4dpi	117#5	*Zea mays* PCO156137 mRNA sequence	80/287	AY103541
Um 4dpi	127#4	*Zea mays* PCO114884 mRNA sequence	193/194	AY103874
Um 4dpi	132#2	*Zea mays* mRNA for translation initiation factor 5A	604/820	Y07920
Um 4dpi	133#3	*Zea mays* clone Contig77 mRNA sequence	235/327	BT016244
Um 4dpi	137#1	*Ustilago maydis* hypothetical protein	372/373	(um00230)
Um 4dpi	138#4	*Mus musculus* chromosome 5, clone RP23-129N14	21/142	AC116715.22
Um 4dpi	141#1	*Zea mays* clone BAC 276N12-123C01	275/325	AY371488
Um 4dpi	148#1	*Zea mays* chloroplast phytoene synthase 1 (PSY1) gene	41/131	AY324431
Um 4dpi	152#3	*Ustilago maydis* between related to COP9 - signalosome complex subunit 4 & conserved hypothetical protein	363/365	(um00849)
Um 4dpi	153#3	*Zea mays* PCO117545 mRNA sequence	162/197	AY105797
Um 4dpi	154#3	*Zea mays* chloroplast 3'part of rpoC2 gene, rps2 gene, atpI gene and 5'part of atpH gene	75/105	X52270
Um 4dpi	160#1	*Ustilago maydis* between related to coenzyme a synthetase & probable AAP1 - alanine/arginine aminopeptidase	42/42	(um00791 & um00792)
Um 4dpi	161#3	*Zea mays* clone EL01N0531G10.d mRNA sequence	91/91	BT018816
Um 4dpi	162#3	*Ustilago maydis* between hypothetical protein & probable MTR4 - involved in nucleocytoplasmic transport of mRNA	86/86	(um01483 & um01484)

Table 3. (Cont.)

Source*	TDF & Clone	Annotation	Blast N identities	Accession number
Um 4dpi	170#3	*Mus musculus* BAC clone RP23-189L19 from chromosome 17	21/325	AC127341.3
Um 4dpi	171#1	*Oryza sativa* (japonica cultivar-group) genomic DNA	105/181	NM_191100
Um 4dpi	172#6	*Zea mays* PCO133003 mRNA sequence	171/172	AY108763
Um 4dpi	173#4	*Zea mays* CL7048_1 mRNA sequence	142/162	AY110237
Um 4dpi	186#1	*Oryza sativa* (japonica cultivar-group) genomic DNA	89/121	XM_475685
Um 4dpi	204#1	*Zea mays* PCO154872 mRNA sequence	234/238	AY103621
Um 4dpi	215#1	Unidentified bacterium clone zdt-9n	259/259	AC150248.3
Um 4dpi	216#5	*Ustilago maydis* hypothetical protein	227/412	(um05004)
Um 4dpi	219#1	*Zea mays* PCO126859 mRNA sequence	122/122	AY104837
Um 4dpi	222#1	*Zea mays* clone Contig286 mRNA sequence	419/431	BT016453
Um 4dpi	223#1	*Zea mays* formate tetrahyrofolate ligase mRNA	160/214	AF439727
Um 4dpi	226#6	*Zea mays* PCO100830 mRNA sequence	54/108	AY103766
Um 4dpi	234#4	*Zea mays* single myb histone 6 (Smh6)	284/333	AY280632
Um 4dpi	235#4	*Zea mays* single myb histone 6 (Smh6)	285/333	AY280632
Um 4dpi	236#5	*Zea mays* single myb histone 6 (Smh6)	285/333	AY280632
Um 4dpi	237#5	*Zea mays* PCO139174 mRNA sequence	82/85	AY108191
Um 4dpi	251#4	*Ustilago maydis* conserved hypothetical protein	237/412	(um04196)
Um 4dpi	252#5	*Zea mays* clone EL01N0417F11.d mRNA sequence	187/211	BT018466
Um 4dpi	253#1	Human DNA sequence from clone RP11-632C17	21/147	AL450405
Um 4dpi	254#1	*Zea mays* clone EL01N0417F11.d mRNA sequence	187/211	BT018466
Um 4dpi	263#2	*Ustilago maydis* between conserved hypothetical protein & probable GTPase Rab7 protein	366/368	(um10348 & um05511)
Um 4dpi	264#1	*Zea mays* PCO100830 mRNA sequence	89/89	AY103766
Um 4dpi	271#2	*Zea mays* clone EL01N0448D09.d mRNA sequence	81/116	BT018598
Um 4dpi	278#1	*Zea mays* single myb histone 5 (Smh5)	500/534	AY280630
Um 4dpi	279#1	*Oryza sativa* (japonica cultivar-group) genomic DNA	21/193	AP008213
Um 4dpi	296#1	*Zea mays* mRNA gs2 for glutamine synthetase	548/553	X65931
Um 4dpi	297#4	*Ustilago maydis* hypothetical protein	219/319	(um01749)
Um 4dpi	308#4	*Zea mays* CL7400_1 mRNA sequence	152/270	AY110160
Um 4dpi	313#4	*Oryza sativa* (japonica cultivar-group)	327/508	NM_187061
Um 4dpi	315#1	*Triticum aestivum* unknown sequence	22/124	AY428632
Sr 3dpi	23#2	*Zea mays* strain NB mitochondrion Features in this part of subject sequence: NADH dehydrogenase subunit 1 NADH dehydrogenase subunit 2	272/386	AY506529
Sr 3dpi	24#1	*Zea mays* GAPC2 glyceraldehyde-3-phosphate dehydrogenase	232/232	L13432
Sr 3dpi	25#1	*Zea mays* PCO073595 mRNA sequence	200/217	AY103693
Sr 3dpi	26#1	*Ustilago maydis* hypothetical protein	19/162	(um03221)
Sr 3dpi	41#11	*Oryza sativa* (japonica cultivar-group)	137/165	XM_481597
Sr 3dpi	42#7	*Ustilago maydis* hypothetical protein	154/156	(um03221)
Sr 3dpi	48#2	*Oryza sativa* (japonica cultivar-group)	292/327	XM_450624
Sr 3dpi	49#2	*Zea mays* PCO137834 mRNA sequence	98/101	AY103926
Sr 3dpi	50#3	*Oryza sativa* (japonica cultivar-group)	32/254	XM_450624
Sr 3dpi	56#6	*Ustilago maydis* probable t-complex-type molecular chaperone, epsilon subunit	132/188	(um03959)
Sr 3dpi	57#5	*Ustilago maydis* related to nitrilase	151/151	(um10444 / um03453)
Sr 3dpi	58#1	*Oryza sativa* (japonica cultivar-group) chromosome 10	19/92	AE017116
Sr 3dpi	59#1	*Zea mays* clone EL01N0531B06.d mRNA sequence	53/53	BT018815
Sr 3dpi	60#2	*Zea mays* clone EL01N0531B06.d mRNA sequence	53/53	BT018815
Sr 3dpi	69#5	*Danio rerio* mRNA for PrP-like protein (stPrP606 gene)	22/197	AJ704630
Sr 3dpi	70#6	*Zea mays* clone EL01N0551C07.d mRNA sequence	138/169	BT018829
Sr 3dpi	71#4	*Ustilago maydis* conserved hypothetical protein	155/156	(um03689)
Sr 3dpi	72#2	*Oryza sativa* (japonica cultivar-group) chromosome 5 clone OJ1735_C10	72/78	AC104284

Table 3. (Cont.)

Source*	TDF & Clone	Annotation	Blast N identities	Accession number
Sr 3dpi	85#5	*Schizosaccharomyces pombe* chromosome I cosmid c17G6	22/23	Z99162.1
Sr 3dpi	94#1	*Oryza sativa* (japonica cultivar-group)	253/688	XM_482964
Sr 3dpi	101#3	*Zea mays* CL501_1 mRNA sequence	438/459	AY109428
Sr 3dpi	102#2	*Zea mays* CL7689_-2 mRNA sequence	219/330	AY111537
Sr 3dpi	103#2	*Oryza sativa* (japonica cultivar-group) cDNA clone:J033030G12	133/182	AK073324
Sr 3dpi	105#2	*Oryza sativa* (japonica cultivar-group) chromosome 10	274/390	AE017114
Sr 3dpi	118#1	*Zea mays* CL539_1 mRNA sequence	103/309	AY109328
Sr 3dpi	119#1	*Zea mays* PCO156137 mRNA sequence	22/369	AY103541
Sr 3dpi	120#3	*Zea mays* PCO149393 mRNA sequence	206/253	AY103798
Sr 3dpi	121#1	*Zea mays* PCO095766 mRNA sequence	151/151	AY108033
Sr 3dpi	128#10	*Zea mays* PCO114884 mRNA sequence	193/194	AY103874
Sr 3dpi	129#2	*Zea mays* 18S ribosomal RNA gene, partial sequence	279/358	U42796
Sr 3dpi	134#4	*Zea mays* strain NB mitochondrion Features in this part of subject sequence: NADH dehydrogenase subunit 1 NADH dehydrogenase subunit 2	274/274	AY506529
Sr 3dpi	135#2	*Ustilago maydis* between related to SSP120 - secretory protein & hypothetical protein	91/142	(um11591 & um05997)
Sr 3dpi	139#5	*Pirellula sp*. strain 1 Features in this part of subject sequence: putative spermidine synthase	20/263	BX294136
Sr 3dpi	145#1	*Ustilago maydis* probable NADH-ubiquinone oxidoreductase 23 kDa subunit precursor	238/404	(um11448 um00633)
Sr 3dpi	149#2	*Ustilago maydis* between two hypothetical proteins	113/225	(um05243 & um05244))
Sr 3dpi	155#2	*Ustilago maydis* hypothetical protein	266/268	(um05765 / umd211-150)
Sr 3dpi	156#5	*Oryza sativa* (japonica cultivar-group) cDNA clone:J033049G04	98/240	AK121627
Sr 3dpi	157#4	*Ustilago maydis* between related to SFT2/YBL102W & probable CCT6 - component of chaperonin-containing T-complex	154/154	(um02349 & um02350)
Sr 3dpi	163#2	*Ustilago maydis* between two conserved hypothetical proteins	122/130	(um01136 & um01137)
Sr 3dpi	174#5	*Zea mays* PCO139346 mRNA sequence	374/730	AY104037
Sr 3dpi	175#5	*Zea mays* chloroplast 3'part of rpoC2 gene, rps2 gene, atpI gene and 5'part of atpH gene	71/71	X52270.1
Sr 3dpi	176#6	*Zea mays* PCO156137 mRNA sequence	88/93	AY103541
Sr 3dpi	187#4	*Zea mays* PCO104800 mRNA sequence	172/214	AY104862
Sr 3dpi	188#3	*Ustilago maydis* hypothetical protein	136/192	(um04719)
Sr 3dpi	189#4	*Ustilago maydis* hypothetical protein	137/192	(um04719)
Sr 3dpi	197#4	*Ustilago maydis* between hypothetical protein & related to Double-strand-break repair protein rad21	331/331	(um02590 & um02591)
Sr 3dpi	198#4	*Zea mays* clone EK07D2311G04.c	164/164	BT017015
Sr 3dpi	199#2	*Zea mays* chloroplast phytoene synthase 1 (PSY1) gene	147/147	AY324431
Sr 3dpi	200#1	*Oryza sativa* clone OSJNBb0008H02	23/83	AC137759.4
Sr 3dpi	205#3	*Ustilago maydis* between related to glutathione s-transferase 3 & related to stress response protein rds1p	456/458	(um05561 & um05562)
Sr 3dpi	207#4	*Zea mays* PCO156137 mRNA sequence	58/149	AY103541
Sr 3dpi	208#4	*Zea mays* PCO131995 mRNA sequence	101/102	AY105969
Sr 3dpi	209#4	*Oryza sativa* clone P0570A02	19/43	AC132490.2
Sr 3dpi	217#4	*Oryza sativa* (japonica cultivar-group)	99/154	XM_468999.1
Sr 3dpi	220#2	*Ustilago tritici* isolate AFTOL-ID 1398 25S ribosomal RNA gene	140/164	DQ094784
Sr 3dpi	238#4	*Zea mays* strain NB mitochondrion	377/450	AY506529
Sr 3dpi	239#1	*Zea mays* strain NB mitochondrion	274/385	AY506529
Sr 3dpi	240#1	*Oryza sativa* (japonica cultivar-group)	213/345	NM_187587
Sr 3dpi	241#6	*Zea mays* PCO139174 mRNA sequence	82/85	AY108191
Sr 3dpi	242#1	*Zea mays* PCO139174 mRNA sequence	73/78	AY108191
Sr 3dpi	243#5	*Zea mays* calmodulin-binding protein mRNA sequence	71/75	L01497.1
Sr 3dpi	255#4	*Ustilago maydis* conserved hypothetical protein	186/186	(um05653)

Table 3. (Cont.)

Source*	TDF & Clone	Annotation	Blast N identities	Accession number
Sr 3dpi	256#1	*Oryza sativa* (japonica cultivar-group)	141/168	XM_481597.1
Sr 3dpi	257#1	*Oryza sativa* (japonica cultivar-group)	141/168	XM_481597.1
Sr 3dpi	265#4	*Zea mays* clone EL01N0449E04.c mRNA sequence	253/407	BT017742.1
Sr 3dpi	266#1	*Oryza sativa* (japonica cultivar-group)	295/327	XM_450624
Sr 3dpi	267#5	*Ustilago maydis* conserved hypothetical protein	151/152	(um00267)
Sr 3dpi	268#	*Ustilago maydis* conserved hypothetical protein	148/152	(um00267)
Sr 3dpi	272#1	*Zea mays* clone EL01N0448D09.d mRNA sequence	75/75	BT018598.1
Sr 3dpi	280#5	*Zea mays* strain NB mitochondrion	324/432	AY506529
Sr 3dpi	281#1	*Oryza sativa* (japonica cultivar-group) genomic DNA	21/194	AP008213
Sr 3dpi	289#4	*Oryza sativa* (japonica cultivar-group)	138/293	XM_469649.1
Sr 3dpi	290#1	*Zea mays* CL3892_1 mRNA sequence	116/125	AY110794
Sr 3dpi	291#2	*Zea mays* CL3892_1 mRNA sequence	116/125	AY110794
Sr 3dpi	298#1	*Ustilago maydis* hypothetical protein	169/358	(um02880)
Sr 3dpi	301#1	*Zea mays* strain NB mitochondrion	377/433	AY506529
Sr 3dpi	302#1	*Ustilago maydis* probable mitochondrial import receptor subunit tom7	256/258	(um10037)
Sr 3dpi	303#1	*Zea mays* PCO136039 mRNA sequence	103/106	AY105116
Sr 3dpi	309#4	*Zea mays* PCO131880 mRNA sequence	73/100	AY104047
Sr 3dpi	316#3	*Ustilago maydis* large subunit ribosomal RNA gene	44/67	AF453938
Sr 3dpi	320#1	*Oryza sativa* (japonica cultivar-group)	144/199	XM_549949.1
Sr 4dpi	27#1	*Zea mays* CL584_1 mRNA sequence	242/684	AY109524
Sr 4dpi	28#2	*Zea mays* PCO073595 mRNA sequence	254/415	AY103693
Sr 4dpi	29#4	*Zea mays* CL584_1 mRNA sequence	208/378	AY109524
Sr 4dpi	30#2	*Oryza sativa* (japonica cultivar-group), mRNA sequence	83/337	XM_478259.1
Sr 4dpi	31#1	*Zea mays* PCO117653 mRNA sequence	203/205	AY106393
Sr 4dpi	43#4	*Ustilago maydis* large subunit ribosomal RNA gene	306/335	AF453938
Sr 4dpi	44#4	*Zea mays* single myb histone 6 (Smh6) mRNA	267/267	AY280632
Sr 4dpi	45#3	*Oryza sativa* (japonica cultivar-group) chromosome 3	36/254	DP000009
Sr 4dpi	46#5	*Zea mays* CL9999_1 mRNA sequence	126/160	AY109995
Sr 4dpi	51#1	*Zea mays* PCO139174 mRNA sequence	82/82	AY108191
Sr 4dpi	61#1	*Neurospora crassa* strain OR74A	20/364	XM_324163.1
Sr 4dpi	62#1	*Ustilago maydis* related to nitrilase	151/152	(um10444 / um03453)
Sr 4dpi	63#1	*Ustilago maydis* between two hypothetical proteins	65/96	(um06266 & umd238-30)
Sr 4dpi	64#3	*Acinetobacter sp*. ADP1 Features in this part of subject sequence: putative FAD/FMN-containing dehydrogenase	20/67	CR543861.1
Sr 4dpi	73#1	*Zea mays* single myb histone 6 (Smh6) mRNA sequence	145/156	AY280632
Sr 4dpi	74#2	*Zea mays* clone Contig565 mRNA sequence	128/129	BT016732.1
Sr 4dpi	75#2	*Ustilago maydis* conserved hypothetical protein	156/156	(um03689)
Sr 4dpi	76#1	*Zea mays* PCO139174 mRNA sequence	79/82	AY108191
Sr 4dpi	82#1	*Ustilago maydis* conserved hypothetical protein	211/274	(um01413)
Sr 4dpi	86#6	*Zea mays* ZmRR10 mRNA for response regulator 10	281/283	AB071695.1
Sr 4dpi	95#1	*Zea mays* PCO154872 mRNA sequence	238/238	AY103621
Sr 4dpi	96#2	*Zea mays* PCO154872 mRNA sequence	238/238	AY103621
Sr 4dpi	104#3	*Zea mays* PCO139346 mRNA sequence	409/417	AY104037
Sr 4dpi	106#2	*Oryza sativa* (japonica cultivar-group) chromosome 10	281/394	AE017114
Sr 4dpi	110#2	*Zea mays* PCO156137 mRNA sequence	95/139	AY103541
Sr 4dpi	122#2	*Zea mays* PCO156137 mRNA sequence	94/378	AY103541
Sr 4dpi	123#1	*Zea mays* PCO156137 mRNA sequence	51/368	AY103541
Sr 4dpi	124#1	*Homo sapiens* 3 BAC RP11-418B12	21/214	AC079910.18
Sr 4dpi	125#2	*Zea mays* homeobox transcription factor KNOTTED1 (kn1) gene	23/163	AY312169

Table 3. (Cont.)

Source*	TDF & Clone	Annotation	Blast N identities	Accession number
Sr 4dpi	130#3	*Ustilago maydis* between related to SSP120 - secretory protein & hypothetical protein	92/138	(um11591 & um05997)
Sr 4dpi	136#4	*Ustilago maydis* putative protein	133/150	(um02101)
Sr 4dpi	140#1	*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 6	37/192	AP008212
Sr 4dpi	142#1	*Ustilago maydis* related to glutathione-S-transferase	268/389	(um01870)
Sr 4dpi	158#4	*Ustilago maydis* between hypothetical protein & conserved hypothetical protein	339/339	(um05618 & um05619)
Sr 4dpi	159#2	*Oryza sativa* (japonica cultivar-group) genomic DNA	228/256	XM_470374.1
Sr 4dpi	165#4	*Ustilago maydis* between two conserved hypothetical proteins	129/130	(um01136 & um01137)
Sr 4dpi	166#5	*Ustilago maydis* between two conserved hypothetical proteins	130/130	(um01136 & um01137)
Sr 4dpi	177#3	*Ustilago maydis* conserved hypothetical protein	641/643	(um01663)
Sr 4dpi	178#1	*Ustilago maydis* related to negative regulator of mitosis	186/188	(um02427)
Sr 4dpi	190#2	*Ustilago maydis* hypothetical protein	163/165	(um03418)
Sr 4dpi	191#4	*Ustilago maydis* hypothetical protein	138/192	(um04719)
Sr 4dpi	192#2	*Ustilago maydis* hypothetical protein	161/164	(um03418)
Sr 4dpi	193#2	*Medicago truncatula* clone mth2-103p8	21/93	AC150843.16
Sr 4dpi	201#3	*Ustilago maydis* between hypothetical protein & related to Double-strand-break repair protein rad21	329/331	(um02590 & um02591)
Sr 4dpi	202#5	*Ustilago maydis* between hypothetical protein & probable HIS3 - imidazoleglycerol-phosphate dehydratase	220/221	(um01118 & um11859)
Sr 4dpi	210#2	*Zea mays* PCO107465 mRNA sequence	336/352	AY104246
Sr 4dpi	211#3	*Ustilago maydis* hypothetical protein	223/225	(um05004)
Sr 4dpi	212#2	*Ustilago maydis* hypothetical protein	223/225	(um05004)
Sr 4dpi	213#1	*Homo sapiens* chromosome 18, clone RP11-36L19	21/54	AC025656
Sr 4dpi	224#6	*Zea mays* PCO114323 mRNA sequence	175/190	AY107331
Sr 4dpi	245#3	*Ustilago maydis* conserved hypothetical protein	39/225	(um01954)
Sr 4dpi	246#4	Mouse DNA sequence from clone RP23-466N19	30/156	AL627125.8
Sr 4dpi	247#4	*Zea mays* PCO139174 mRNA sequence	82/82	AY108191
Sr 4dpi	258#2	*Zea mays* PCO089998 mRNA sequence	303/305	AY105934
Sr 4dpi	259#6	Zebrafish DNA sequence from clone CH211-23E22	19/65	CR376773.7
Sr 4dpi	269#2	*Shigella flexneri* 2a str. 301	221/222	AE005674
Sr 4dpi	273#4	*Oryza sativa* (japonica cultivar-group) genomic DNA	68/252	AP008213
Sr 4dpi	282#2	*Ustilago maydis* related to WD40 repeat protein CreC	764/775	(um02846)
Sr 4dpi	283#1	*Zea mays* clone EL01T0205D04.d mRNA sequence	151/219	BT018885.1
Sr 4dpi	287#4	*Zea mays* PCO100830 mRNA sequence	253/257	AY103766
Sr 4dpi	288#4	*Zea mays* PCO100830 mRNA sequence	255/257	AY103766
Sr 4dpi	299#1	*Ustilago maydis* probable PHO89 - Na+/phosphate co-transporter	290/559	(um03475)
Sr 4dpi	304#1	Contiguous genomic DNA sequence comprising the 19-kDa-zein gene family from *Zea mays*	210/268	AF546188
Sr 4dpi	317#4	*Ustilago maydis* probable MGM101 - mitochondrial genome maintenance protein	73/124	(um04831)
Sr 4dpi	322#4	*Oryza sativa* (japonica cultivar-group) genomic DNA	63/336	AP008214

* Um 3dpi = *U. maydis* 3 days post infection, Um 4dpi = *U. maydis* 4 days post infection, Sr 3dpi = *S. reilianum* 3 days post infection, Sr 4dpi = *S. reilianum* 4 days post infection

Table 4. List of differentially occurring fragments of fungal origin as identified by cDNA-AFLP transcript profiling of the *U. maydis*-maize and *S. reilianum*-maize pathosystems.

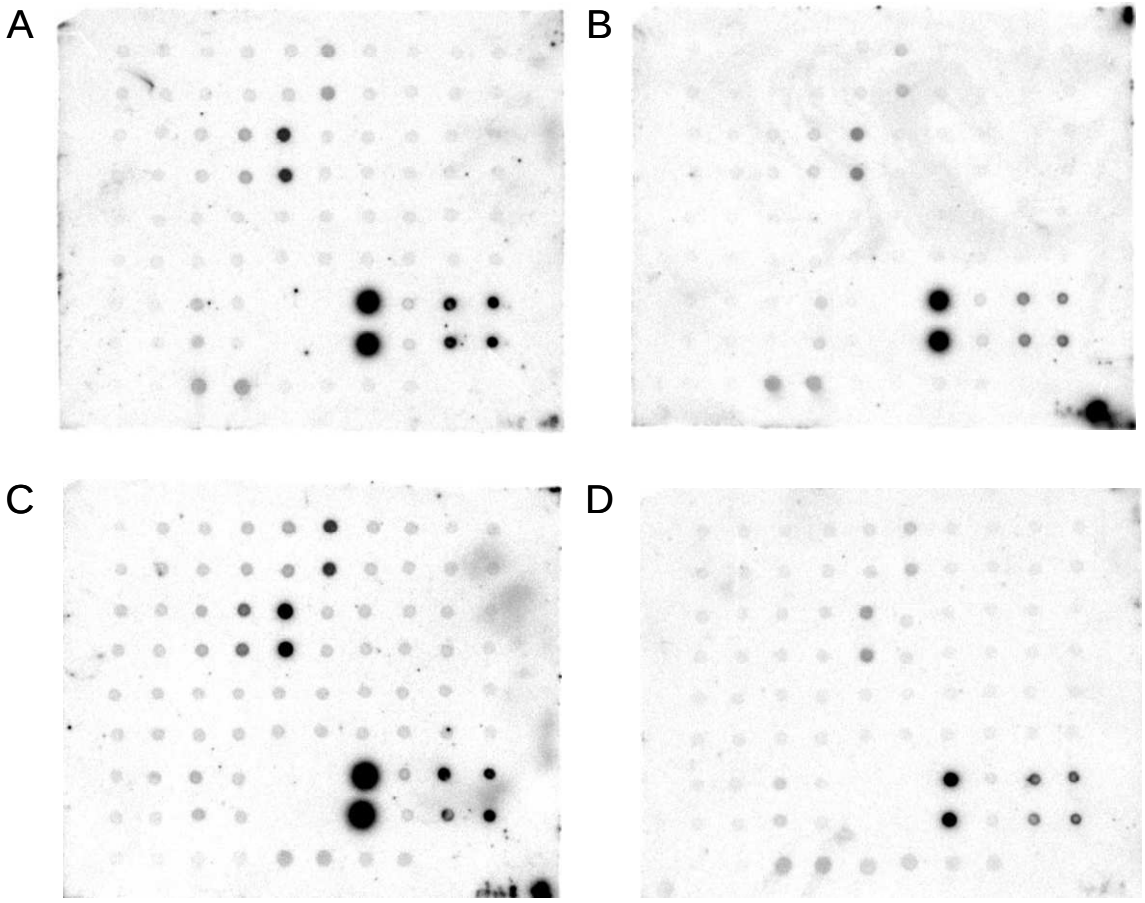
Treatment	TDF	Um Blast hit[1]	Sr Blast hit[2]
Um 3dpi	12	Conserved hypotheitcal protein (um 02952)	Conserved hypothetical protein (sr13744)
	15	Between related to SFT2/YBL102W & probable CCT6 (um02349 & um02350)	None
	66	Probable RLI1-Protein promoting preinitiation complex assembly (um03351)	Probable RLI1-Protein promoting preinitiation complex assembly (sr14352)
	131	Hypothetical protein (um05920)	Probable PPA1 - H+-ATPase 23 KD subunit (sr16543)
	233	Gene for small subunit ribosomal RNA	None
	305	Related to Acetoacetyl-CoA synthetase (um10088)	Related to Acetoacetyl-CoA synthetase (sr11637)
	314	Related to Na-K-Cl cotransporter (um10987)	Related to Na-K-Cl cotransporter (sr15487)
Um 4dpi	47	Between conserved hypothetical protein & probable GTPase Rab7 protein (um05510 & um05511)	None
	137	Hypothetical protein (um00230)	Conserved hypothetical protein (sr11581)
	152	Between related to COP9 (um00848) & conserved hypothetical protein (um00849)	None
	160	Between related to coenzyme a synthetase & probable AAP1 - alanine/arginine aminopeptidase (um00791 & um00792	None
	162	Between hypothetical protein & probable MTR4 - involved in nucleocytoplasmic transport of mRNA (um01483 & um01484)	None
	251	Conserved hypothetical protein (um04196)	Conserved hypothetical protein (sr15081)
	297	Hypothetical protein (um01749)	Conserved hypothetical protein (sr12825)
Sr 3dpi	26	Hypothetical protein (um03221)	None
	145	Probable NADH-ubiquinone oxidoreductase 23 kDa subunit precursor (um11448 / um00633)	Between conserved hypothetical protein & probable NADH-ubiquinone oxidoreductase 23 kDa subunit precursor (sr11911 & sr11912)
	149	Between two hypothetical proteins (um05243 & um05244)	Between conserved hypothetical Ustilaginaceae-specific protein & conserved hypothetical protein (sr14681 & sr14682)
	155	Hypothetical protein (um05765)	None
	268	Conserved hypothetical protein (um00267)	Conserved hypothetical protein (sr11618)
	298	Hypothetical protein (um02880)	Conserved hypothetical protein (sr13934)
	302	Probable mitochondrial import receptor subunit tom7 (um10037)	Probable mitochondrial import receptor subunit tom7 (sr11490)
Sr 4dpi	10	Hypothetical protein (um04961)	None
	63	Between two hypothetical proteins (um06266 & umd238-30)	Between conserved hypothetical Ustilaginaceae-specific protein & conserved hypothetical protein (sr14681 & sr14682)

Table 4. cont.

Treatment	TDF	Um Blast hit[1]	Sr Blast hit[2]
	82	Conserved hypothetical protein (um01413)	Conserved hypothetical protein (sr12480)
	136	Putative protein (um02101)	Conserved hypothetical protein (sr13324)
	142	Related to glutathione-S-transferase (um01870)	Related to glutathione-S-transferase (sr10584)
	158	Between hypothetical protein & conserved hypothetical protein (um05618 & um05619)	None
	177	Conserved hypothetical protein (um01663)	Conserved hypothetical protein (sr12732)
	178	Related to negative regulator of mitosis(um02427)	None
	202	Between hypothetical protein & probable HIS3 - imidazoleglycerol-phosphate dehydratase (um01118 & um11859)	None
	245	Conserved hypothetical protein (um01954)	Conserved hypothetical protein (sr10707)
	299	Probable PHO89 - Na+/phosphate co-transporter (um03475)	Probable PHO89 - Na+/phosphate co-transporter (sr14465)
	317	Probable MGM101 - mitochondrial genome maintenance protein (um04831)	Between conserved hypothetical Ustilaginaceae-specific protein & conserved hypothetical protein (sr14681 & sr14682)

1. Comparison to *Ustilago maydis* genome sequences using MUMDB Blast
2. Comparison to *Sporisorium reilianum* genome sequences using MSRDB Blast

A validation of the differentially identified TDFs was required to confirm the differences in expression profiles exhibited by cDNA-AFLP. The fungal TDFs listed in Table 4 were analysed by reverse Northern analysis by spotting the cloned fragments on nylon membranes and probing with the cDNA used for the initial expression profiling, i.e. total cDNA from maize infected with *U. maydis* 3 or 4 dpi (Fig. 17A and 17B) and maize infected with *S. reilianum* 3 and 4 dpi ((Fig. 17 C and D). Unfortunately, none of the TDFs appeared to be differentially expressed as the same hybridisation pattern was obtained with all four of the cDNA samples used as seen in Fig. 17. However, two TDFs (TDF63 and TDF149) did have slightly stronger hybridisation signals and these were checked by Northern analysis. The Northern analysis confirmed that the expression patterns of both TDF63 and TDF149 were not specific (Fig. 17F). Since none of the TDFs could be confirmed to be differentially expressed they were not further investigated.



E

10	12	15	26	47	63	66	82	169	131
10	12	15	26	47	63	66	82	169	131
136	137	142	145	149	152	155	158	160	162
136	137	142	145	149	152	155	158	160	162
177	178	202	233	245	251	268	297	298	299
177	178	202	233	245	251	268	297	298	299
302	305	314	317			Plant	Um	Sr	cDNA
302	305	314	317			Plant	Um	Sr	cDNA
vector	vector	mig2-6	mig2-6	Sr cbx	Sr cbx	Um cbx	Um cbx		

F

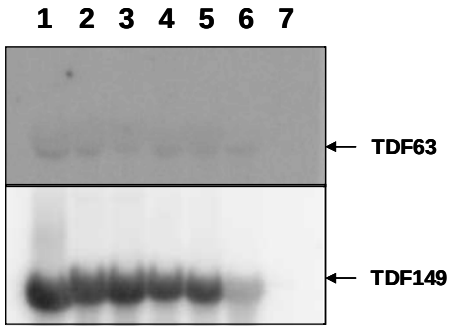


Figure 17. Reverse Northerns (dot-blots) and Northern blot of differentially expressed fragments of fungal origin as identified by cDNA-AFLP. 250 ng of the cloned TDFs and respective controls were spotted onto nylon membranes as illustrated in the grid (**E.**). Each TDF is colour-coded according to its origin TDFs in red are derived from *U. maydis* infected maize 3 dpi (Um 3 dpi), green TDFs from *U. maydis* infected maize 4 dpi (Um 4 dpi), blue TDFs from *S. reilianum* infected maize 3 dpi (Sr 3 dpi) and pink TDFs from *S. reilianum* infected maize 4 dpi (Sr 4 dpi). Total cDNA was used as respective probes. **A.** Dot-blot probed with total cDNA from Um 3 dpi. **B.** Dot-blot probed with total cDNA from Um 4 dpi **C.** Dot-blot probed with total cDNA from Sr 3 dpi **D.** Dot-blot probed with total cDNA from Sr 4 dpi **F.** Northern blot probed with TDF63 and TDF149. Lanes 1 = Uninfected maize RNA, 2 = *U. maydis* FB1xFB2 infected maize 3dpi total RNA, 3 = *U. maydis* FB1xFB2 infected maize 4dpi total RNA, 4 = *S. reilianum* SRZ2xSRZ1 infected maize 3dpi total RNA, 5 = *S. reilianum* SRZ2xSRZ1 infected maize 4dpi total RNA, 6 = *U. maydis* fungal RNA, 7 = *S. reilianum* fungal RNA.

4.3 Characterisation of a nitrilase deletion strain and its role in auxin biosynthesis in *U. maydis*

In the previously described cDNA-AFLP screen to identify genes with a possible function in tumour formation, a gene related to nitrilase (um10444) was identified as being differentially expressed in the *U. maydis*-maize pathosystem. The fungal nitrilase gene was only found in infected tissue isolated from *U. maydis* 3 dpi and not in 4 dpi tissue (Table 3). This was extremely interesting as the *U. maydis* genome revealed three predicted nitrilase genes (um10444, um11973 and um05032) but um10444 showed the highest amino acid sequence similarities (43%) to the plant nitrilase genes *ZmNit2* (maize), *NIT1* and *NIT2* (Arabidopsis). These plant nitrilase genes are involved in auxin (indole-3-acetic acid) biosynthesis (Schmidt *et al*., 1996; Normanly *et al*., 1997; Kriechbaumer *et al*., 2006). The enzyme *ZmNit2* catalyses the conversion of indole-3-acetonitrile to indole-3-acetic acid in maize plants (see Fig. 3). Auxin production by *U. maydis* has been postulated to be associated with tumour formation (Moulton, 1942; Wolf, 1952; Basse *et al*., 1996; Martinez *et al*., 1997; Sosa-Morales *et al*., 1997) so we were intrigued to investigate the possible role of the nitrilase gene, um10444, in tumour development of *U. maydis*.

A previous study investigating the role of certain key enzymes in the biosynthesis of indole-3-acetic acid (IAA) demonstrated that the deletion of *iad1*, one of two indole-3-acetaldehyde dehydrogenase genes substantially reduced IAA formation (Basse *et al*.,2003), while a double deletion of *iad1* and *iad2* dramatically reduced IAA formation (Reinecke *et al*. 2008). The residual IAA detected in *iad1* deletion strains could be attributed to the action of a nitrilase gene. To test the influence of the nitrilase gene (um10444) on pathogenicity, the gene was deleted in the *U. maydis* solopathogenic strain SG200 and used to infect maize seedlings. No difference was observed in pathogenicity between the deletion strain SG200Δ10444-11 and wild type strain SG200 (not shown). Since the effect of the residual IAA production in strains containing deletions of the two key IAA biosynthetic enzymes, *iad*1 and *iad*2, was ascribed to the action of a nitrilase, the gene um10444 was therefore also deleted in the previously constructed strains GRN7 and GRN8, both carrying double deletions of *iad1* and *iad2*. Triple deletion mutants were assayed for their mating ability and pathogenicity in the plant. Strains containing the triple deletion were not compromised in their ability to mate on charcoal plates (Fig. 18A). As with the single deletion strains, no difference between the deletion strains and wild type strains was

observed in pathogenicity as determined by plant infection assays (Fig. 18B). Slightly fewer matured spores were observed in a comparable amount of tumour material in the triple deletion strains G7#7 and G8#13 as compared to wild type strains FB1 and FB2 (Fig. 18C). However, this may be attributed to a delay in spore formation as no distinct difference in tumour size was evident (Fig. 18B).

Figure 18. Deletion of the nitrilase gene, um10444, has no effect on mating, pathogenicity or spore formation in *U. maydis*. **A.** Mating reaction test for the formation of dikaryotic filaments (fuzzy white colony) on charcoal-containing plates; **B.** plant infection assay for the formation of tumours; **C.** microscopic analysis of tumour material for the presence of teliospores. Bar = 30µm

To investigate the possible role of the nitrilase gene in auxin production in *U. maydis in planta*, an analysis of the IAA content in tumour material was made. Maize seedlings were infected with compatible wild type strains, triple deletion strains as well as with sterile water as a negative control. Representative samples of tumour material and corresponding sections of water infected leaves were excised at five different time points and used for the chemical extraction of IAA for measurement by HPLC. The time points covered the early to late tumour formation stages and were taken at: 0 dpi (corresponding to 12 h post infection); 2 dpi; 4 dpi; 6 dpi and 9 dpi. The amounts of IAA measured for each strain over the previously-mentioned time course is illustrated by Fig. 19A. No significant difference in the amount of IAA in the plant material was observed between the water control and the wild type (FB1 x FB2) or triple deletion (G7#7 x G8#13) infections on 0 dpi and 2 dpi. However, on 4 dpi a substantially greater amount of IAA could be observed in both the wild type (FB1 x FB2) infection and in the triple deletion (G7#7 x G8#13) infection. Plants infected with the triple deletion mutants appeared to have a higher IAA level. On 6 dpi and 9 dpi IAA amounts declined in both wild type and triple deletion infections as compared to

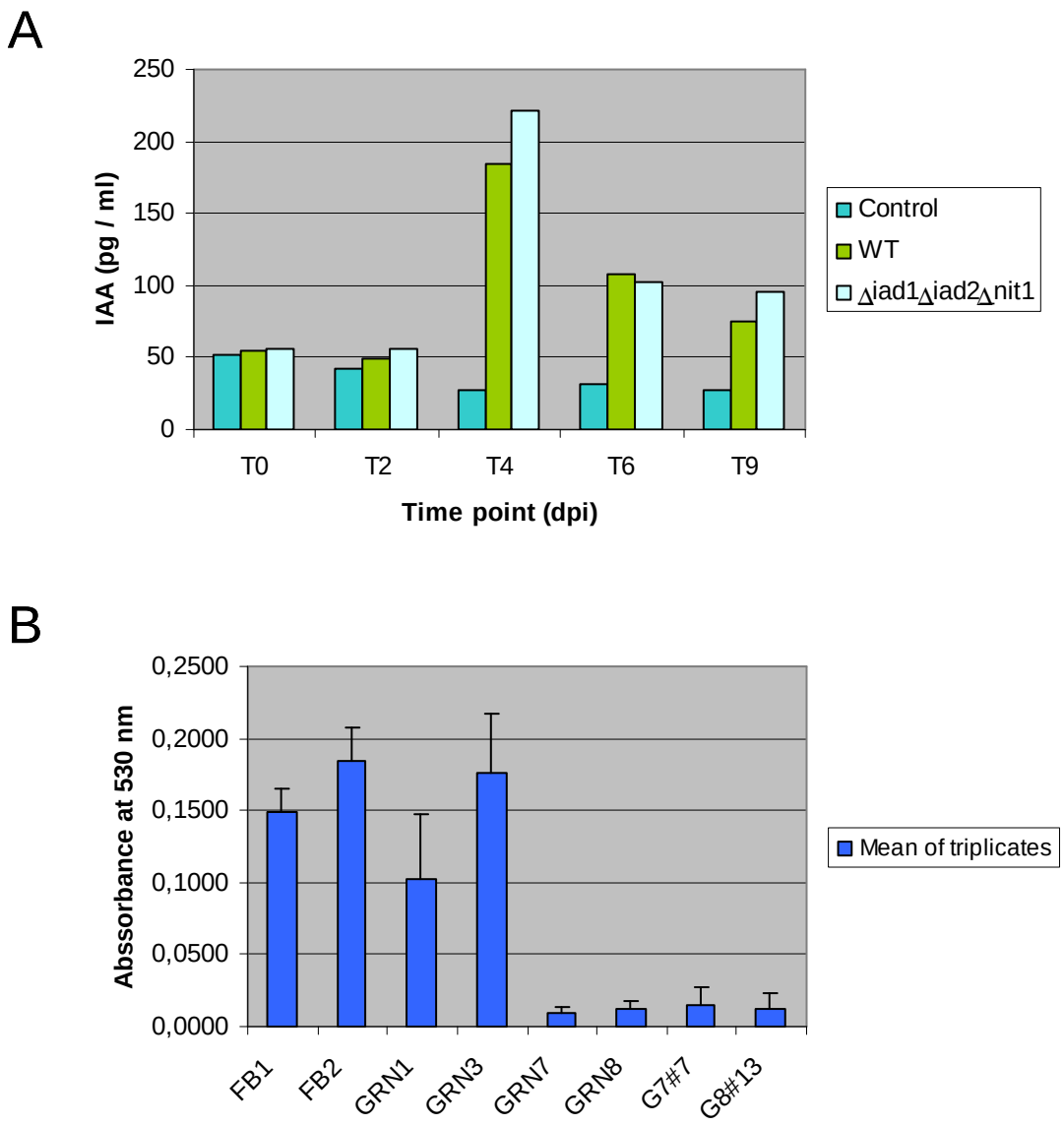


Figure 19. Indole-3-acetic acid (IAA) measurements of maize leaves before and after infection with *U. maydis* and *in vitro* IAA production by haploid cells. **A**. IAA levels in maize leaf / tumour material over five time intervals following infection with water (control), wild type or a triple deletion (Δ*iad*1Δ*iad*2Δ*nit*1) strain. **B**. Colorimetric reaction using Salkowski reagent for IAA produced by haploid *U. maydis* cells *in vitro.* FB1 and FB2 = wild type strains; GRN1 and GRN3 = *iad*2 deletion strains; GRN7 and GRN8 = *iad*1 and *iad*2 double deletion strains; G7#7 and G8#13 = *iad*1, *iad*2 and nitrilase triple deletion strains. Vertical bars represent the standard deviation.

that measured on 4 dpi. However, these levels still exceeded those of the water control by approximately 2.5 fold (Fig. 19A).

Since the IAA levels measured in infected material may be a result of endogenous IAA produced by the plant, IAA produced by the fungus or an effect of the fungus inducing the plant to produce IAA, it was necessary to determine the levels of IAA produced by the fungus *in vitro*. Wild type strains (FB1 and FB2), *iad*2 deletion strains (GRN1 and GRN3), *iad*1 and *iad*2 double deletion strains (GRN7 and GRN8) and *iad*1, *iad*2 and nitrilase triple deletion strains (G7#7 and G8#13) were analysed

for their ability to produce IAA *in vitro*. Strains were grown in CM-Glu medium containing tryptophan and the supernatants tested by colorimetric reaction using Salkowski reagent for the levels of IAA produced (Fig. 19B). Wild type cells as well as *iad*2 single deletion strains are capable of producing a significant amount of IAA in tryptophan containing medium, although a noticeable reduction is evident in strain GRN1 as compared to the corresponding wild type strain FB1. The effect of the double deletion on IAA production is very evident in both GRN7 and GRN8 as IAA levels are just detectable. The additional deletion of the nitrilase um10444 did not abolish IAA production as the IAA levels measured in strains G7#7 and G8#13 were comparable to those for the double deletion strains. The nitrilase gene um10444 therefore does not appear to contribute to the residual IAA formed in the absence of *iad*1 and *iad*2. The IAA levels in plant tissue infected with the triple deletion strains were comparable to wild type, even though these triple deletion strains were dramatically reduced in their capacity to produce IAA. This indicates that the increased IAA may be due to the stimulation of plant auxin production by the presence of the fungus.

To investigate a possible cause of the increased levels of IAA during tumour formation, we analysed the expression levels of certain key genes involved in plant auxin biosynthesis (*ZmNit*2), transport (*ZmAux*1) and downstream effectors (*ZmSAUR*2 and *ZmExpA*4). The maize actin gene (*ZmActin*) was initially included as a control for constitutive expression. In addition, a fungal gene *Umi*2a (Basse, 2005) was included as a control for genes induced during tumour formation. These genes were analysed by Northern analysis and quantitative real time RT-PCR (QRT-PCR). Expression profiles obtained for each gene by Northern analysis are shown in Fig. 20A and the corresponding QRT-PCR data is displayed in Fig. 20B. Northern analysis revealed that *ZmActin* and *ZmExpA*4 genes were most highly induced on 4 dpi and 6 dpi respectively. This is quite surprising as *ZmActin* was selected as a control gene considered to be constitutively expressed. *ZmAux*1 also showed an induction in expression on 6 dpi as compared to the control, although the level was not as high as that observed in *ZmExpA*4 or *ZmActin.* Both *ZmNit*2 and *ZmSAUR*2 showed relatively constitutive expression levels in both infected and control tissue. *ZmNit*2 however, had higher expression levels than that observed for *ZmSAUR*2 and therefore was subsequently used as control for constitutive expression. As expected the *Umi*2a gene showed an induction of expression at 4 dpi, which increased on 6 dpi as compared to the control where no expression was detected, confirming the previously observed induction of *Umi*2a during tumour formation (Basse, 2005). To

normalise the QRT-PCR data, each gene was compared to the *ZmNit*2 transcript and not the *ZmActin* since the *ZmActin* expression appeared to be developmentally regulated (Fig. 20A). Patterns of expression for all of the genes reflected those obtained by Northern analysis, although absolute values were much higher since QRT-PCR is a far more sensitive technique. The expression data is however, not conclusive to explain the cause for the increased IAA biosynthesis during tumour formation.

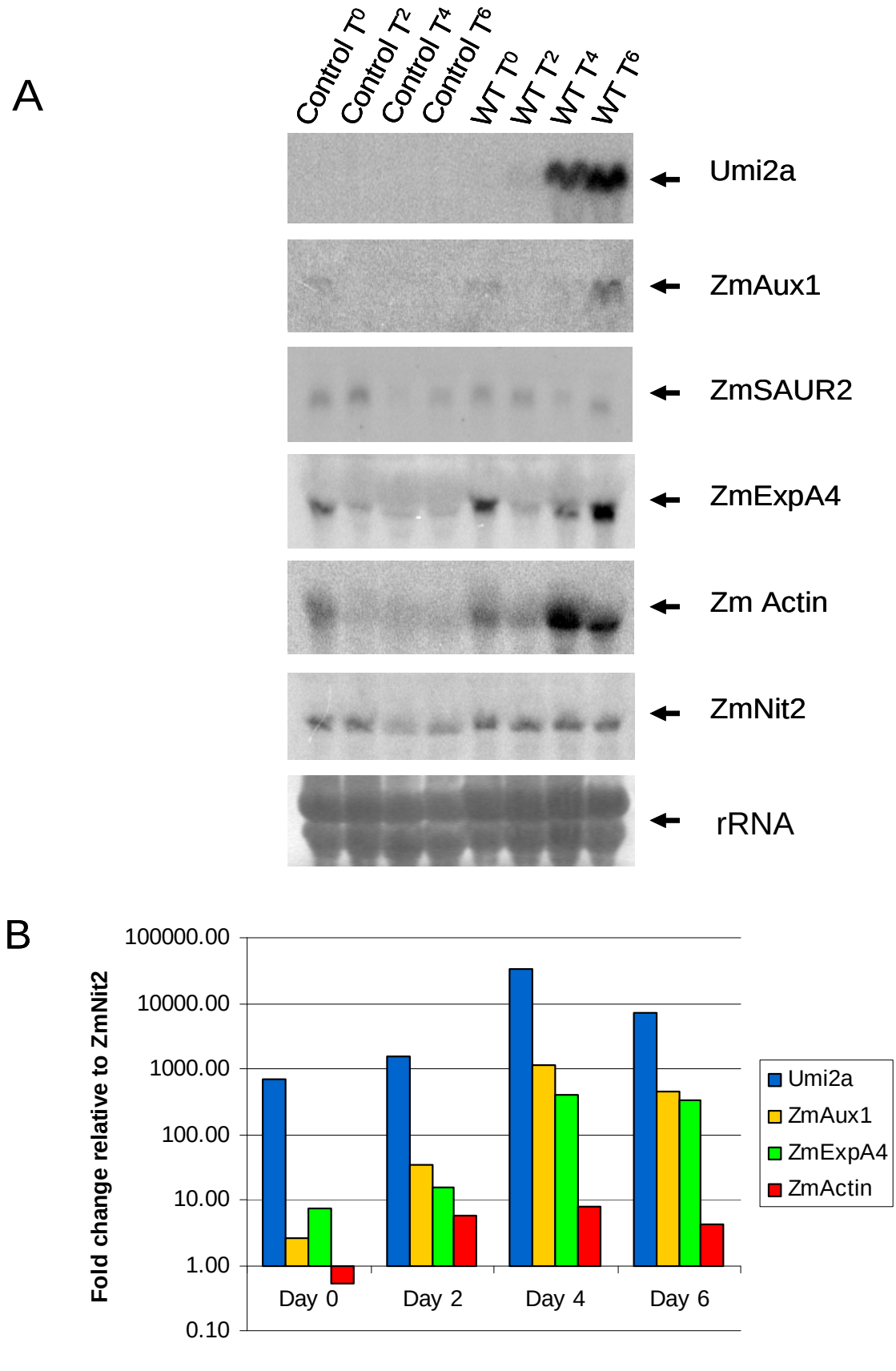


Figure 20. Expression analysis of maize auxin-related genes in tumour material derived from wildtype infection (FB1 x FB2) and uninfected leaves (water control). **A**. Northern blot analysis of maize auxin responsive genes. Methylene blue stain served as an indicator of equal amounts of RNA. **B**. Quantitative real time RT-PCR analysis of the expression levels of the maize auxin responsive genes during the course of tumour development in *U. maydis* FB1 x FB2 infected tissue. Fold change was calculated using the 2-ΔΔCt method relative to ZmNit2 expression.

Discussion

5.1 Mating in *Sporisorium reilianum* compared to *Ustilago maydis*

In this study the independent *a* and *b* mating type loci of *S. reilianum* have been molecularly characterised, thus confirming the tetrapolar mating system of the fungus first described by Hanna (1929). Contrary to the biallelic *a* locus found in *U. maydis* and other members of the smut fungi, this work revealed that the *a* locus of *S. reilianum* exists in three alleles and displays the unique feature of having two pheromone genes and one pheromone receptor. All pheromone genes code for active peptides. All six pheromones contain a CaaX (C, cysteine; A, aliphatic; X, any amino acid residue) sequence, a signal of isoprenylation with a farnesyl group at the cysteine residue (Clarke, 1992) typical of fungal pheromones characterised so far (Caldwell, 1995). The two mature pheromones with the same specificity are predicted to have exactly the same amino acid sequence. This is supported by functional tests conducted by J. Schirawski, which demonstrate that the pheromones encoded by *mfa1.2* and *mfa3.2* are specifically recognised by Pra2. Similarly, the pheromones specified by *mfa1.3* and *mfa2.3* are only recognised by Pra3, while the genes *mfa2.1* and *mfa3.1* both encode pheromones activating Pra1 (Schirawski *et al*., 2005). This is a novel feature observed in a smut fungus since the well characterised smut fungi *U. maydis* and *U. hordei* both have bi-allelic *a*-loci that allow individuals to be mating compatible with only one partner. The presence of three *a*-alleles containing one receptor and two pheromone genes, as found in *S. reilianum*, allows more individuals in a population to be mating compatible with one another. This characteristic is more common in other basidiomycetes. In the homobasidiomycete mushroom species *Coprinopsis cinerea* and *Schizophyllum commune*, even more complex mating type loci are known. The *B* locus in these fungi is comparable to the *a* locus in *U. maydis* and *S. reilianum*. Three independent *B* loci were identified in *C. cinerea*, each encoding three receptors and six pheromones (O'Shea *et al*., 1998). While the nine Bα and nine Bβ specificities found in *S. commune* contain up to eight pheromone genes able to induce the same receptor (Wendland *et al*., 1995; Vaillancourt et., 1997; O'Shea *et al*., 1998; Fowler *et al*., 1999; Halsall *et al*., 2000). However, in these cases, the pheromones have distinct specificities (with some of them being

able to activate more than one receptor) and are unrelated in primary amino acid sequence (Fowler *et al*., 2001). Multi-allelic mating type genes of mushrooms are presumed to be generated by gene duplications before becoming divergent (Casselton, 2002; Kothe *et al*., 2003). The gene order in the *a1* locus of *S. reilianum* shows nearly perfect synteny to that in *U. maydis a1*, with the exception that the *a1* locus of *S. reilianum* carries an additional pheromone precursor gene (Fig. 6A). The same is true for the *a2* locus of *S. reilianum*, where the gene order is highly conserved in comparison to that in the *a2* locus of *U. maydis*. Interestingly, the second *S. reilianum* pheromone precursor gene, *mfa2.3*, occupies a position in the *a2* locus at which in *U. maydis* a pheromone precursor pseudogene is located (Urban *et al*., 1996). This might indicate that in *U. maydis*, like in *S. reilianum*, formerly three (or more) different *a* alleles existed. In this case all but two must have disappeared during evolution, and the second unused pheromone precursor genes in the *a1* and *a2* loci were either lost or accumulated mutations rendering them non-functional or non-recognisable. As a consequence, the *a1* and *a2* loci of *U. maydis* do not share significant regions of sequence identity. Alternatively, the presence of the pseudogene in the *U. maydis a2* locus might indicate an early step towards *a3* locus evolution in this species in this case. If the invading pheromone precursor gene (gene transferred from a close relative) led to self-stimulated strains, then its conversion into a pseudogene might have been selected for since it leads to a block in sexual development and therefore a waste of energy. The presence of the third *a* locus in *S. reilianum* has not been observed in other smut fungi such as *U. hordei* or *S. scitamineum*, although *pra*3 has been detected in other smut species (D. Bergerow, pers. comm.). This supports the idea of the acquisition of the *a*3 locus through interspecies mating with a close relative, as interspecies mating has been demonstrated among several smut fungi (Bakkeren *et al*.1993). Additional evidence for this is shown by several large regions in the three *a*-loci that show more than 95% nucleotide sequence similarity (not shown). However, it is not known whether the third *a*-locus is present in other smut fungi or is a feature unique to *S. reilianum*.

The distribution of the *a*-alleles appears to correspond with the history of maize, since maize is thought to have originated in Mexico and then spread to the rest of North and South America. Entry into Europe was achieved by Spanish and Portugese explorers who brought the plants from the Americas amongst their treasures. Propagation then spread through rest of Europe into Africa and Asia (Maiti & Wesche-Ebeling, 1998). All three *a*-alleles were detected in samples obtained from the USA and China with both showing very similar distribution of genotypes. Since

maize was more recently grown in China, the presence of a similar genotype distribution to that of the USA is indicative of the propagation of *S. reilianum*-infected maize obtained from the USA. This is not surprising as the proximity between the two countries is closer than that of Europe and Africa as well as the fact that the USA and China are the world's largest producers of maize. The spread of *S. reilianum* in European (Germany and France) maize appears to have been by a single spore infection as only two *a*-alleles were found in the European samples and perfect Mendelian distribution of the genotypes. Similarly, a single spore of *S. reilianum* must have been introduced into maize in South Africa as only two *a*-alleles were detected in the samples. These were different from the *a*-alleles found in the European samples, indicating that the infected maize did not originate from Germany or Europe. It is more likely that S. reilianum spores or infected maize was introduced in Southern Africa in the late 17th century when maize was either brought directly from Portugal by Bartholomew Dias as he circumnavigated the southern part of Africa or by Jan van Riebeck who established a Dutch trading post at the southern tip of Africa.

In addition to the *a*-loci previously described, this study determined that the *b* locus of *S. reilianum* is multi-allelic and displays the same features as the *b* locus of *U. maydis*. Five different *b* alleles of *S. reilianum* have been identified and sequenced. All five alleles show perfect synteny to the *b* locus of *U. maydis* with respect to gene order, orientation, and intron position. This holds true also for the adjacent regions where the same genes are present in *U. maydis* and *S. reilianum* and are even more conserved than the *b* genes themselves. This highlights the highly polymorphic nature of the mating type genes and represents a general feature of self/ non-self discrimination systems thought to be maintained by balancing selection (Richman, 2000). The greatest genotypic variation was observed in the isolates from China and the USA. This could be ascribed to the fact that China and USA are the world's largest producers of maize and therefore have a wide area under cultivation. Interestingly, the *b1* and *b2* alleles, which were found in *S. reilianum* isolates from France and Germany, carried a transposon insertion downstream of the *bE* gene. The transposon is absent in the corresponding genic region of the *b3*, *b4*, and *b5* strains from China, the United States of America (USA), and South Africa. The presence of the transposon exclusively in the European strains indicates that it was acquired only recently. It is possible that the disease, which became prominent in Europe only since the beginning of the 1980s (Martinez *et al*., 1999), was spread

through Europe by spores originating from one infected crop. However, the presence of the transposon in other European *b1* and *b2* alleles needs to be established.
The *b4* and *b5* alleles were found only in the spore samples from South Africa, possibly indicating their recent development. However, the spore sample size was small and a broader investigation would be required to confirm this.

Multi-allelic *b*-loci as found in *S. reilianum* and *U. maydis* are not common among smut fungi, though not unusual in basidiomycetes. The high number of allelic mating genes allows enhanced out breeding and tetrapolar mating systems lead to decreased inbreeding, a maximum of 25% among offspring. This is enabled by the location of the two mating loci on separate chromosomes unlike the bipolar system in other smut fungi such as *U. hordei* that are located on a single chromosome but separated by ~ 500 kb (Bakkeren and Kronstad, 2004). The bipolar mating system of *U. hordei* functions as if there was a single mating type locus because of the suppression of recombination across the mating locus. Lack of recombination in this region has been shown to be due to the accumulation of repetitive sequences as seen in *Microbotryum violaceum* (Hood, 2005) and *U. hordei* (Bakkeren *et al*., 2006) or DNA regions marked by high G + C base pair composition as seen in *Cryptococcus neoformans* (Hsueh *et al*., 2006). Suppression of recombination in the sex chromosomes is also a feature in mammals (Fraser and Heitmann, 2004). However, heterologous expression studies revealed that the *U. hordei b* mating genes are functional in *U. maydis* (Bakkeren and Kronstad, 1993), which proves their evolutionary relatedness. Some authors propose the bipolar system to be ancestral as it is present in both ascomycetes and basidiomycetes (Fraser and Heitmann, 2004). On the other hand, Bakkeren *et al*. (2006) propose that the bipolar system evolved from the tetrapolar system due to the presence of genes not associated with mating and the accumulation of repetitive sequences in the mating locus of bipolar systems. Investigation of the mating type loci of related smut fungi would help to elucidate the matter.

5.2 Factors determining infection specificity in *U. maydis* and *S. reilianum*

Protoplast fusion of a *U. maydis b*-null strain and a *S. reilianum* solopathogenic strain succeeded in achieving genetic complementation between these two organisms from different genera, generating hybrids. The hybrids demonstrated that tumour formation in *U. maydis* is dominant while symptom expression solely in

flowers is determined by *S. reilianum*. Putative hybrids of *U. maydis* and *S. reilianum* obtained were found to contain all the genetic markers tested for by PCR as well as the phenotypic markers of resistance to both antibiotics. Phenotypic screening confirms the functionality of the integrated marker. This was evident in the case of the hybrid JA1-1 where the PCR result for the *bE*2 marker was negative (Table 2) but the strain grew filamentously on a mating plate assay indicative of the presence of the *bE*2 gene. Further evidence for the hybrid nature was the alteration in cell and colony morphology as shown in Fig. 9. Cell morphology of the hybrids resembled one or the other parent but intermediate types were also found as was expected (Peberdy, 1979; Anne, 1983; Tamaki, 1986), however morphologies resembling only one parent have also been reported (Gera *et al*., 1997; Santopietro *et al*., 1997). The number of putative hybrids obtained from the fusion of *U. maydis* and *S. reilianum* was very low (Table 2) but this is not uncommon for protoplast fusions (Peberdy, 1979; Anne, 1983; Tamaki, 1986). The low rate of hybrid formation may be due to somatic and / or nuclear incompatibility between the two organisms since normal development and metabolism within the organism are dependent on coordinated interaction of several genes throughout the genome and not solely on single genes (Anne, 1983; Carlile *et al*., 2001). Alternatively, the fusion products may have failed to grow after having been exposed to too great a stress of simultaneously regenerating a cell wall and adjusting to the presence of foreign DNA (Spencer *et al*., 1989).

Cell morphology of the hybrids resembled one or the other parent but intermediate types were also found as was expected (Peberdy, 1979; Anne, 1983; Tamaki, 1986), however morphologies resembling only one parent have also been reported (Gera *et al*., 1997; Santopietro *et al*., 1997). FACS analysis revealed that the progenitor strain JS333 is a diploid, this correlated with a longer cell length compared to the haploid strains SRZ1 and SRZ2 (J. Schirawski, pers. comm.). In their analysis of several stable hybrids from the fusion of *S. cerevisiae* and *Kluyveromyces marxianus,* Witte *et al*. (1989) discovered that the *K. marxianus* progenitor strain was triploid. The haploid nature of hybrid JA1-1 suggests that only the *b*-locus and the cbx resistance cassette or a small portion of the genome of *S. reilianum* were incorporated into the *U. maydis* AB1 strain. While the diploid nature of hybrids JA1-2 and JA1-3 suggest that similarly only the antibiotic resistance marker or a small portion of the *U. maydis* genome may have been incorporated into the *S. reilianum* JS333 strain. Incorporation of unequal amounts of DNA from parent strains has been reported for several other hybrids. Diploid hybrids of *Yarrowia lipolytica* and *Kluyveromyces lactis*

contained mostly *Y. lipolytica* nuclear DNA as revealed by cesium chloride density gradients (Groves and Oliver, 1984). The fusion products of *S. cerevisiae* and *K. marxianus* contained a large proportion of *K. marxianus* DNA as determined by hybridisation analysis (Witte *et al*., 1989). Spencer *et al*. (1989) developed a method for obtaining fusants that contain mostly *S. cerevisiae* DNA as seen in the fusion with *Hansenula canadensis*. Interspecies hybrids of entomopathogenic fungi *Beauveria bassiana* and *Beauveria sulfurescens* were also found to contain only partially heterozygous genomes (Viaud *et al*., 1998). In the mammalian fusion of human and mouse cells, only a small portion of the human genome is kept - the human chromosomes are preferentially lost over several generations (Latron *et al*., 1988).

Karyotyping of the hybrids by PFGE analysis did not reveal any differences between the hybrids and their progenitor strains. This however does not conclusively prove the absence of heterozygous DNA present in the hybrids. In addition, the identical electrophoretic behaviour of progenitor strains and their hybrids does not necessarily indicate the presence of identical chromosome sets, which can only be confirmed by hybridisation analysis and physiological experiments (Witte *et al*., 1989). Analysis may be impeded if the progenitor strains contain chromosomes of similar length, which would not reveal any changes in the karyotypes of the strains (Witte *et al*., 1989; Selmecki *et al*., 2006). Hybrids showing a mixed chromosomal banding pattern do not necessarily contain the full genomic complement of both progenitor strains as shown in the hybrids of *K. marxianus* and *S. cerevisiae* that had mixed banding patterns but contained mostly *K. marxianus* DNA (Gera *et al*., 1997).

Chromosomal length polymorphisms (CLPs) were observed in the hybrid progeny B7 and T2-22 (Fig. 12) and this may be due to the *de novo* chromosomal rearrangements known to occur in interspecies hybrids (O'Neill *et al*., 2001) although CLPs have been reported to be present in natural isolates of several fungi such as *A. nidulans* and *U. hordei* (Fierro and Martin, 1999). Despite the great variability in pathogenicity observed among the hybrids and their progeny, no distinct correlation between the electrophoretic pattern and pathogenicity could be shown. The origin of the re-arranged chromosomes could not be determined by microarray analysis using the Ustilago chip since the hybridisation signals produced by the *S. reilianum* strain JS333 were not strong enough to allow proper comparison. Ideally, molecular probes specific to several chromosomes of each of the parent strains should be used to identify the nature of the hybrid DNA content (Witte *et al*., 1989). Sufficient sequence information of *S. reilianum* was lacking at the time to facilitate such a comparison, however, the recent completion of the genome sequence should allow for a more

complete determination of the DNA contribution of each progenitor strain in the hybrids using chromosome-specific probes. The available sequence information should also allow the determination of the probable chromosomal regions containing the factors responsible for tumour formation as well as the site-specific symptom development observed in the hybrids. This can be accomplished by a genomic subtraction of the hybrid and one progenitor strain to isolate the DNA inherited from the other progenitor strain (Wieland *et al*., 1990).

Plant infection assays revealed a very low infection rate in the hybrids. This may be due to problems encountered due to conflicting or asynchronous regulation of the complex cascade of genes involved in pathogenicity and symptom expression. Symptom expression in the form of tumour development exclusively in the cob is proposed to be due to the hybrids expressing the tumour specific trait of *U. maydis* and the site of symptom development trait specific to *S. reilianum*. Possible contamination by *U. maydis* spores from another experiment in the glasshouse can be excluded since the germinated spores produced progeny that contained *S. reilianum* specific genes. Most of the progeny contained both antibiotic markers suggesting aberrant segregation behaviour. Almost all the hybrid progeny were non-viable after passage to fresh medium, which has been reported for other hybrid progeny (Tamaki, 1986). Peberdy (1979) suggested that this instability may be due to aneuploidy (an unequal number of chromosomes) in the hybrid progeny.

Hybrids have been of substantial benefit to improving strains used in industrial processes. Strains have been obtained with improved traits such as increased ethanol production and thermotolerance in the fusion of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* (Gera *et al*., 1997); increased production of carotenoids by hybrids of *S. cerevisiae* and *Phaffia rhodozyma,* and of *S. cerevisiae* and *Cryptococcus laurentii* (Santopietro *et al*., 1997); utilisation of a novel range of carbon sources as shown by the fusion of *Yarrowia lipolytica* and *Kluyveromyces lactis* (Groves and Oliver, 1984) and that of *S. cerevisiae* with *Schwanniomyces castelli* (Tamaki, 1986) to mention but a few.

5.3 Early stages in the infection process

The microscopic observations during the early stages of infection of *U. maydis* and *S. reilianum* in maize leaves revealed that both fungi form appressoria and penetrate the leaf surface. No differences are evident in the hyphal growth within the plant on 1 dpi and 2 dpi between the two fungi. However, from 3 dpi to 5 dpi distinct differences

in hyphal appearance are evident in *U. maydis* as compared to *S. reilianum*. Phytopathogenic fungi are known to undergo complex developmental and hence a series of metabolic events to enable them to penetrate formidable plant surface barriers such as plant host cuticles and invade the living plant tissue, thereby causing disease (Tucker and Talbot, 2001). No difference was observed between *S. reilianum* and *U. maydis* in their ability to form appressoria and penetrate maize leaf tissue 24 h post infection. This may indicate that *S. reilianum* uses the same strategy as *U. maydis* and *U. hordei* to penetrate the leaf by entering between epidermal cells directly (Snetselaar and Mims, 1992; Hu *et al*., 2002). The mode of hyphal growth within the plant leaf during the first two days of infection is very similar for the two fungi. There is virtually no distinction between the two pathogens, which both show intra- and inter-cellular branching hyphal growth. Another striking similarity is the fact that no visible damage is observed to the host cells, although increased hydrogen peroxide accumulation and auto-fluorescence are observed *in planta* containing older sections of fungal hyphae as well as increased expression of plant defence genes (G. Döhlemann, pers. comm.). This is suggestive of both pathogens' ability to evade the plant's defence responses, which Luttrel (1987) attributed to the high degree of compatibility between smut fungi and their hosts. The fungi might achieve this high degree of compatibility through the expression of effectors that may shield the fungus from or interfere with plant defence response. Evidence for this is the presence of many clusters of small secreted proteins containing multiple gene copies in *U. maydis* (Kämper *et al*., 2006) and *S. reilianum* (J. Schirawski, pers. comm.). Heath (1995) suggested that rust fungi, during hyphal growth within the host plant, release suppressors that promote compatible host-pathogen interaction.

An alteration in hyphal growth began very early in *U. maydis* characterised by prolific hyphal branching as early as three days post inoculation while no change was observed in *S. reilianum* (Fig. 14). *U. maydis* may possibly respond to plant cues that *S. reilianum* is insensitive to or suppresses until floral induction where its mode of growth is altered to a more prolific one (Fullerton, 1970; Langdon and Fullerton, 1975; Matyac, 1985; Martinez *et al*., 1999; 2002). Subsequent hyphal growth of *U. maydis* appeared to be both inter- and intracellular, with all cell layers invaded by the fungus. Early signs of sporogenesis were evident 5 dpi characterized by hyphae appearing to round off (Fig. 14). *S. reilianum* also appeared to grow both inter- and intra-cellularly but did not show much branching or any increase in proliferation as observed in *U. hordei* infection of barley (Hu *et al*., 2002). The later stages of fungal development within maize have previously been described for *U. maydis* (Snetselaar

and Mims, 1992; 1993) and *S. reilianum* (Langdon and Fullerton, 1975; Matyac, 1985; Martinez *et al*., 1999; 2002). Hanna (1929) investigated the physiology and cytology of the germinating spores of both fungi in addition to the mating behaviour and pathogenicity of the resultant sporidia. Several studies have focussed on the infection process of the individual fungi. A detailed microscopic study was made by Snetselaar and Mims (1992; 1993; 1994) of the infection of maize seedlings and stigmas by *U. maydis*. A microscopic overview of the lifecycle of *S. reilianum* is described by Martinez *et al*. (2002) but they focused on the atypical root penetration reported by Martinez *et al*. (2000), while Martinez *et al*. (1999) described the fungal development within the shoot apex. The report of infection via maize roots should be regarded as an atypical infection as the root-infected plants did not develop any symptoms (Martinez *et al*., 2000; 2002). A similar atypical root infection was demonstrated by Sesma and Osbourn (2004) for the rice blast fungus *Magnaporthe grisea* that normally infects rice leaves through the formation of appressoria. These authors however, observed that the root infections proceeded not by appressoria formation but by hyphal swellings or hyphopodia leading to typical symptom development in the plant (Sesma & Osbourn, 2004). This therefore indicates that *M. grisea* is capable of infecting rice both via roots or leaves using different developmental strategies, whereas the same cannot be concluded for *S. reilianum* since the root infection does not lead to typical symptom development. Most reports on *S. reilianum* infection of maize used young seedlings that were infected by hypodermic syringes (Potter, 1914; Hanna, 1929; Matyac, 1985). *S. reilianum*, like most other smut fungi, is known to infect maize at the seedling stage and invade the meristematic tissue (Potter, 1914; Hanna, 1929; Halinsky, 1962; Matyac, 1985). However, it is more likely that the fungal growth merely follows the meristem as the presence of the fungus in the meristematic cells would lead to morphological alterations in the plant that are not observed (V. Walbot, pers. comm.).

5.4 Expression profiling of two fungal pathosystems

The identification of genes that are differentially expressed between *U. maydis*- and *S. reilianum*-infected plants was achieved by comparative expression profiling using the cDNA-AFLP technique. This comparative screen facilitated the discrimination of basal host responses to wounding, mock infection using non-pathogenic haploid strains and pathogen-induced or infection specific genes elicited in either host or pathogen. Two time points were selected during the early stages of infection, namely,

three and four days post infection. A total of 322 fragments were identified as being differentially expressed. Sixty percent of these were identified as being of plant origin while 22 % were of fungal origin. Forty percent of fragments of fungal origin were of genes coding for hypothetical proteins of unknown function, while 30 % were identical to intergenic regions. Those of known function coded for genes involved in basic metabolism or transport.

Differences in pathogenesis are mostly due to specific differences in gene regulation (Ahmad *et al*., 2006) in which pathogens subvert host defences or metabolic pathways for their own benefit (de Torres *et al*., 2003). Transcript profiling of material obtained *in planta* containing both host and fungus provides a rich source of genes that may only be expressed during the infection process. Using different time points allows the identification of stage-specifically expressed genes. However, since the infections of *U. maydis* and *S. reilianum* are not completely synchronous, comparison of different time points would lead to an inaccurate comparison of the stages of infection. Instead, a more accurate comparison could be made by comparing the specific stages of infection in these two fungi. For example, by using the stage prior to spore formation for comparison should be more effective, as the time interval at which this occurs after infection differs between *U. maydis* and *S. reilianum* by several weeks. Thus, by selecting a broader time frame whereby different stages of infection can be compared could supply a more detailed representation of the actual differences in infection between *U. maydis* and *S. reilianum*.

Putatively differentially expressed fragments identified during this study could not be confirmed by dot-blot hybridisation or by Northern hybridisation analysis. This may be due to the insensitivity of using labelled total cDNA as a probe that often does not label efficiently due to complex formation problems as observed by Taylor and Harrier (2003). Alternatively, the inability to detect these cDNA species may result from the low abundance of the mRNA species. The use of more sensitive techniques such as quantitative RT-PCR or real-time RT-PCR should be able to demonstrate any differences in expression levels. The criteria used for selection of amplified fragments between the two pathosystems may also have contributed to the lack of differential expression by Northern analysis. Perhaps it would be more prudent to select fragments that show differences in expression levels rather than presence or absence, thereby possibly identifying differences in regulation of key genes associated with pathogenicity rather than identifying unique genes.

The cDNA-AFLP technique has been widely used to identify genes associated with pathogenicity (Dellagi *et al*., 2000; Qin *et al*., 2000; van der Biezen *et al*., 2000; Jeney *et al*., 2004; Santaella *et al*., 2004). In genome expression analysis of *Saccharomyces cerevisiae* cDNA-AFLP was shown to produce expression data that correlated well with microarray and Genechip data (Reijans *et al*., 2003). The redundancy of fragments identified during the screen has also been reported by other authors and is considered within the expected range of up to 15% (Bachem *et al*., 1996; van der Biezen *et al*., 2000; Craciun *et al*, 2003). Bachem *et al*. (1996) found that mismatched primed PCR of highly abundant transcripts led to amplified fragments with the same mobility in fingerprints obtained with primers having similar sequence extensions. Redundancy could also result from mispriming during PCR amplification generating PCR products representing either different alleles from the same gene or different instances of multicopy genes (Craciun *et al*., 2003). More than 40% of the amplified fragments of fungal origin were identified as hypothetical proteins and about 20% were between two genes (intergenic). This displays the difficulty in annotating genes in higher eukaryotes and that the *U. maydis* genome, like that of *Arabidopsis thaliana*, is not yet fully annotated (Reijans *et al*., 2003).

5.5 Nitrilase and its role in auxin production and tumour formation

Serendipitously, a nitrilase gene was identified in the cDNA-AFLP screen as being expressed in *U. maydis* on 3 dpi and not 4 dpi while it was found in *S. reilianum* as being expressed on both days. Thus the nitrilase initially appeared to be differentially regulated in *U. maydis* but not in *S. reilianum*. This however, could not be confirmed by Northern analysis and as the gene is not represented on the Ustilago chip (J. Kämper, pers. comm.) no microarray data is available either. Nonetheless, the nitrilase was investigated for its possible role in the production of the auxin, indole-3-acetic acid (IAA) and subsequent association in tumour formation. The nitrilase could not be shown to be involved in the synthesis of IAA in *U. maydis* since the deletion of the nitrilase in an *iad*1/*iad*2 double deletion strain did not abolish IAA production. In addition, deletion of the nitrilase gene had no effect on tumour formation besides a possible delay in spore formation. This suggests that the ability of the fungus to produce high levels of IAA is not a prerequisite for the formation of tumours although the possible influence of another nitrilase gene cannot be excluded. Preliminary

analysis of several maize auxin-related genes indicated an indirect influence of *U. maydis* on increased levels of auxin in tumour tissue.

Although high IAA levels are often found in diseased plants, especially where neoplasmas (tumours) are formed, the exact role of IAA in fungus-plant interactions has not been determined (Robinson *et al*., 1998). Tumour development in plants infected with *U. maydis* is characterised by increased mitotic divisions and an enlargement of cells (Christensen, 1963; Callow and Ling, 1973). Other phytopathogens that cause neoplastic diseases, such as *Agrobacterium tumefaciens*, have been demonstrated to produce high amounts of auxin (Gruen, 1959) and require this ability to induce disease symptoms in the infected host plant (Costacurta and Vanderleyden, 1995). Substantially elevated levels of auxin have been demonstrated in *U. maydis* infected tumour tissue as compared to healthy maize tissue (Moulton, 1942; Wolf, 1952). Since *U. maydis* was shown to produce the auxin indole-3-acetic acid (IAA) when grown in culture in the presence of tryptophan (Wolf, 1952), it is highly possible that the IAA produced by *U. maydis* is concomitant to the formation of tumours. However, wide varieties of plants are also capable of producing IAA from tryptophan, so it is possible that the increased IAA levels observed in infected plant tissue may be a result of an alteration in IAA accumulation by the plant. Although IAA has been detected in plants over a century ago, the biochemical routes of its synthesis have not been completely elucidated to date. The proposed biosynthetic routes observed in both plants and microorganisms are outlined in Fig. 21. Briefly, tryptophan (TRP) can be converted to IAA via indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA), tryptamine (TAM) or indole-3-acetaldoxime (IAOx). In bacteria TRP is converted to IAM by tryptophan monooxygenase (*iaaM*) and IAM is hydrolysed to IAA by indole acetamide hydrolase (*iaaH*). In plants and fungi aminotransferase activity (TAA1 / TAR or TAM1) results in the conversion TRP to IPA, which is subsequently decarboxylated to indole-3-acetaldehyde (IAAld) by either of the two indole-3-acetaldehyde dehydrogenases *iad1* and *iad2*. IAAld can also be converted to indole-3-ethanol in a reversible reaction. TAM is formed by the decarboxylation of TRP by tryptophan decarboxlase (*TDC*). In *Arabidopsis,* hydroxylation of TAM results in N-hydroxyl-TAM by the action of *YUCCA* / *FLOOZY* (flavin monooxygenase). N-hydroxyl-TAM is converted to IAA via the two intermediates IAOx and IAAld. TRP can also be converted to IAOx by two cytochrome P450 enzymes, *CYP79B2* and *CYP79B3.* IAOx is first converted to indole-3-S-alkyl-thiohydroximate by *SUR2* (*CYP83B1*) and then to indole-3-

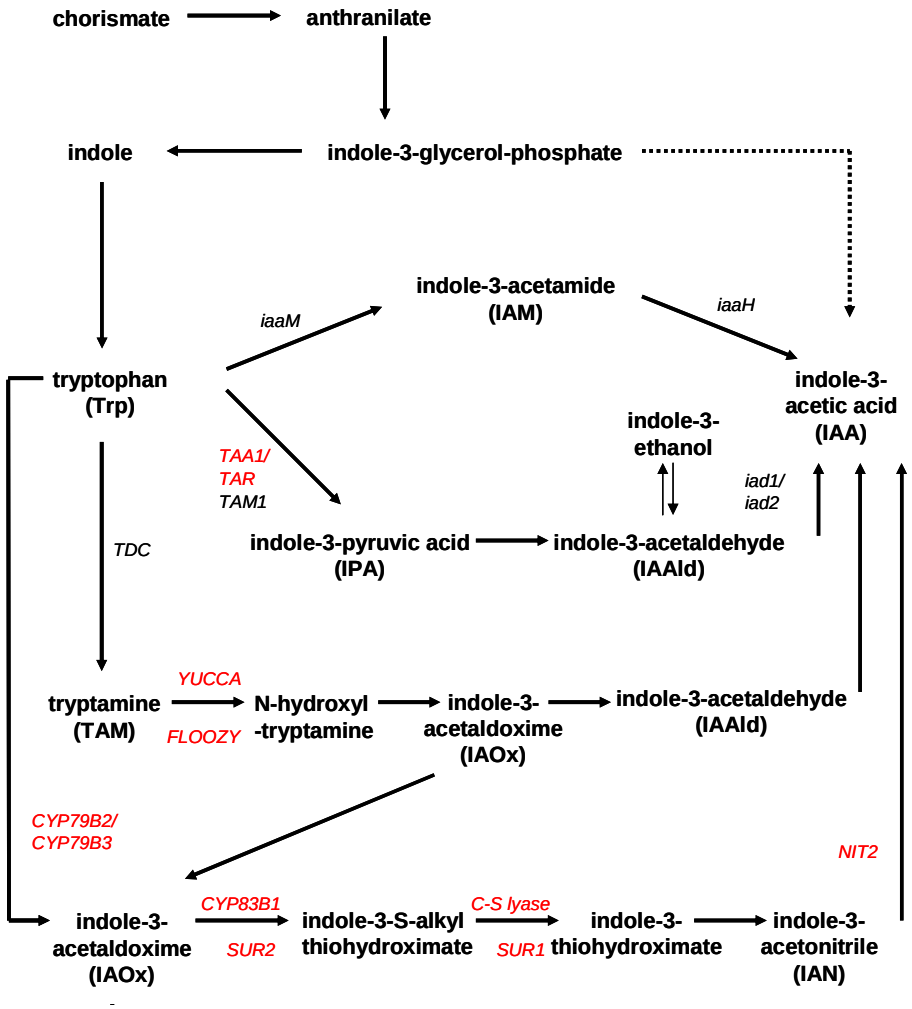


Figure 21. Tryptophan-dependent and proposed tryptophan-independent indole-3-acetic acid (IAA) biosynthesis routes in plants and microorganisms. Auxin biosynthetic genes identified in plants are in red and those from bacteria or fungi are in black. Schematic diagram modified from Chandler, 2009.

thiohydroximate by *SUR1* (*C-S lyase*). Indole-3-thiohydroximate is converted to indole-3-acetonitrile (IAN), which in turn is converted to IAA by a nitrilase (*Nit*2). In a proposed tryptophan-independent route, IAA is produced via indole-3-glycerol-phosphate derived from anthranilate and chorismate (Ljung *et al*., 2002; Kriechbaumer *et al*., 2006; Chandler, 2009).

Tumours are the product of cell division and subsequent elongation of the newly formed cells (Ingram & Tommerup, 1972). Mani (1964) found that exogenous applications of auxins to plants were not able to induce the formation of tumours of complex morphologies and did not produce structures that exhibited the degree of hyperplasia found in most tumours caused by pathogens. In *U. maydis* tumour formation has been suggested to be related to the fungus' production of IAA, although no direct correlation has been found (Moulton, 1942; Wolf, 1952; Basse *et al*., 1996; Martinez *et al*., 1997; Sosa-Morales *et al*., 1997). Basse *et al*. (1996) were

able to show that the deletion of a key enzyme involved in the conversion of IAA from indole-3-acetaldehyde (IAAld), an IAAld dehydrogenase (*iad*1), dramatically reduced the amount of IAA produced by the fungus *in vitro*. No difference was observed in tumour formation in the absence of this gene. Analysis of a second IAAld dehydrogenase (*iad*2) demonstrated that *in vitro* IAA production was further reduced (Reinecke *et al*., 2008). However, IAA production was not completely abolished when both genes were deleted. Residual IAA produced was then suggested to be attributed to the action of a nitrilase, which could produce IAA via another pathway, through indole-3-acetonitrile (Basse *et al*., 1996; Reinecke *et al*., 2008). The biosynthetic pathway through which IAA is produced by *U. maydis* has not been completely elucidated, although it has been suggested to proceed from tryptophan through indole-3-pyruvic acid (IPA) (Zuther *et al*., 2008) and IAAld (Wolf, 1952; Navarre, 1990) and tryptamine (Basse *et al*., 1996; Reinecke *et al*., 2008). In *Colletotrichum gloeosporioides f. sp aeschynomene* IAA biosynthesis is also tryptophan dependent and Robinson *et al*. (1998) were the first to report the conversion of tryptophan to IAA via the indole-3-acetamide (IAM) pathway in this fungus. This was the first time that the IAM pathway had been reported in fungi. *Colletotrichum acutatum*, which causes lime anthracnose in citrus, has been shown to synthesise IAA via the IPA pathway and the IAM pathway described for *C. gloeosporioides f. sp aeschynomene* (Chung *et al*., 2003). Yamada *et al.* (1990) reported that in virulent fungal species such as *Taphrina wiesneri*, *Taphrina deformans* and *Taphrina pruni*, which cause hyperplastic diseases in plants such as peach, cherry and plum, respectively, IAA is synthesised from tryptophan not only via IPA and IAAld but also from IAN by a nitrilase. For many phytopathogenic bacteria the ability to produce IAA is tightly linked to pathogenicity (Fett *et al*., 1987). Gall induction by *Pseudomonas syringae pv. savastanoi* is caused by the IAA produced by the bacterium itself, while other gall-forming bacteria such as *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have the IAA biosynthesis genes encoded on a plasmid (Smidt and Kosuge. 1978; Comai *et al*., 1982; Liu *et al*., 1982; Thomashow *et al*., 1984; Surico *et al*., 1985; van Onckelen *et al*., 1985). In the gall-forming bacterium *Erwinia herbicola pv gypsophilae* inactivation of the IAM pathway reduced gall formation by 40% but little effect was observed when the IPA pathway was inactivated (Manulis *et al*., 1998) demonstrating the primary route of IAA biosynthesis via IAM. Elucidation of the IAA biosynthetic pathways in *U. maydis* would permit the assessment of their contributions to IAA production during tumour formation.

Prusty *et al*. (2004) found that the ability to perceive the plant hormone IAA caused *S. cerevisiae* to differentiate into an invasive form. Perception of IAA may also apply to *U. maydis* but instead it could cause *U. maydis* to stimulate an increased production of IAA by the host plant by manipulating the plant's auxin biosynthetic genes. This may then be advantageous to *U. maydis* since it was shown that auxin down-regulates certain genes involved in plant-defence responses (Dominove *et al*., 1992; Navarro *et al*., 2006).

This study revealed that the presence of *U. maydis* leads to an increase in expression of the maize actin gene, an auxin transporter and an expansin gene. Up-regulation of these genes could imply the manipulation of host auxin levels to induce hyperplasia (tumourous growth), which is normally associated with increased expression of expansins (Devos *et al*., 2005) and possibly actin. A possible explanation for the lack of differential regulation for the auxin responsive gene *ZmSAUR*2 is that the time points used to isolate the RNA were not appropriate, since this gene was shown to display a very rapid (10 - 25 min) and transient alteration in expression level in response to auxin (Quint and Gray, 2006). Activation of *ZmSAUR2* therefore, occurs long before the onset of other physiological responses such as cell elongation (Yamada, 1993). This indicates that *U. maydis* does not influence the plant's auxin production via *ZmNit2*, as the expression level of the IAA biosynthetic gene *ZmNit*2 was not affected. However, this analysis does not exclude an influence of the fungus on plant auxin production, as *ZmNit*2 is only involved in one of several IAA biosynthetic pathways present in maize. An indication of the possible influence of *U. maydis* is seen in the induction of genes responsive to the auxin produced such as the auxin transporter *ZmAux*1 and the expansin gene *ZmExpA*4.

5.6 Outlook

The molecular dissection of the genetic factors that enable fungi to invade and proliferate in their hosts has intrigued many researchers (Osbourn, 2001). This study has attempted to elucidate the molecular determinants causing differences in symptom development in the smut fungi *U. maydis* and *S. reilianum*. Subsequent experiments are required to verify that tumour formation in *U. maydis* is dominant while symptom development in the flowers is determined by *S. reilianum*. One could employ BAC libraries containing genomic regions of *U. maydis*, transform them into

S. reilianum and screen for the ability for tumour formation. The genomic regions can then be sequenced and the specific genes responsible identified.

The adaptation of *U. maydis* to its host has been attributed to the secretion of proteins of unknown function that are encoded in clusters (Kämper *et al*., 2006). The availability of the recently determined genome sequence of *S. reilianum* should facilitate a comparative genomics approach to identify differences in secreted effector proteins and these could be good candidates for determining differences in symptom development.

Materials and Methods

6.1 Materials

6.1.1 Media, Solutions, Enzymes and Kits

Chemicals

Chemicals used for all the experiments were of molecular biology grade supplied by Sigma, Merck, Fluka, Bio-Rad, Difco or Roth. Exceptions are noted in the respective formulations.

Buffers and Solutions

All standard buffers and solutions were prepared according to Ausubel *et al.* (1987) and Sambrook *et al.* (1989), exceptions or modifications are noted in the respective methods or listed below.

Media

E. coli was cultivated in either dYT or LB liquid medium and YT solid medium (Ausubel *et al*., 1987 and Sambrook *et al*., 1989). Unless otherwise noted, these media were supplemented, when necessary, with the following: Ampicillin (100 µg/ml), Chloramphenicol (25 µg/ml), Kanamycin (40 µg/ml), Tetracycline (25 µg/ml) und X-Gal (40 µg/ml).
U. maydis and *S. reilianum* were cultivated using the following media:

YEPS$_L$-Medium (modified Tsukuda *et al*., 1988):
- 10 g Yeast Extract
- 10 g Peptone
- 10 g Saccharose
- Volume adjusted to 1 l with H_2O

CM Complete medium (Banuett and Herskowitz, 1989):
- 1,5 g NH_4NO_3
- 2,5 g Casamino Acids
- 0,5 g DNA
- 1 g Yeast Extract
- 10 ml Vitamin solution (see below)
- 62,5 ml Salt solution (see below)
- Volume adjusted to 960 ml with H_2O
- Adjusted to pH 7,0 with NaOH and autoclaved
- After autoclaving: Glucose solution added (f. c. 1%)

PD medium:
- 24 g Potato Dextrose Broth
- Volume adjusted to 960 ml with H_2O and autoclaved

NM Nitrate minimal medium (Holliday, 1974):
- 3 g KNO_3
- 62,5 ml Salt solution (see below)
- Volume adjusted to 980 ml with H_2O
- Adjusted to pH 7.0 with NaOH and autoclaved
- after autoclaving, supplemented with:
- 20 ml 50% (w/v) Glucose (f. c. 1%)

Salt Solution (Holliday, 1974):
- 16 g KH_2PO_4
- 4 g Na_2SO_4
- 8 g KCl
- 4 g $MgSO_4$ x 7 H_2O
- 1,32 g $CaCl_2$ x 2 H_2O
- 8 ml Trace elements solution
- Volume adjusted to 1l with H_2O and filter sterilised

Trace Elements Solution (Holliday, 1974):
- 60 mg H_3BO_3
- 140 mg $MnCl_2$ x 4 H_2O
- 400 mg $ZnCl_2$
- 40 mg $NaMoO_4$ x 2 H_2O
- 100 mg $FeCl_3$ x 6 H_2O
- 40 mg $CuSO_4$ x 5 H_2O
- Volume adjusted to 1l with H_2O and filter sterilised

Vitamin Solution (Holliday, 1974):
100 mg Thiamin
50 mg Riboflavin
50 mg Pyridoxin
200 mg Calcium pantothenate
500 mg p-Aminobenzoic acid
200 mg Nicotinic acid
200 mg Cholin chloride
1000 mg myo-Inositol
adjusted to 1l with H_2O and filter sterilised

Regeneration medium (Schulz *et al*., 1990):
a) Top-Agar:
1.5% (w/v) Bacto-Agar
1 M Sorbitol in YEPS Medium (see above)
b) Bottom-Agar:
as in a), with the addition of double concentration of antibiotic

PD-CC activated charcoal containing PD Medium:
24 g Potato Dextrose Broth
10 g Activated charcoal
20 g Bacto-Agar
Volume adjusted to 1 l with H_2O and autoclaved

Water agar
1% Bacto-agar in water, autoclaved

Media were supplemented when necessary with the following antibiotics unless stated otherwise: Carboxin was used at 5 µg/ml for *S. reilianum* and 2 µg/ml for *U. maydis*, Hygromycin (200 µg/ml), ClonNAT (150 µg/ml) and Phleomycin (40 µg/ml).

Enzymes

Restriction enzymes were supplied by New England Biolabs (NEB), unless stated otherwise all other enzymes were supplied by Invitrogen with the exception of the in-house (self-made) *Taq* DNA Polymerase.

Commercial kits and miscellaneous materials

RNeasy Plant Mini Kit (Qiagen) for the isolation of highly pure total RNA; QiaQuick PCR Purification Kit (Qiagen) for the purification of PCR products; QiaQuick Gel Extraction Kit (Qiagen) for the purification of PCR products and restriction digested fragments; JETquick Plasmid Miniprep Kit (Genomed) for the isolation and purification of plasmid DNA; TOPO TA Cloning Kit (Invitrogen) for cloning of PCR products, NEBlot Kit (NEB Biolabs) for radioactive labeling of DNA fragments, Microspin S-300 columns (Amersham Pharmacia Biotech) for the purification of radioactively-labeled DNA probes; Quantitect cDNA synthesis kit (Invitrogen) for the synthesis of gDNA-free cDNA for real time qPCR; Platinum SYBR Green Supermix (Invitrogen) for real time qPCR; AFLP Analysis System I - Core Reagent and Starter Prirmer kits (Invitrogen). Other materials utilised are listed with the respective methods.

6.1.2 Oligonucleotides

The oligonucleotides were synthesised by Sigma Genosys and MWG. Lyophilised oligonucleotides were resuspended with sterile TE buffer to a final concentration of 100µM and stored at -20°C. The sequence of the oligonucleotides is entered in a 5'- 3' direction.

Table 5. List of oligonucleotides used in this study

Primer name	Sequence	Remarks
E-HIIIb	GATGAACCATAGCGTGAGCTGATG	from Joerg, to amplify the variable regions of bW and bE, hybridizes within homeodomain of bE (bp 3498-3475 of bW2bE2) nested
ITS1	TCCGTAGGTGAACCTGCGG	hybridizes within 18SrRNAgene, used with ITS4 to screen for fungal DNA
ITS4	TCCTCCGCTTATTGATATGC	hybridizes within 23SrRNAgene, used with ITS1 to screen for fungal DNA
Tnatr	GCATGGGCCCAGATCTGTCATGATG	G. Reinicke - hybridizes within nat-cassette 5' region
oAN23	AAGTCGGTCTTGGTGCGG	A.Brachmann
oAN24	GCTCGATCTCGTTGAGGC	A.Brachmann
oAN74	GAGCAGTTCATGATGGTAAG	A.Brachmann - hybridizes within *U. maydis* Cbx
oAN75	TTCGAGCTGGTTGCCTGC	A.Brachmann - hybridizes within *U. maydis* Cbx
oAN76	CTATGCGGCACTAGAGCAG	A.Brachmann - hybridizes within *U. maydis* Cbx
oAN77	TTCGCTCTACCGATGCCTT	A.Brachmann - hybridizes within *U. maydis* Cbx
oAN78	TCTCCAAGCCACGGTTCC	A.Brachmann - hybridizes within *U. maydis* Cbx
oBH077f	GCAGGCACTTTGATCAGAGG	sequencing of SRZ a1 locus (3382 – 3363)
oBH1	CCCTAGCTGGCATTCCC	used to amplify right border probe for a-locus identification (pHLN10Ba1, 6708-6692)
oBH2	CCTTGAGACCGGGATAGCC	used to amplify right border probe for a-locus identification (pHLN10Ba1, 5641-5659
oBH3	TTCACATTTGGACGCATCG	used to amplify left border probe for a-locus identification (pHLN10Ba1, 11976-11958)
oBH4	TTCACATTTGGACGCATCG	used to amplify left border probe for a-locus identification (pHLN10Ba1, 11976-11958)
oBH5	TAGGTACGCAGGAATTCTC	used to create SacI-linker with oBH6

Primer name	Sequence	Remarks
oBH6	TCGAGAGAATTCCTGCGTACCTA	used to create SacI-linker with oBH5
oBH7	GCTCTCAGCGTGATTAGACC	sequencing of SRZ a2 locus (5191 – 5210)
oBH8	CCGCTTCGTTCTGGCAAATC	sequencing of SRZ a2 locus (5575 – 5556)
oBH9	TCCGGATTTGCCAGAAC	sequencing of SRZ a2 locus (5552 – 5568)
oBH10	ACACAAGGCGGATTTGGAAG	sequencing of SRZ a2 locus (6135 -6154)
oBH13	TGGGCATTGGTGGTGGAGAG	sequencing of SRZ a2 locus (9532 – 9551)
oBH14	ATGCCCACCACCTCCACCAATG	sequencing of SRZ a2 locus (9519 – 9498)
oBH15	TCTGTGCTTTCAGGCGAGAG	sequencing of SRZ a2 locus (9767 – 9748)
oBH16	GCGTTCGGATGTGGCAATCG	sequencing of SRZ a2 locus (9696 – 9715)
oBH17	GTTGGGTTGATTCCGTAGG	sequencing of SRZ b2-locus
oBH18	GAGAGTGTTCGCGATATGG	sequencing of SRZ b2-locus
oBH19	ATATGGCCTGAGTGGCCGGGATCACTCACG	sequencing of SRZ b2-locus
oBH20	TCACGCGCTCAGATCAGTCC	sequencing of SRZ b2-locus
oBH21	ATAAGGCCGCGTTGGCCTCGCCCTTGCTCACC	sequencing of SRZ b2-locus
oBH22	AAGGGAGAAAGGCGGACAGG	sequencing of SRZ b2-locus
oBH23	TCTTGTGCCAGACGCGAACC	S3B7rev (Pair)
oBH24	GCTCAGCAGCAACAGCATCC	S3B7rev (Pair)
oBH25	GCTGGTGGAATCAAGTACG	S4B0uni (Pair)
oBH26	CATCTGCGCTAACTACTCG	S4B0uni (Pair)
oBH27	TTCGGGCTTGAATCCGTTCG	S5B3rev (Pair)
oBH28	ACAGCAGCAAGTGGCAAAGG	S5B3rev (Pair)
oBH31	CGATCATGTAGGCAGAGTTACC	used to amplify ORF of ZmAux-1 (forward)
oBH32	CGCCGATGATCAACTTCTTCC	used to amplify ORF of ZmAux-1 (reverse)

Primer name	Sequence	Remarks
oBH43	CCACGGCGATGATGTTCTCC	used to amplify ORF of ZmExpA4 (forward)
oBH44	ACTTGAACCAGGGCGAGACG	used to amplify ORF of ZmExpA4 (reverse)
oBH45	CCAAGAAGTGGCAGAGGATGG	used to amplify ORF of ZmSAUR2 (forward)
oBH46	TGCAGCCTTGCAGGATTCG	used to amplify ORF of ZmSAUR2 (reverse)
oBH51	GCAAGCGGTTGCTGCAAAGG	used to amplify ORF of ZmNit2 (forward)
oBH52	CGGCAGACGTGAAAGACACAGG	used to amplify ORF of ZmNit2 (reverse)
oBH5211	TGATCGAGTCGCATCTCAAC	sequencing of SRZ a1 locus (13271 – 13252)
oBH5211f	TCTAGCCTTGGCACCTTCG	sequencing of SRZ a1 locus (12612 – 12594)
oBH5211r	GCTTGCCCTCGTTGAGATGC	sequencing of SRZ a1 locus (13242 – 13261)
oBH527	GTCCTATCCAGCCGTAGCAC	sequencing of SRZ a1 locus (551 – 570)
oBH527f	ATGCACCTGCATGTCGAACC	sequencing of SRZ a1 locus (1253 – 1272)
oBH527r	GTGCTACGGCTGGATAGGAC	sequencing of SRZ a1 locus (570 – 551)
oBH67	CACGGCGATGATGTTCTCC	used to amplify ORF of ZmEXPA4 (forward)
oBH68	CGGGCATTCACGTACATTCC	used to amplify ORF of ZmEXPA4 (reverse)
oBH73	ACCTCACCGACCACCTAATG	Maize actin real time primer F
oBH74	ACCTGACCATCAGGCATCTC	Maize actin real time primer R
oBH77	ATCCACGTGTACCTCGATCTC	ZmAux1 real time primer F
oBH78	ACTCCCTGTCGAGTTTGCTC	ZmAux1 real time primer R
oBH79	TACTTGAACCAGGGCGAGAC	ZmExpA4 real time primer F
oBH80	TACTTGAACCAGGGCGAGAC	ZmExpA4 real time primer R
oBH91	GGTGCTATGTTCTGCGTTTG	Umi2a real time primer
oBH92	TCTGCTCACATGCTTCATCC	Umi2a real timer primer R
oBH93	TGGAGTACGCCATGTGTTTG	ZmSAUR2 real time primer F

Primer name	Sequence	Remarks
oBH94	AACAGCGATCTGATGTGTGG	ZmSAUR2 real time primer R
oBH99	AGCCTCCATGACACACATTG	ZmNit2 real time primer F
oBH100	AATGATAACGCTGCCTCCAG	ZmNit2 real time primer R
oBHpra1	CTGCCTGAAACTGACGCCGA	sequencing of SRZ a1 locus (7699 – 7718)
oBHrba1	CTGATTTACGCCGAGCATAC	sequencing of SRZ a1 locus (8852 – 8833)
oBHrba2	TGTCGAGGTCGTCGGTCTTC	sequencing of SRZ a1 locus (9457 – 9476)
oBHrba3	CGGCGTATGCAATGTAAGGC	sequencing of SRZ a1 locus (9376 – 9395)
oJS6	CTTCATACAGACGCTCTGAGGTG	Um a2 locus at 7848 to7870 upstream of BamH I site within rga2. Used for making a probe to look for SRZ a-locus.
oJS7	GATTCGACGAGCACAGTCACAG	Um a2 locus at 8731 to 8752 downstream of Sal I site downstream of rga2. Used for making a probe to look for SRZ a-locus.
oJS8	ATGCTGTCCCAGTCCTCCTC	hybridizes within bE of *S. reilianum*, facing towards 5'-end of bE gene
oJS9	AGGACTGGGACAGCATGCTC	hybridizes within bE of *S. reilianum*, facing towards 3'-end of bE gene
oJS10	GGATCCTAGTAGCTAGCTAGGATCCA	designed to hybridize with oJS11 to insert a BamH I-linker fragment into pCR4topo
oJS11	GGATCCTAGCTAGCTACTAGGATCCA	designed to hybridize with oJS10 to insert a BamH I-linker fragment into pCR4topo
oJS12	GAGGTTGTCGCTGAGAACGC	hybridizes within bE of *S. reilianum*, facing towards 5'-end of bE gene, downstream of oJS8
oJS13	GCTGGATCAAGTACGGCGTC	hybridizes within bE of *S. reilianum*, facing towards 3'-end of bE gene, downstream of oJS9
oJS18	TGTGATGATGTAAGCCCACGC	used for sequencing SRZ SRZ1 a-locus (7180 – 7160)
oJS19	TGAAGCGCACTATAGACTCTCC	used for sequencing SRZ SRZ1 a-locus (6126 – 6147)
oJS20	TCCATCTTCACTCAGACCATCC	used for sequencing SRZ SRZ1 a-locus (4993 – 4972)
oJS21	TGTCCCATCATCATAGCAGCTC	used for sequencing SRZ SRZ1 a-locus (4278 – 4299)
oJS22	AGAGCTGCTATGATGATGGGAC	used for sequencing SRZ SRZ1 a-locus (4300 – 4279)
oJS23	TGACCACCTCGATGTCTGC	used for sequencing SRZ SRZ1 a-locus (7204 – 7222)

Primer name	Sequence	Remarks
oJS24	TTGACGACTCGGTAGCTCTC	used for sequencing SRZ SRZ1 a-locus (6470 – 6451)
oJS25	AACGAGATCAGACATCCTAGCG	used for sequencing SRZ SRZ1 a-locus (6781 – 6802)
oJS26	ATGACTCTGGCTTCGATGGC	used for sequencing SRZ SRZ1 a-locus (5094 – 5113)
oJS27	TCAGTGACCGGATTTGGTGC	used for sequencing SRZ SRZ2 b-locus (1084 – 1065)
oJS28	AAACCACGACGAAGCACACG	used for sequencing SRZ SRZ2 b-locus (970 – 989)
oJS29	AGAAGGAGCTGCTGTCTGG	used for sequencing SRZ SRZ2 b-locus (1447 – 1465)
oJS30	AGGCAGAGTAAGCAGCAGC	used for sequencing SRZ SRZ2 b-locus (640 – 622)
oJS31	CACATACTTCGCGGAAGCC	used for sequencing SRZ SRZ2 b-locus (514 – 532)
oJS32	AGGAACCAACTCCCAGATGG	used for sequencing SRZ SRZ2 b-locus (1856 -1837)
oJS33	CAAGCGAGGCTTCTTGAGG	used for sequencing SRZ SRZ2 b-locus (2156 – 2174)
oJS34	CTCGTCTGACTACCAGCTG	used for sequencing SRZ SRZ2 b-locus
oJS35	TGACATCCCGACATGTTCTCC	used for sequencing SRZ SRZ2 b-locus (3663 – 3683)
oJS36	GGATGTCAAGAAGCTCTGATCG	used for sequencing SRZ SRZ2 b-locus (3100 – 3079)
oJS37	GAAGAAAGTCTCATCGGGTTGC	used for sequencing SRZ SRZ2 b-locus (2972 – 2993)
oJS38	CCAAACTCTATCCAGACAACGC	used for sequencing SRZ SRZ2 b-locus (8343 – 8364)
oJS39	TGAACGAGGCTGAGAGCG	used for sequencing SRZ SRZ2 b-locus (8434 – 8417)
oJS40	AAGAGTTGCGGCTGAGTCG	used for sequencing SRZ SRZ2 b-locus (4889 – 4871)
oJS41	ACACAGACGAAAGCGCAACC	used for sequencing SRZ SRZ2 b-locus (4778 – 4797)
oJS47	TGTTGTGAGCTTGCCAAAGCAG	used for sequencing SRZ SRZ2 a-locus (537 – 558)
oJS48	ACCCATACATCGACCTGTGC	used for sequencing SRZ SRZ2 a-locus (1439 – 1420)
oJS49	TGTGCTGCTGAGTGAAACAAGC	used for sequencing SRZ SRZ2 a-locus (2036 – 2057)
oJS50	TGAGTACGTGACAACCCATGC	used for sequencing SRZ SRZ2 a-locus (2303 – 2323)
oJS51	TGCTGCTTTGTCGTGTTCGC	used for sequencing SRZ SRZ2 a-locus (2129 – 2110)

Primer name	Sequence	Remarks
oJS52	GCTCAGACAAGCTCACTAACG	used for sequencing SRZ SRZ2 a-locus (4479 – 4459)
oJS53	TAAGGCCTCTCCTCTCTTGG	used for sequencing SRZ SRZ2 a-locus (5006 – 4987)
oJS54	TTCTTGGCTGGAGTTTGGCG	used for sequencing SRZ SRZ2 b-locus (5474 – 5493) used for sequencing SRZ SRZ1 b-locus
oJS55	AGGAACTGTAGAAGTGCTCGTC	used for sequencing SRZ SRZ1 b-locus
oJS56	TTCGACACCTCTACCTCAACG	used for sequencing SRZ SRZ1 b-locus
oJS57	AGTGCTTGCGCATGTGATACG	used for sequencing SRZ SRZ2 b-locus (3901 – 3881) used for sequencing SRZ SRZ1 b-locus
oJS58	ACAGACTTCGGACCTTGTCG	used for sequencing SRZ SRZ1 b-locus
oJS59	TCTGAATGGTGACGGTGATGG	used for sequencing SRZ SRZ2 b-locus (5576 – 5556) used for sequencing SRZ SRZ1 b-locus
oJS60	CAAGAGCTGCTATGATGATGGG	used for sequencing SRZ SRZ2 a-locus (614 – 593)
oJS61	AGGACTGCTCTCTCAATCGG	used for sequencing SRZ SRZ2 a-locus (1040 – 1059)
oJS62	TCGACAGTCTGTCTGCTGAC	used for sequencing SRZ SRZ2 a-locus (1515 – 1534)
oJS63	TCGTGCACTTACCATGTCGG	used for sequencing SRZ SRZ2 a-locus (2634 – 2653)
oJS64	TCGTGAGAAAGTTGTCACAGCC	used for sequencing SRZ SRZ2 a-locus (3789 – 3810)
oJS65	CTTAGATGAGAGAGGGAAGGC	used for sequencing SRZ SRZ2 a-locus (5030 – 5050)
oJS66	TCTGGTCTGTTCTCCTATGGG	used for sequencing SRZ SRZ2 a-locus (3922 – 3902)
oJS67	AGTGCACGACTTCCACCTC	used for sequencing SRZ SRZ2 a-locus (2642 – 2624)
oJS69	TGAGCTGTTCGTAGTTGTGCG	used for sequencing SRZ SRZ2 b-locus (8488 – 8468) used for sequencing SRZ SRZ1 b-locus
oJS70	ACGTAGTTCCCGTAGTTGCG	used for sequencing SRZ SRZ2 b-locus (5407 – 5426) used for sequencing SRZ SRZ1 b-locus

Primer name	Sequence	Remarks
oJS71	ACGTGTTGTGGATGCAGTGG	used for sequencing SRZ SRZ2 b-locus (8034 – 8015) used for sequencing SRZ SRZ1 b-locus
oJS72	TGCGTATCTCACACAGCTGC	used for sequencing SRZ SRZ2 b-locus (7957 – 7976) used for sequencing SRZ SRZ1 b-locus
oJS73	TCAGAAGGCAACAGACGAGG	used for sequencing SRZ SRZ1 b-locus
oJS74	AGATCCTCAGCTTGCTACCG	used for sequencing SRZ SRZ1 b-locus
oJS77	GGTCCCTACAATCAGTGTGG	used for sequencing SRZ SRZ2 a-locus (427 – 408)
oJS78	TGTTCGTCTTCCACCTGTGG	used for sequencing SRZ SRZ2 a-locus (3172 – 3153)
oJS79	CACAGAATGGAAGCCAGGAG	used for sequencing SRZ SRZ2 a-locus (3130 – 3149)
oJS80	ACTTGCGCAAGATGTGAGTCC	used for sequencing SRZ SRZ2 b-locus (4075 – 4055)
oJS81	ACCCTTCGAGTGCTCTTGG	used for sequencing SRZ SRZ2 b-locus (4643 – 4625)
oJS82	TCACAGATCCTCTTGCCACC	used for sequencing SRZ SRZ2 b-locus (5538 – 5557) used for sequencing SRZ SRZ1 b-locus
oJS83	ACTGCTCTCTGCTGCTTGC	used for sequencing SRZ SRZ2 b-locus (7330 – 7312) used for sequencing SRZ SRZ1 b-locus
oJS84	AGCAAGGAAGAAGTCATCGGC	used for sequencing SRZ SRZ2 b-locus (7243 – 7263) used for sequencing SRZ SRZ1 b-locus
oJS85	AGGTCCAAGCCAATCGAAGG	used for sequencing SRZ SRZ2 a-locus (3449 – 3468)
oJS86	TCCAAGCTCCAGCTCAGTG	used for sequencing SRZ SRZ2 b-locus (7429 – 7447) used for sequencing SRZ SRZ1 b-locus
oJS87	TGACTGATGTGCTGCCCTG	used for sequencing SRZ SRZ2 b-locus (7632 – 7650) used for sequencing SRZ SRZ1 b-locus
oJS88	AGTTGGTTGCGGTCACCTG	used for sequencing SRZ SRZ2 b-locus (7143 – 7125)

Primer name	Sequence	Remarks
		used for sequencing SRZ SRZ1 b-locus
oJS89	TCCAGGCAACAGAGAGACC	used for sequencing SRZ SRZ2 b-locus (6229 – 6247) used for sequencing SRZ SRZ1 b-locus
oJS90	ACCTTGCTGATGAGTCTTGCC	used for sequencing SRZ SRZ2 b-locus (6331 – 6311) used for sequencing SRZ SRZ1 b-locus
oJS95	ACCTCCACTATCCTGAGAGAG	used for sequencing SRZ SRZ2 b-locus (7736 – 7716) used for sequencing SRZ SRZ1 b-locus p171
oJS96	AAGACCAACTGCAGGCATCG	used for sequencing SRZ SRZ2 b-locus (6951 – 6970) used for sequencing SRZ SRZ1 b-locus
oJS99	AAGCGTTGACTGGAGTGAGG	used for sequencing variable regions of SRZ b3
oJS100	AGGTATCATGTCAGCGACCG	used for sequencing variable regions of SRZ b3
oJS101	TGCTCCAAGATTCGGATCGC	used for sequencing variable regions of SRZ b3
oJS102	TCCGAATCTTGGAGCAAGCC	used for sequencing variable regions of SRZ b3
oJS103	TCGCATTCCGCGGAGTTTCGGCTGGAAACGG	used to amplify *U. maydis* Cbx^R gene in combination with oJS141
oJS105	TCTTCCCAAACTTCTCCGAGC	used to amplify SRZ a1 in combination with oJS195
oJS114	AGAAGAAGCGCAAATCTTACGG	used for sequencing constant region of SRZ b3
oJS115	ACAACGTTCTCAGCGACAACC	used for sequencing constant region of SRZ b3
oJS124	ACTACCGCTGCCTTCTCCAC	used to amplify *S. reilianum* Cbx^R gene in combination with oJS140
oJS136	TGCCTACAATTTGAAGCCTAGC	used for sequencing SRZ SRZ1a locus (9756 – 9777)
oJS137	CAGCACAGCAACGCATTCAG	used for sequencing SRZ SRZ1a locus (3182 – 3163)
oJS138	TCTGCATCCACGCGATGAGC	used for sequencing SRZ SRZ1a locus (1947 – 1966)
oJS139	GTTGGCGTGAGACAAGAGG	used for sequencing SRZ SRZ1a locus (12161 – 12143)
oJS140	GATCCGAAGTGCGAACATGC	used to amplify *S. reilianum* Cbx^R gene in combination with oJS124

Primer name	Sequence	Remarks
oJS141	AAGCGCACAGGATGCACTCG	used to amplify *U. maydis* Cbx^R gene in combination with oJS103
oJS152	CAGGAAGGACTGCTCCATTCG	used for sequencing SRZ SRZ1a locus (12659 – 12679)
oJS153	TCTCACGCTTCTGCCTTCTTCC	used for sequencing SRZ SRZ1a locus (12289 – 12310)
oJS154	CTGTTCTCACGCCTTTGTCTGG	used for sequencing SRZ SRZ1a locus (11777 – 11798)
oJS155	AGACAAAGGCGTGAGAACAGC	used for sequencing SRZ SRZ1a locus (11796 – 11776)
oJS156	AACGCGCTTGCCGGTATTCC	used for sequencing SRZ SRZ1a locus (2514 – 2495)
oJS157	GTGGATGAAGGACACCTTTACC	used for sequencing SRZ SRZ1a locus (2164 – 2143)
oJS158	TGATCCGTACAGGACGTTTACC	used for sequencing SRZ SRZ1a locus (1519 – 1498)
oJS159	CTAAAGATGGCGTTGAGTCTGG	used for sequencing SRZ SRZ1a locus (1114 – 1093)
oJS160	GTTGCCATTGACGTACAGTTCC	used for sequencing SRZ SRZ1a locus (2593 – 2614)
oJS161	AGCCGTGGTTGAGCAAATCG	used for sequencing SRZ SRZ1a locus (2964 – 2983)
oJS162	CATCAACGCCATTCGCTAATCG	used for sequencing SRZ SRZ1a locus (3570 – 3591)
oJS163	ACCAACGCAGATGAGAAGTTCG	used for sequencing SRZ SRZ1a locus (7973 – 7994)
oJS164	AGGGCCTCTTGCTGATATCTGC	used for sequencing SRZ SRZ1a locus (8538 – 8559)
oJS165	TATTCCCGTCTTGCGAGTCC	used for sequencing SRZ SRZ1a locus (9122 – 9141)
oJS166	CAACGCGCCAATCCAAACTCG	used for sequencing SRZ SRZ1a locus (9624 – 9644)
oJS167	GCCCTTGCTCAAGGTTCTCG	used for sequencing SRZ SRZ1a locus (9893 – 9912)
oJS181	AGCAGGGTTGACAAAGATCG	used for sequencing SRZ SRZ1a locus (10605 – 10624)
oJS182	AGCAGTCGAAACGCCTACC	used for sequencing SRZ SRZ1a locus (10160 – 10142)
oJS183	GAAGACCTCGCTTCGTATCC	used for sequencing SRZ SRZ1a locus (10589 – 10570)
oJS184	CCTCCACCTCTTCACAAACC	used for sequencing SRZ SRZ1a locus (11081 – 11062)
oJS185	TTCATCGGGCCAAGATCTGC	used for sequencing SRZ SRZ1a locus (11184 – 11203)
oJS186	TCCACCGTGAGCTCGTTTCC	used for sequencing SRZ SRZ1a locus (13667 – 13648)

Primer name	Sequence	Remarks
oJS187	CAGCTCGACCAACTCAAATACG	used for sequencing SRZ SRZ1a locus (13221 – 13200)
oJS188	CGAGTCGGATGAGGATTGG	used for sequencing SRZ SRZ1a locus (10803 – 10821)
oJS189	CATCGACAATCTCTTGCTAGG	used for sequencing SRZ SRZ1a locus (9792 – 9772)
oJS190	TGCGGCATTCTTTGTCCC	used for sequencing SRZ SRZ1a locus (5829 – 5846)
oJS191	GCAGGCACTTTGATCAGAGG	used for sequencing SRZ SRZ1a locus (3382 – 3363)
oJS197	TGGCGAGGTTCGAAATCTCAGC	used for sequencing SRZ b4
oJS198	TGCCTTGCTCAGTCTCTACG	used for sequencing SRZ b5
oJS199	AGAAGACGTCCCGTTTGAGG	used for sequencing SRZ SRZ2a locus (6794 – 6775)
oJS200	ACGCAGATTCAATCTCAATCCC	used for sequencing SRZ SRZ2a locus (7268 – 7289)
oJS201	ATCAGTCAGTGGCTCCTACC	used for sequencing SRZ b4/b5
oJS202	ACTGGATGTCGGTCATTTCG	used for sequencing SRZ b4/b5
oJS203	TTCGCCCATGTTGAGCTGGAGG	used for sequencing SRZ b4/b5
oJS204	TGCCTGCAACCAACAAATCC	used for sequencing SRZ b4
oJS205	CTTGACGCCGTACTTGATCC	used for sequencing SRZ b4/b5
oJS207	ACGCAATCCACGCAATCCC	used for sequencing SRZ b4/b5
oJS208	AGTGACCCGCAGAGAAGAGG	used for sequencing SRZ b4/b5
oJS209	GACATCGTGGGCACAAAGC	used for sequencing SRZ b4/b5
oJS210	TGGACATCTTCGCATCAACC	used for sequencing SRZ b5
oJS211	CCTCTGTCCAGGCTGTTTCG	used for sequencing SRZ SRZ2a locus (6912 – 6931)
oJS212	ATAGGCGCATCGCGGATCAAGG	used for sequencing SRZ SRZ2a locus (7173 – 7152)
oJS213	TGCCACTTTCTCGTTCTGAAGG	used for sequencing SRZ SRZ2a locus (8157 – 8178)
oJS214	GGAGGACAGGCTCTATTCG	used for sequencing SRZ SRZ2a locus (8115 – 8097)
oJS215	TGAGGGATGTGAGGGATACG	used for sequencing SRZ b5

Primer name	Sequence	Remarks
oJS216	TCGCGACCTTGGACAATCCC	used for sequencing SRZ b5
oJS217	TAATGTGGGCGCTTGGACCG	used for sequencing SRZ b5
oJS218	ACAACTCTTCCCGCCCTACG	used for sequencing SRZ b4
oJS219	TCCGAGCGCGAAATCGTAGG	used for sequencing SRZ b4
oJS220	TCGGGCTCTGAAGAAGATCG	used for sequencing SRZ b4
oJS221	ATTGGGCGTGAGAAATGAGG	used for sequencing SRZ SRZ2a locus (9873 – 9854)
oJS222	TACGTGAACAGGGCCATAGC	used for sequencing SRZ SRZ2a locus (7425 – 7444)
oJS223	TGCCGACTTGAAGGCTAAGG	used for sequencing SRZ SRZ2a locus (8793 – 8774)
oJS224	TCGATGGCAGCCTCCTTAGC	used for sequencing SRZ SRZ2a locus (8761 – 8780)
oJS225	ACGAATCCCTCTAGCAATCC	used for sequencing SRZ SRZ2 a locus (9203 – 9184)
oJS226	TGTCGGGCTGAATGATGTGG	used for sequencing SRZ SRZ2a locus (11391 – 11410)
oJS228	ACGCGTCATAGTCTATTGGC	used for sequencing SRZ b4
oJS229	TCGGCCTCTGTTCTTAGACC	used for sequencing SRZ SRZ2a locus (9655 – 9636)
oJS235	ACACCAGATGTCACCTCAAACG	used for sequencing SRZ SRZ2 a locus (6762 – 6783)
oJS236	CGTGCCAAGTCGAATGACTG	used for sequencing SRZ a3 locus
oJS237	GGCGAGAACAGGAAAGAGC	used for sequencing SRZ a3 locus
oJS238	TCATTCACGGCCCTTTGTGG	used for sequencing SRZ a3 locus
oJS239	ACGCTTGACCCAGTGTTCG	used for sequencing SRZ a3 locus
oJS240	CGTCAGCCCTAGAGTCATCC	used for sequencing SRZ a3 locus
oJS241	CCCGCAACGATGATGATAGC	used for sequencing SRZ a3 locus
oJS242	CCTCAAGCTGCCATTTCTCC	used for sequencing SRZ a3 locus
oJS243	GTTGCCAACTTGAAGGCTAAGG	used for sequencing SRZ a3 locus
oJS244	ATGCCGCTGTCACATAGTGG	used for sequencing SRZ a3 locus

Primer name	Sequence	Remarks
oJS252	ACTGAGCGCTGCAAAGTTCC	used for sequencing SRZ a3 locus
oJS253	AGACTACGACCCTGCTACG	used for sequencing SRZ a3 locus
RevI	CAGGAAACAGCTATGACCATG	standard primer for sequencing
SR1a	CAGGTTATGTATGGGCCG	Xu *et al.* (2000) used with SR1b to specifically amplify a 960 bp fragment from *S. reilianum*
SR1b	TTGAGCGATGACCATTCC	Xu *et al.* (2000) used with SR1a to specifically amplify a 960 bp fragment from *S. reilianum*
T5h	CACGATTCAGGCATTTCGCAGATGC	G. Reinecke - hybridizes to the *iad2* 5' region
U13koF	GCTGCTCGAACAGTATGGCGAGGAG	G. Reinecke - hybridizes within *iad2* ORF
U13R	GGCTTTGGGGATCCAGAGTGCTTG	G. Reinecke - hybridizes within *iad2* ORF
UM11a	GAACCTTTCTGGCCTCCTTT	Xu *et al*. (2000) used with UM11b to specifically amplify a 900 bp fragment from *U. maydis*
UM11b	CCTTGGTTTCCGTTCCGTAC	Xu *et al*. (2000) used with UM11a to specifically amplify a 900 bp fragment from *U. maydis*
UniI	CGTTGTAAAACGACGGCCAGT	standard primer for sequencing
UsbE4	CGCTCTGGTTCATCAACG	Albert & Schenk (2000) used with UsbE8 to amplify a 450 bp fragment of the *S. scitaminea* bE
UsbE8	TGCTGTCGATGGAAGGTGT	Albert & Schenk (2000) used with UsbE4 to amplify a 450 bp fragment of the *S. scitaminea* bE
W-IntHIII	GAGATCATGCACTCACCCAGATAG	J. Kämper - used to amplify the variable regions of bW and bE, hybridizes within homeodomain of bW (2164 - 2187 of bW2bE2)

6.1.3 Bacterial and Fungal Strains

E. coli Strains

For cloning purposes, the *Escherichia coli* K-12 derivatives DH5α (Bethesda Research Laboratories) and Top10 (Invitrogen) were used.

U. maydis and *S. reilianum* Strains

S. reilianum spores were kindly obtained from L. Claflin (USA), C. Roux and B. Garet (France), N. McLarren (South Africa), M. Piepenbring and T. Lübberstedt (Germany), and X. Xianchun (China).

Table 6. *U. maydis* and *S. reilianum* strains used.

Strain	Organism	Genotype	Resistance*	Reference
FB1	*U. maydis*	*a1 b1*	-	Banuett und Herskowitz, 1989
FB2	*U. maydis*	*a2 b2*	-	Banuett und Herskowitz, 1989
FBD11	*U. maydis*	*a1a2 b1b2*	-	Banuett und Herskowitz, 1989
SG200	*U. maydis*	*a1mfa2 bW2bE1*	P	Bölker *et al*., 1995
AB1	*U. maydis*	*a1 Δb*	P	Brachmann, 2001
AB2	*U. maydis*	*a2 Δb*	P	Brachmann, 2001
SRZ1 (SRZ1)	*S. reilianum*	*a1b1*	-	Schirawski *et al*., 2005
SRZ2 (SRZ2)	*S. reilianum*	*a2b2*	-	Schirawski *et al*., 2005
SRZ3 (4-2)	*S. reilianum*	*a1b2*	-	Schirawski *et al*., 2005
SRZ4 (4-1)	*S. reilianum*	*a2b1*	-	Schirawski *et al*., 2005
SRZCXII2	*S. reilianum*	*a3b1*	-	Schirawski *et al*., 2005
SRZCXI1	*S. reilianum*	*a3b2*	-	Schirawski *et al*., 2005
SRZCXI2	*S. reilianum*	*a3b3*	-	Schirawski *et al*., 2005
SRZSAW11	*S. reilianum*	*a1b4*	-	Schirawski *et al*., 2005
SRZSAW21	*S. reilianum*	*a3b5*	-	Schirawski *et al*., 2005
JS333	*S. reilianum*	*a1 bW2bE1*	C	J. Schirawski (unpublished)
JA1-1	*U. maydis / S. reilianum*	*Hybrid AB1+JS333*	C, P	This study
JA1-2	*U. maydis / S. reilianum*	*Hybrid AB1+JS333*	C, P	This study
JA1-3	*U. maydis / S. reilianum*	*Hybrid AB1+JS333*	C, P	This study
B7	*U. maydis / S. reilianum*	*Progeny JA1-1*	C, P	This study
T2-21	*U. maydis / S. reilianum*	*Progeny JA1-3*	C, P	This study
T2-22	*U. maydis / S. reilianum*	*Progeny JA1-3*	C, P	This study
GRN1	*U. maydis*	*a1b1Δiad*1	N	Reinecke, 2003
GRN3	*U. maydis*	*a2b2Δiad*1	N	Reinecke, 2003
GRN7	*U. maydis*	*a1b1Δiad1Δiad2*	N	Reinecke, 2003
GRN8	*U. maydis*	*a2b2Δiad1Δiad2*	N	Reinecke, 2003
G7#7	*U. maydis*	*a1b1Δiad1Δiad2Δnit*1	N, H	This study
G8#13	*U. maydis*	*a2b2Δiad1Δiad2Δnit*1	N, H	This study
SG200Δ10444#11	*U. maydis*	*a1mfa2 bW2bE1Δnit*1	C, H	This study

*C = carboxin, H = hygromycin, N = nourseothricin, P = phleomycin

6.1.4 Plasmids and Plasmid Constructs

All the plasmids used carried ampicillin resistance for selection in *E. coli*. The plasmids were analysed by restriction analysis to confirm the introduction of heterologous DNA. Constructs were sequenced to confirm that no mistakes were introduced by PCR.

Plasmids used or constructed

Table 7. List of plasmids used or created

Name	Insert	Reference / Plasmid	Antibiotic Resistance*
pCR2.1	Cloning vector	Invitrogen	Amp, Kan
pCR4-Topo	Cloning vector	Invitrogen	Amp, Kan
pCR4-BamHI	Cloning vector	Schirawski *et al*., 2005	Amp, Kan
pBS(+)SKII	Cloning vector	Stratagene	Amp
pBS-Hyg(+)	Cloning vector	Brachmann *et al*. 2004	Hyg
pNEB-Cbx (+)	Cloning vector	Brachmann, 2001	Cbx
pNEBUH	Freely replicating plasmid	Weinzierl, 2001	Hyg
pG10	5.5kb SRZ2 a-locus fragment	pCR4-Topo	Amp
p13A1	4kb SRZ1 a-locus fragment	pCR4-Topo	Amp
p17-1	9kb SRZ1 b-locus	pCR4-BamHI	Amp
p114-1	9kb SRZ2 b-locus	pCR4-BamHI	Amp
p10SRZ2	2kb SRZ1 a-locus PCR fragment	pCR2.1-Topo	Amp
p105-3	2kb SRZ1 a-locus PCR fragment	pCR2.1-Topo	Amp
p105-5	2kb SRZ1 a-locus PCR fragment	pCR2.1-Topo	Amp
pSRZ1#7	8kb SRZ1 a-locus fragment	pCR4-BamHI	Amp
pSRZ1#11	8kb SRZ1 a-locus fragment	pCR4-BamHI	Amp

* Amp = ampicillin; Kan = kanamycin; Hyg = hygromycin; Cbx = carboxin

6.2 General Methods

6.2.1 *Escherichia coli*

Cultivation of *E. coli* strains

E. coli strains were grown on solid medium or as liquid cultures with constant agitation of 200 rpm at 37℃. Liquid cultures were inoculated from cultures grown on solid YT medium supplemented with the specific antibiotic. Glycerol preserved strains stored at -80℃ were resuscitated by first streaking onto solid YT medium containing the appropriate antibiotic.

Determination of *E. coli* Cell Densities

Cell densities of liquid cultures were determined photometrically at 600 nm using a Novospec II Photometer (Pharmacia Biotech). To ensure that a linear correlation was established for the determination of optical density at 600 nm (OD_{600}) the culture were diluted to a measured value of below 0.8 after determining the zero value with the pure medium in which the cells were cultivated. An OD_{600} = 1 is equivalent to 1-5x10^7 *U. maydis* cells / ml or 1-5x10^9 *S. reilianum* cells / ml.

Colony Hybridisation

Thomas Walter and Hans-J Holtlke in Colloquium (Molecular Biologists Newsletter, Boehringer Mannheim)
Bacteria were spread on plates and incubated until colonies were approximately 1-2 mm in diameter. The plates were chilled at 4℃ for 1 h before the nylon membranes were placed over the colonies and adsorbed for 5 min. Membranes were carefully lifted off the plates with colonies adhering to the surface and left to dry on blotting paper with the colonies facing up. Sheets of blotting paper were saturated with solutions of denaturing, neutralising or 2x SSC. Dried colony covered membranes were placed on the sheet saturated with denaturing solution for 15 min. The membranes were then transferred to neutralisation solution for 15 min followed by 2x SSC for 15 min. Membranes were then placed in a container containing a 3x SSC / 0.1% SDS solution and incubated at 68^{o}C for 1 h with constant shaking. Cellular debris was removed from the membrane by gently rubbing the top of the membranes with gloved hands and membranes left to air dry. The DNA was crosslinked to the membrane by UV exposure. The membranes were either used directly for hybridisation or stored for later use.

Denaturing solution
0.5N NaOH
1.5M NaCl

Neutralisation solution
1.5M NaCl
0.5M Tris-HCl (pH 7.4)

20x SSC
3M NaCl
0.3M Na-citrate (pH 7.0)

RbCl Transformation of *E. coli*

This is a modified version of Cohen *et al*. (1972). For the preparation of competent bacterial cells, an overnight culture of DH5α was used to inoculate 100 ml LB medium. The culture was incubated with constant shaking at 37℃ until an $OD_{600} \approx 0.5$ was reached. The culture was centrifuged at 3000rpm for 15 min at 4℃. After discarding the supernatant, the cells were resuspended in 33 ml ice cold RF1 solution and incubated on ice for 30 min. Thereafter the cells were centrifuged again at 3000 rpm for 15 min at 4℃. The cell pellet was resuspended in 5 ml ice-cold RF2 solution and incubated on ice for 15 min and finally aliquoted in 50µl and flash-frozen in liquid nitrogen and stored at -80℃.
For the transformation cells were thawed on ice and then mixed with 3-5 µl of the ligation mixture or 10 ng plasmid DNA and incubated on ice for 15 min. The cells were heat-shocked at 42℃ for 40 sec and then supplemented with 250 µl SOC medium and incubated at 37℃ for 1 h and constant shaking at 500 rpm. The

transformation mixture was then plated onto YT plates containing 100 µg/ml ampicillin and incubated at 37℃ overnigh t.

RF1 solution:

100 mM RbCl
50 mM $MnCl_2$. 4 H_2O
30 mM K-Acetate
10 mM $CaCl_2$ .2 H_2O
15% (v/v) Glycerol
in $H_2O_{bid.}$
Adjusted to pH 5.8 with HCl and filter sterilised

RF2 solution:

10 mM MOPS
10 mM RbCl
75 mM $CaCl_2$. 2 H_2O
15% (v/v) Glycerol
in $H_2O_{bid.}$
Adjusted to pH 5.8 and filter sterilised

Electro Transformation of *E. coli*

For the preparation of electro-competent cells, a single colony of the desired *E. coli* strain was used to inoculate SRZ20 ml of dYT and grown overnight at 37℃ with constant shaking. This culture was used to inoculate 500 ml of dYT to a 1:100 dilution and incubated at 37℃ with constant shaking until an OD_{600} = 0.5-0.8 was reached. The culture was then cooled on ice for 15-30 min and the cells subsequently pelleted at 3000rpm for 15 min at 4℃. Pelleted cells were washed in 500 ml sterile cold water and centrifuged at 3000 rpm for 15 min at 4℃. A second wash was with 250 ml sterile cold water followed by centrifugation as before. Cells were then re-suspended in 10 ml ice-cold 10% glycerol, centrifuged as before and then finally resuspended in 500 µl ice-cold 10% glycerol. The cells were dispensed in 40 µl aliquots, flash-frozen in liquid nitrogen and stored at -80℃.
Electro-competent cells were thawed on ice, carefully mixed with 5 µl ligation reaction (previously dialysed) and incubated for 5 min on ice. The transformation mixture was transferred to a pre-chilled electroporation cuvette and then placed in the electroporation machine (Gene Pulser, Bio-Rad). Thereafter the cells were subjected to an electric pulse (25 mF, 2.5 kV, 200 W) for 4-5 msec. After removing the cuvette, 500 µl SOC medium was added to the cells and then transferred to a 1.5 ml microcentrifuge tube. Cells were incubated at 37℃ for 1h with constant shaking, spread onto YT-Amp plates containing 1% X-gal and incubated at 37℃ overnight.

6.2.2 Fungal Strains

Cultivation of Fungal Strains

Fungal strains were grown on solid medium or as liquid cultures with constant agitation of 200 rpm at 28℃. Liquid cultures were inoculated from cultures grown on solid medium supplemented with the specific antibiotic. Glycerol preserved strains stored at -80℃ were resuscitated by first streaking onto solid medium containing the appropriate antibiotic. *S. reilianum* strains cultivated in PD broth were incubated at 22℃ with constant agitation of 200 rpm.

Determination of Cell Densities

Cell densities of liquid cultures were determined photometrically at 600 nm using a Novospec II Spectrophotometer (Pharmacia Biotech). To ensure that a linear correlation was established for the determination of optical density at 600 nm (OD_{600}) the cultures were diluted to a measured value of below 0.8 after determining the zero value with the pure medium in which the cells were cultivated. An OD_{600} = 1 is equivalent to 1-5x10^7 *U. maydis* cells / ml or 1-5x10^9 *S. reilianum* cells / ml.

Fungal Transformation

This method is a modification of Schulz *et al*. (1990) and Gillissen *et al*. (1992). A single fungal colony was used to inoculate 2 ml $YEPS_L$ liquid medium and incubated at 28°C for 8 – 12 h. This culture was used at a dilution of 1:300 or 1:500 to inoculate 50 ml of $YEPS_L$, which was incubated at 28°C with constant shaking at 200 rpm. Cells were grown to a measured OD_{600} = 0.6 – 0.8 and subsequently pelleted at 3500 rpm (Beckmann Biofuge) for 5 min. The supernatant was discarded and the pellet resuspended in 25 ml SCS buffer. Cells were pelleted again at 3500 rpm for 5 min and the supernatant discarded. Protoplasts were induced by resuspending the cells in 2 ml of Novozyme solution and incubating at room temperature for 5 – 10 min. The formation of protoplasts was confirmed by microscopic analysis. Protoplasting was stopped by the addition 20 ml SCS buffer and centrifuging the solution at 2300 rpm for 10 min. The supernatant was discarded and the pellet washed twice by carefully resuspending the pellet in 20 ml SCS buffer and centrifuging at 2300 rpm for 10 min. The pellet was carefully resuspended in 20 ml STC buffer and centrifuged at 2400 rpm for 10 min and the supernatant discarded. Protoplasts were re-suspended in 500 µl ice-cold STC buffer and separated into 70 µl aliquots and either used directly for transformation or stored at -80°C.

A 70 µl aliquot of protoplasts was mixed with 5 µg DNA and 1 µl heparin and kept on ice for 10 min. Protoplasts were then carefully mixed with 500 µl of cold STC/PEG solution and incubated for a further 15 min on ice. The entire mixture was plated onto regeneration medium containing the selective antibiotic. Plates were incubated at 28℃ for at least four days or until distinct colonies appeared. Single colonies were picked using sterile toothpicks and streaked onto PD-plates containing the selective medium to obtain single colonies. Putative transformants were picked as single colonies and verified by Southern analysis.

SCS:

20 mM Na-Citrate (pH 5.8)
1 M Sorbitol
in $H_2O_{bid.}$ filter sterilised

STC:

10 mM Tris-Cl (pH 7.5)
100 mM $CaCl_2$
1 M Sorbitol
in $H_2O_{bid.}$ filter sterilised

STC/PEG:

15 ml STC
10 g PEG4000

Protoplast fusion

Protoplast fusion was as described by Gu & Ko, 2000.
Equal numbers of protoplasts of each culture (*U. maydis* and *S. reilianum*) were mixed and pelleted at 1000 rpm for 10 min. The supernatant was aspirated and the pellet resuspended in 400 µl fusion solution containing 40% PEG 6000, 50 mM CaCl and 20 mM Tris and incubated at room temperature for 30 min. The protoplast mixture was then plated onto regeneration medium and incubated at 28℃ for one week. Single colonies were streaked onto fresh PD plates containing both selective antibiotics and incubated at 28℃ for four days. Single colonies were selected to inoculate 2 ml $YEPS_L$ medium and grown overnight at 28℃ with constant agitation. DNA was extracted and used in PCR to check for the presence of various markers.

Isolation Fungal Genomic DNA

A single colony of the desired strain was used to inoculate 2 ml $YEPS_L$ and incubated overnight at 28℃ with constant shaking or until confluent. The culture was transferred to a 2 ml micro-centrifuge tube containing 200 µl glass beads. Cells were pelleted at 13000 rpm for 2 min and the supernatant discarded. Five hundred microliters of lysis buffer and an equal amount of phenol/chloroform was added to cells and then vortexed for 10min. Thereafter the phases were separated by centrifuging at 13000 rpm for 15-30 min. The upper phase was transferred to a new tube containing 1ml of absolute ethanol and mixed well. The DNA

was pelleted at 13000 rpm for 10 min and washed with an equal amount of 70% ethanol and centrifuged at 13000rpm for 5min. After discarding the ethanol, the pellet was air dried and resuspended in 50µl TE + RNase and incubated at 55℃ for 10min. DNA is quantified and stored at 4℃.

Lysis Buffer:

100 mM NaCl

10 mM Tris-HCl (pH 8.0)

1 mM EDTA

2% Triton X-100

1% SDS

Mating Test for Dikaryotic Filaments

U. maydis strains were inoculated in $YEPS_L$ medium and incubated at 28℃ with constant shaking until an OD_{600} of 0.5 - 1.0 was reached. The culture was centrifuged at 3500rpm for 5min at RT. Pelleted cells were resuspended in H_2O to a calculated OD_{600} = 3.0. An equal volume (10 µl) of each strain was mixed in a microtiter plate and 5 µl of this mixture and 5 µl of the individual strains were dropped onto a PD-CC plate. The plate was sealed with Parafilm and incubated at RT overnight. Plates were analysed by eye or binocular microscope for the presence of white fuzzy colonies indicative of dikaryotic filaments.

S. reilianum strains were inoculated in PD broth and incubated at 22℃ with constant shaking until an OD_{600} of 0.5 - 1.0 was reached. The culture was centrifuged at 3500 rpm for 5 min at RT. Pelleted cells were resuspended in H_2O to a calculated OD_{600} = 3.0. An equal volume (10 µl) of each strain was mixed in a microtiter plate and 5 µl of this mixture and 5 µl of the individual strains were dropped onto a water agar plate. The plate was sealed with Parafilm and incubated at RT overnight. Plates were analysed by eye or binocular microscope for the presence of white fuzzy colonies indicative of dikaryotic filaments.

Plant Pathogenicity Test

To test the *on planta* growth and appressorium formation and penetration ability of modified strains, maize plant pathogenicity test were used. Maize kernels of Early Golden Bantam variety and Gaspe Flint variety were placed in potting soil Type T and Type P (supplier) and grown under standard glasshouse conditions to a three leafed-seedling stage. Mixtures of the strains were inoculated into the leaf whorl using a syringe and 10 gauge needle. The plants were not watered for a 24 h period. Leaf samples were taken at 24 h and 48 h to observe filamentous growth on planta, appressorium formation and plant penetration by microscopy using calcofluor and chlorazole black E staining.

Spore formation and Segregation analysis

Infected plants are assayed for their symptoms using a standardised disease index. Tumours are excised from the plants and are macerated and dried at 37℃ for two weeks. The spores are mixed in water and centrifuged at 13000 rpm for 5 min at RT and the supernatant and plant debris is removed. The washing in water is repeated if necessary. Spore suspensions were serially diluted and plated out on PD plates containing ampicillin, chloramphenicol, kanamycin and tetracyclin to reduce bacterial contamination. Plates are incubated at 28℃ for two days to one week to allow the spores to germinate. Colonies were streaked out twice on PD plates to single colonies. Colonies were then grown to test in mating tests.

Light Microscopy and Image Processing

For *in vivo* observations, cells from logarithmically growing cultures were placed on a thin 1% agarose-layer and immediately observed using a Zeiss Axioplan II microscope (Zeiss, Jena, Germany). Epifluorescence was observed using filter sets for FITC (BP500/20, FT515, BP535/30) and DsRed (HQ565/30). All microscopic observations were done using a CoolSNAP-HQ CCD camera (Photometrics,

Tucson, AZ, USA) controlled by the imaging software MetaMorph (Universal Imaging, Downing Town, PA, USA). All measurements and image processing, including adjustment of brightness, contrast and gamma-values and 2D-deconvolution were performed with MetaMorph and Photoshop (Adobe Systems GmbH, München, Germany).

Calcofluor Staining

The third leaf of infected maize plants was removed at the leaf stalk and rinsed briefly in sterile water, thereafter immersed in calcofluor solution (100 µg/ml) for approximately 1 min. After rinsing briefly in sterile water, the leaf was cut to size and placed on a glass slide and covered with a cover slip. The presence of fungal material was observed by microscope using the DAPI filter.

Calcofluor stock solution:
10 mg / ml Fluorescent Brightener 28 (Sigma F-3543) in DMSO
Keep in the dark and store at –20°C

Working solution:
Dilute stock 1:100 in 0.2 M Tris pH 8
Keep in the dark and store at –20°C

Chlorazole Black E staining

For the visualisation of fungal hyphal infection structures in leaves of *Zea mays.* An appropriate fraction of the leaf (usually the third leaf, up to 3 cm below the injection hole) was soaked in ethanol overnight. The ethanol was discarded and the leaf segments rinsed once with water. A 10% KOH solution was added and incubated at 90°C for about 3 - 4 hours. After carefully discarding the KOH solution and rinsing with water, the leaves were incubated in chlorazole black E (CBE) staining solution at 60°C overnight. The CBE solution was appropriately discarded and the samples were allowed to destain in 50% glycerol overnight or several days (longer destaining leads to better contrast). Samples were carefully placed on glass slides and visualised by microscope using the DIC filter.

CBE staining solution:
0.03 % Chlorazole Black E (Sigma) in a 1:1:1 solution of water, lactic acid and glycerol. Dissolve the dye in water first, and then add lactic acid and glycerol.

NB. CBE is toxic and needs to be handled and discarded appropriately
.

6.3 Standard Molecular Biology Methods

Standard techniques such as purification, precipitation, restriction and electrophoretic separation of DNA, or cloning techniques were as described by Ausubel *et al*., 1987; Sambrook *et al*., 1989 . The concentrations of nucleic acids were determined by a photometer either the Lambda Bio UV-Spectrophotometer (Perkin Elmer) or NanoDrop (man).

6.3.1 Isolation of Nucleic Acids

Isolation of plasmid DNA from *E. coli*

Plasmid isolation is achieved by the boiling lysis method of Sambrook *et al*. (1989). A 2 ml overnight culture of the relevant *E. coli* strain was pelleted at RT for 1 min at 13000 rpm in a benchtop centrifuge (Heraeus Biofuge 15). After decanting the supernatant the cell pellet was thoroughly resuspended in 300 µl STET buffer. Cells were lysed with 20 µl lysozyme (10 µg/µl) and heating at 99°C for 30sec. The lysis mixture was then centrifuged at RT for 15 min at 13000 rpm and the clear supernatant transferred to a clean 1.5 ml microcentrifuge tube. Plasmid DNA was precipitated by the addition of 40 µl 3 M Na-acetate (pH5.3) and 400 µl isopropanol. After mixing by inverting the tube several times, the plasmid DNA was pelleted at RT for 10 min at 13000 rpm. The supernatant was aspirated and the pellet washed with 500 µl 70% ethanol and

recentrifuged for another 5 min. After removing the ethanol the pellet was air dried for 5 min followed by resuspension in 50 µl TE buffer containing RNase A (20 µg/ml). The DNA was either used directly or stored at 4℃

STET buffer:

50 mM Tris-Cl (pH 8.0)
50 mM Na_2-EDTA
8% (w/v) Saccharose
5% (v/v) Triton X-100
in $H_2O_{bid.}$

Lysozyme solution:

10 mg/ml Lysozyme
10 mM Tris-Cl, pH 8,0
in $H_2O_{bid.}$

Genomic DNA isolation of *U. maydis* and *S. reilianum*

This is a modified version of the method by Hoffman and Winston (1987).
A 2 ml overnight culture grown in YEPSL was pelleted together with 0.3 g of glass pearls at 13000 rpm for 1 min at RT. Supernantant was aspirated and the cells resuspended in 400 µl *Ustilago* lysis buffer and 400 µl TE-Phenol/Chloroform. Mixtures were vortexed for 10 min in a vibrax shaker. After centrifuging at 13000 rpm, 10 min, RT, the upper phase was transferred to a fresh tube. The DNA was precipitated with the addition of 1 ml of ethanol and pelleted at 13000 rpm, 10 min, RT. The DNA pellet was resuspended in 50 µl TE plus 20 µg/ml RNAse A at 50℃ for 5 min and stored at -20℃.

Ustilago lysis buffer:

50 mM Tris-Cl (pH 7.5)
50 mM Na_2-EDTA
1% (w/v) SDS
in $H_2O_{bid.}$

TE-Phenol/Chloroform:

A 1:1 mixture of phenol (equilibrated withTE buffer) and chloroform

Highly Pure Genomic DNA Extraction

One hundred milliliters of $YEPS_L$ medium was inoculated with 2 ml overnight culture of the desired strain and grown at 28℃ with constant shaking until an optical density of OD600 = 0.8 was achieved. The cells were pelleted by centrifuging for 7 min at 3500 rpm, washed with 60 ml SCS buffer and re-centrifuged. Protoplast formation was induced by re-suspending the cells in 5 ml SCS buffer containing Novozyme (12 mg/ml) and incubated at room temperature for 5 -10 min. Cells were checked by microscopy to confirm the formation of protoplasts. The protoplasts were then carefully mixed with 20 ml SCS buffer and centrifuged at 2800 rpm for 10 min. This washing step was repeated twice to remove all traces of the enzyme. Protoplasts were completely re-suspended in 15 ml G2 buffer containing 300 µl RNAse A (10mg/ml) thereafter 400 µl Proteinase K (20 mg/ml) was added and the cell suspension mixed well and incubated at 50℃ for 2 h to lyse the cells. Centrifugation at 5000*g* for 10 min pelleted cellular debris. The supernatant was loaded onto a G-100 column and separated by gravity flow. Purification of the DNA was as described by the manufacturer (Qiagen). DNA was finally resuspened in 200 - 300 µl TE buffer and incubated at 37℃ overnight.

RNA Isolation from Liquid Fungal Cultures

This is a modified version of Schmitt *et al*. (1990). Twenty milliliters of $YEPS_L$ medium was inoculated with 2 ml overnight culture of the desired strain and grown at 28℃ with constant shaking until an optical density of OD600 = 0.5 was achieved. The cells were pelleted by centrifuging for 10 min at 3000 rpm and resuspended in 600 µl AE buffer and 1% (w/v) SDS. Six hundred microliters of AE-phenol was added and the mixture vortexed briefly to mix thoroughly. Cell lysis was achieved by incubating the mixture at 65℃ for 4 min with slight agitation. The mixture was placed at -80℃ for 10 min and then left at RT for 3 min followed by centrifugation at 4℃ for 20 min at 22000 rpm (Beckmann Avanti30 centrifuge). The top phase was then

transferred to a fresh tube and mixed with an equal volume of AE-phenol / chloroform and centrifuged again at 4℃ for 20 min at 22000 rpm. After transferring the top phase to a fresh tube the RNA was precipitated by the addition of 40 µl Na-acetate (pH5.3) and 1 ml ethanol and centrifugation at 4℃ for 20 min at 22000 rpm. The pellet was washed with 80% ethanol centrifuged again and air dried briefly. The RNA was resuspended in 20 µl RNase-free water and stored at -80℃. The integrity of the RNA was confirmed by separating 1 µl of RNA on a 1% TBE agarose gel and quantified by spectrometry.

AE buffer:
50 mM Na-Acetate, pH 5.3
10 mM Na_2-EDTA
in $H_2O_{bid.}$

AE-Phenol/Chloroform:
A 1:1 mixture of phenol (equilibrated with AE buffer) and chloroform

6.3.2 Manipulation of Nucleic Acids

Denaturing Gel Electrophoresis of RNA

Denaturation of RNA was by the addition of glyoxal and DMSO. Total RNA (10 – 30 µg) was mixed with 1 M Glyoxal, 50% DMSO and MOPS buffer in a final volume of 24 µl. The mixture was incubated at 50℃ for 1 h and ice-quenched. Six microliters of RNA loading dye was added and the RNA separated on a 1% MOPS agarose gel at 8 V/cm for 2 h. The gel was turned upside down and polarity switched every 30 min to keep the pH of the buffer constant

MOPS buffer:
200 mM MOPS (pH 7.0)
80 mM Na-Acetate
10 mM Na_2-EDTA
in $H_2O_{bid.}$

RNA loading dye:
50% (w/v) Saccharose
0,25% (w/v) Bromphenol Blue
0,25% (w/v) Xylene Cyanol FF
in MOPS buffer

Transfer of DNA (Southern-Blot)

The transfer of DNA separated by gel electrophoresis onto nylon membrane by capillary action was performed according a modified version of Southern (1975). After gel electrophoretic separation of the DNA, the gel was incubated in 0.25 M HCl for 15 min with gentle agitation. After rinsing briefly with distilled water, the gel was incubated in 0.4 M NaOH for 15 min with gentle agitation to denature the DNA. Transfer of the DNA onto a positively charged nylon membrane (Hybond-N^+ or -XL, Amersham/GE Healthcare) was by capillary transfer overnight using 0.4 M NaOH as transfer buffer. Positions of the lanes were marked on the membrane with a pencil. Membranes were air-dried and crosslinked by UV exposure (302 nm, 120 mJ/cm^2).

Radioactive Hybridisation - DNA

The Hybond-N^+ or -XL membranes (Amersham Pharmacia Biotech) were pre-hybridised for 30 min in 10 ml hybridisation buffer at 65℃ for specific probes or 55℃ for heterologous probes. Labeled probes were added to fresh hybridization buffer and denatured at 95℃ for 10 min before being added to the pre-hybridised membranes and incubated overnight. The membranes were then washed twice with wash buffer for 15 min at either 55℃ or 65℃. Membranes were then sealed in plastic bags and exposed to a phosphor screen (Molecular Dynamics) for several hours to one week. Screens were scanned using a STORM840 Phosphorimager (Molecular Dynamics) and signals quantified using the IMAGEQUANT programme (Molecular Dynamics).

Hybridisation buffer:
50 mM Na-Phosphate buffer, pH 7.0
50 mM PIPES
100 mM NaCl
1 mM Na_2-EDTA

5% (w/v) SDS
in $H_2O_{bid.}$

Wash buffer:
1x SSC
0.1% (w/v) SDS
in $H_2O_{bid.}$

Transfer of RNA (Northern-Blot)

The transfer of RNA separated by gel electrophoresis onto was onto nylon membrane (Hybond-NX, Amersham/GE Healthcare) by capillary action using 20x SSC as transfer buffer. MOPS-RNA gels were incubated in 20x SSC for 15 min with gentle agitation. Capillary transfer of the RNA onto the membrane was allowed to proceed overnight. Positions of the lanes were marked on the membrane with a pencil. Membranes were air-dried and crosslinked by UV exposure (302 nm, 120 mJ/cm^2).
To confirm the successful transfer of RNA, membranes were stained in methylene blue solution (200 mg/l in 300 mM Na-acetate) for 5 min, followed by several washes with distilled water and air-dried.

20x SSC:
300 mM Na-citrate (pH 7.0)
3 M NaCl
in $H_2O_{bid.}$

Radioactive Hybridisation - RNA

The Hybond-N$^+$ membranes (Amersham Pharmacia Biotech) were pre-hybridised for 30 min in 10 ml hybridisation buffer at 60℃. Labeled probes were added to fresh hybridization buffer and denatured at 95℃ for 10 min before being added to the pre-hybridised membranes and incubated overnight. The membranes were then washed twice with wash buffer for 20 min at 60℃. Membranes were then sealed in plastic bags and exposed to a phosphor screen (Molecular Dynamics) for several hours to one week. Screens were scanned using a STORM840 Phosphorimager (Molecular Dynamics) and signals quantified using the IMAGEQUANT programme (Molecular Dynamics).

Hybridisation buffer:
50 mM Na-Phosphate buffer, pH 7.0
50 mM PIPES
100 mM NaCl
1 mM Na_2-EDTA
5% (w/v) SDS
in $H_2O_{bid.}$

Wash buffer:
1x SSC
0.1% (w/v) SDS
in $H_2O_{bid.}$

Probe labeling

A maximum concentration of 1 µg of DNA was denatured at 95℃ for 5 min and ice-quenched. Labeling buffer (NEB) containing random primers and a dNTP mix without dCTP was added. Klenow enzyme (Roche) was used for the radioactive labeling of the DNA with dCTP[^{32}P] at 37℃ for 1 h. Unincorporated nucleotides were removed from the reaction with S300 Microspin columns (Pharmacia). The reaction was purified by loading onto a spin column and centrifuging at 2800 rpm for 2 min. Labeled DNA was added to 10ml hybridization buffer, denatured in a boiling waterbath for 10min and thereafter added to a membrane pre-wet with hybridization buffer.

Dot-Blot Hybridisation Analysis

This method was used to screen the differentially expressed fragments identified by cDNA-AFLP. Two hundred and fifty nanograms of plasmid DNA containing the cloned fragment was spotted in 1µl aliquots on a nylon membrane (Hybond-XL) and left to air-dry. The membranes were placed on Whatmann paper soaked in denaturation solution for 15 min, then neatralising solution for 5 min and finally on renaturation solution for 15 min. Membranes were air-dried and crosslinked by UV exposure (302 nm, 120 mJ/cm^2). Membranes we probed with radioactively-labeled DNA as described above.

Denaturation solution
0.5N NaOH
1.5 M NaCl

Neutralisation Solution

1.5 M NaCl

0.5 M Tris-HCl (pH 7.4)

Renaturation Solution

3 M NaCl

0.3M Na-citrate (pH 7.0)

PFGE of intact cells

Plugs of intact cells for PFGE were prepared according to McCluskey *et al*. (1990).
U. maydis and *S. reilianum* cultures were grown in 10 ml of PD broth until an OD600 = 0.6 – 0.8. Cells were pelleted by centrifuging for 5min at 3000rpm and resuspended in 1 ml sterile distilled water. The cell suspension was mixed with an equal volume of 2% agarose and cast into plugs. The plugs were transferred to 10 ml of solution containing 1 mg/ml proteinase K in 0.45 M EDTA pH8.0 and 1% SDS and incubated at 50℃ for 24 h. The plugs were rinsed three times with 0.5 M EDTA and incubating at 50℃ for two hours. Plugs were stored in 0.5 M EDTA at 4℃.

For PFGE the plugs were rinsed in 0.01 M EDTA at room temperature for 1 hr with constant agitation. The running buffer was used to equilibrate the plugs by shaking at room temperature for 1 hr. Electrophoresis was performed using a Bio-Rad CHEF-DR III system using 0.5x TBE cooled to 14℃. The run parameters were using 120℃ angle, 6 V and 17 hr with a switch time of 60 sec followed by 9 hr with a switch time of 120 sec. Gels were stained with 10 mg/ml ethidium bromide in 0.5X TBE and de-stained in 0.5X TBE. For southern analysis, gels were soaked in distilled water for 30min. Depurination of the DNA was achieved by gently shaking the gel in 0.25 M HCl for 30 min. The gel was rinsed with distilled water and denatured in 0.4 M NaOH for 30 min with gentle shaking. Alkaline transfer was in 0.4 M NaOH onto positively-charged nylon membrane for 24 h. Membranes were air-dried and crosslinked by UV exposure (302 nm, 120 mJ/cm^2).

Genomic DNA hybridization on high-density oligonucleotide arrays

Hybridisation of DNA on oligonucleotide arrays was as described by Winzeler *et al*. 1998.
Ten micrograms of highly pure fungal genomic DNA was digested with 0.15 U deoxyribonulease I (DNAse I) in 1X One-Phor-All buffer for 5 min at 37℃ and heat inactivated at 70℃ for 20min. The DNA fragments were then end-labeled in the same buffer with the addition of 25 U terminal transferase and 1 nmol biotin N-11-dideoxyadenosine triphosphate (N-11-ddATP) and incubated at 37℃ for 1 h. The entire sample was hybridized to the array in a 200 µl volume as described by the manufacturers (Affymetrix). Washing scanning and analysis of the array were according to the standard procedure described by the manufacturer (Affymetrix).

6.3.3 Sequence and Bioinformatic Analysis

DNA Sequencing

All sequencing reactions were done through the services of ADIS at the MPI for Plant Breeding in Cologne. Purified PCR products or plasmid DNA and the respective primers were sent in the required concentrations. The electropherograms obtained were manually edited using the freeware program CHROMAS (supplier??)

Sequence Analysis

The following programs were used:

BLAST2 (Altschul *et al*., 1990; 1997; Gish and States, 1993; Madden *et al*., 1996) for the identification of homologous DNA or protein sequences in public databases.

Chromas for manually editing sequence electropherograms for sequencing errors.

Clone Manager 7 (Scientific and Educational Software) for the construction and

manipulation of plasmid and genomic sequences; used in the determination of cloning strategies.

Bioinformatic analysis

Nucleotide and protein sequences were downloaded from public databases (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). For Blast search of homologous nucleotide and / or protein sequences, sequences were blasted against the public *Ustilago* database http://mips.gsf.de/genre/ proj/ustilago/. For phylogenetic analyses they were aligned using Clone Manager multiway analysis and the PAM250 residue weight table with default parameters.

Accession numbers

Nucleotide sequence data of *S. reilianum* can be found in GenBank data libraries under the accession numbers: *a1* (AJ884588), *a2* (AJ884589), *a3* (AJ884590), *b1* (AJ884583), *b2* (AJ884584), *b3* (AJ884585), *b4* (AJ884586) and *b5* (AJ884587)

6.3.4 PCR Techniques

Standard PCR Reactions

For all standard reactions a modified method of Innis *et al*. (1990) was used. A typical PCR reaction volume was 25 µl containing 1x PCR buffer (containing Mg^{++}), 2 µM final concentration of each primer, 1 U of Taq polymerase (in-house purified), 5% DMSO and 1 µl of 100-fold diluted gDNA as template. For the amplification of fragments of up to 1 kb the following thermal profile was used in a PTC-200 (Bio-Rad) thermocycler:

Initial denaturation at 94℃ for 2 min
30 cycles of
denaturation at 94℃ for 1 min
annealing at 55 - 65℃ for 1 min
extension at 72℃ for 1 min.
Final extension at 72℃ for 5 min.

For the exact annealing temperature, a standard of T_m – 5 was used to calculate the annealing temperature of the primer-pair used (where T_m is the melting temperature of the primer).

PCR Buffer (*Taq* DNA-Polymerase):
10 mM Tris-Cl, pH 8.3
50 mM KCl
1.5 mM $MgCl_2$
in $H_2O_{bid.}$

For the amplification of DNA fragments larger than 4 kb or for fragments used in the creation of deletion constructs, Phusion polymerase (Finnzymes) was used as prescribed by the manufacturer.

Quantitative Real Time PCR

Reverse Transcription Reaction

The following methodology was taken from Rajeevan *et al*., 2001.
The same source of total RNA used in primary gene
expression profiling should be used in validation experiments. The SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to synthesize cDNA in a 20 µl reaction containing 1 mg of DNase I-treated total RNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl2, 10 mM dithiothreitol (DTT), 0.5 µg oligo (dT) 12–18, 0.5 mM each of dATP, dGTP, dCTP, and dTTP, and 200 U SuperScript II Reverse Transcriptase. In terms of the order of adding reaction components, RNA and oligo (dT) were mixed first, heated to 70℃ for 10 min, and placed on ice until addition of the remaining reaction components. The reaction was incubated at 42℃ for 1 h, and terminated by heat inactivation at 70℃ for 15 min. The cDNA product was treated with 2 U of RNase H (Invitrogen) for 20 min at 37℃, followed by

heat inactivation of the enzyme at 70℃ for 15 min. An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA (no-RT control). The cDNA was stored at -20℃ until ready to use. Purification of cDNA prior to real-time PCR was not necessary.

Basic Strategy

To achieve the flexibility required to validate the large numbers of genes we used the Light Cycler-based real-time PCR with product detection using generic SYBR Green I dye. In addition, a relative standard curve was constructed to permit comparison between samples. The relative standard curve is simply constructed using serial dilutions of cDNA prepared from one sample identified in the gene profiling technique as having a high expression level of the gene to be validated. While still requiring the design and synthesis of gene-specific primers, the expense of the special chemistry required for synthesis of gene-specific fluorogenic internal probes is eliminated. In addition, the specificity of the reaction is monitored by determination of the product melting temperature (T_m: defined as the temperature at which half of DNA helical structure is lost). Reaction specificity is improved by "hot-start" PCR and by acquiring signal at a temperature 1–2℃ below the T_m of specific product, avoiding the nonspecific signal from primer–dimers that usually melt at lower temperatures.

Quantification using the $2^{-\Delta\Delta C}{}_T$ method

Calculation of change in expression level was based on the method described by Livak and Schmittgen (2001).
Real Time-PCR was conducted on cDNA samples in triplicate and the C_T values generated were displayed in a spreadsheet (Microsoft Excel). To calculate the change in expression of the target gene normalised to the actin or calibrator gene, the data were analysed using the following formula

$$\Delta\Delta C_T = (C_{T,Target} - C_{T,Actin})_{Time\ x} - (C_{T,Target} - C_{T,Actin})_{Time\ 0}.$$

where Time *x* is any time point and Time 0 represents the 1X expression of the target gene normalized to the actin or calibrator gene. The mean, SD and CV are determined from the triplicate samples at each time point. Using this analysis the mean fold change at time zero should be very close to one (i.e. 2^0 = 1). A value that is very different from one suggests a calculation error in the spreadsheet or a very high degree of experimental variation.

6.3.5 Enzymological methods

Feeding Experiments

Pre-cultures of the various *U. maydis* strains were grown in CM liquid medium containing 1% arabinose or glucose at 28℃ overnight. The OD was first adjusted to OD600 = 1 and then 100 µl used to inoculate 3 ml of fresh CM liquid medium containing 1 % Arabinose and 20 mM IAAld and grown for 14 hr at 28℃. One milliliter of the culture was transferred to a 2 ml centrifuge tube and pelleted at 3000 rpm for 5 min. The supernatant (500µl) was transferred to a fresh tube and the pH adjusted to pH3.5 with HCl. A volume of 750µl ethyl acetate (HPLC grade) was added to the supernatant and the mixture homogenized on a vibrax for 2 min. The upper organic phase was transferred to a fresh tube and evaporated in a SpeedVac. Samples were then analysed by HPLC or stored at -20℃.

IAA Detection using Salkowski Reagent

The method used was as described by Gordon and Weber (1951)
Approximately 50µl of the ethyl extracted supernatant (see above) is mixed with 2X the volume of Salkowski reagent in a 96 well plate and left at RT for 20 min. For colorimetric quantification, the samples are read in a

photometer at 530nm to determine the intensity of coloration. As a blank control, distilled water is mixed with the Salkowski reagent as above. A standard curve is calculated using serial dilutions of IAA.

Salkowski Reagent

$H_2O_{bid.}$, 70 % $HClO_4$, 0,5 M $FeCl_3$

in a ratio of 25:25:1, (freshly prepared)

6.4 cDNA-AFLP Fingerprinting

This method was first described by Bachem *et al*. (1996) who modified the original method described by Vos *et al*., (1995) which is based on a genomic DNA template. In this method total RNA is first reverse transcribed and a second strand of cDNA generated to produce the double-stranded template used for restriction digests. An outline of the method is given in Fig. 21.

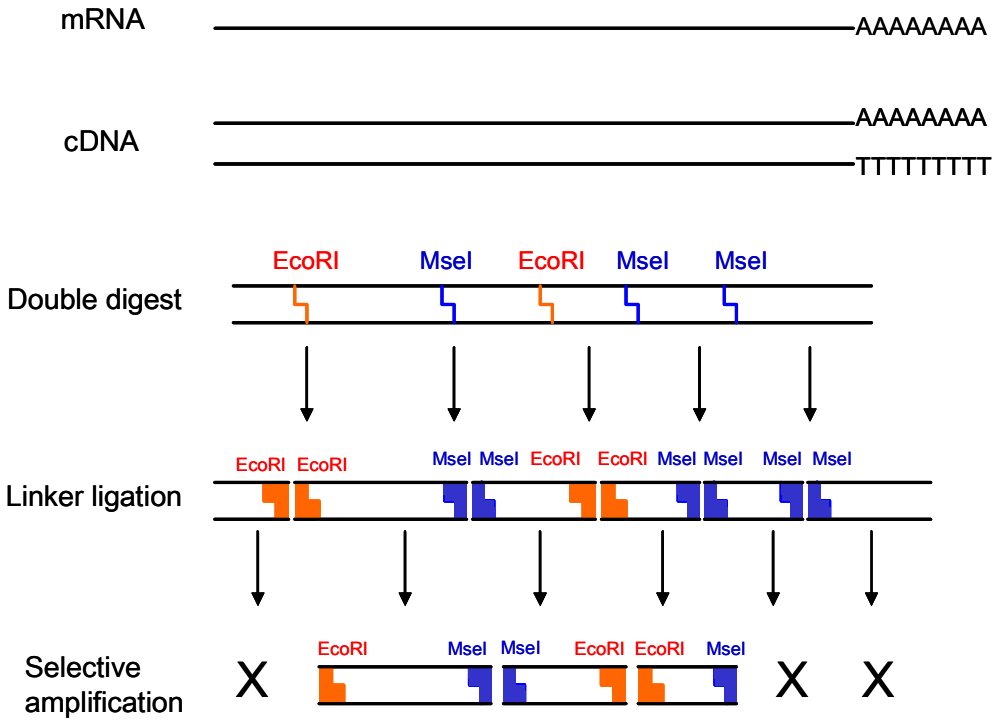


Figure 21. Outline of the cDNA-AFLP technique.

Total RNA or mRNA is reverse transcribed into double-stranded cDNA and subsequently digested with two restriction endonucleases. Linkers containing the respective recognition site overhangs are ligated to the restricted DNA. The sequence of the linkers and the adjacent restriction site serve as primer binding sites for subsequent PCR amplification. Selective nucleotides extending into the restriction fragments are added to the 3' end of the PCR primers so that only a subset of the restriction fragments is recognised. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified.

6.4.1 Plant inoculation and post-harvest treatment

Maize seeds of Early Golden Bantam variety (Old Seeds) were germinated and grown in soil under standard glasshouse conditions. Seven day-old plants were then inoculated by injecting approximately 200 µl of a sporidial suspension of compatible strains of either *U. maydis* or *S. reilianum* into the leaf whorl. A single sporidial culture of *U. maydis* or *S. reilianum* inoculated into plants served as negative controls to subtract any wounding induced genes. Uninfected plants were also kept as a control. Plants were kept in the glasshouse until harvested three and four days post infection. The third leaf of 16-20 plants per

infection was removed and flattened onto a glass slide containing double-sided tape. A thin layer of liquid latex was spread over the surface of the leaf and allowed to dry. The latex layer was peeled off thus removing any fungal material on the surface. Stripped leaves were flash-frozen in liquid nitrogen, pooled and ground to a fine powder and either used for RNA extraction or stored at -80℃. Uninfected plants were also subjected to latex treatment.

6.4.2 RNA isolation and cDNA-AFLP method

Total RNA was isolated from 0.5 g frozen ground leaf tissue using the RNeasy Plant Mini Extraction Kit (Qiagen). Multiple samples were pooled and the integrity of the RNA checked on a 1.5% agarose gel. Poly(A) RNA was isolated from the total RNA (75 µg) using oligo(dT) coupled to paramagnetic beads (Dynal A.S.) according to the manufacturer's instructions. A slightly modified version of Bachem *et al*. (1996) was used for the cDNA-AFLP. First and second strand cDNA was synthesised from 500n g poly(A) RNA using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primer, followed by DNA polymerase and RNaseH (Invitrogen) according to the manufacturer's instructions. The resulting double-stranded cDNA was then used as the template using the AFLP Analysis System I Kit (Invitrogen) following the manufacturer's instructions. Briefly, double-stranded cDNA was digested with restriction endonucleases *EcoR* I and *Mse* I and ligated to linkers containing *EcoR* I and *Mse* I compatible ends (Table 7). Pre-amplification was carried out using primers corresponding to the *EcoR* I and *Mse* I linkers and the standard PCR reaction and cycling parameters. For radioactive detection, *EcoR* I primers were end-labelled with γ-^{33}P ATP and T4 kinase. A 1:20 dilution of the PCR product was used in the selective amplification using *EcoR* I and *Mse* I primers containing three selective base extensions at the 3' end in a standard AFLP touchdown-selective amplification programme. Sixty four primer combinations were used. Selective amplification products were boiled with loading dye (containing 50% formamide) and separated on 5% polyacrylamide gels run at 55 W, 50℃ for 2.5 h on a Sequi-Gen GT (Bio-Rad). Gels were transferred to blotting paper, covered with cellophane and dried under vacuum at 80℃ for 2 h and exposed to X-ray film (Kodak) for approximately 3 days at room temperature. Films were positionally marked on the gels before being developed.

Table 8. List of linker and primer sequences used in cDNA-AFLP method

Function	Name	Sequence (5'-3')
AFLP linkers	*EcoR* I 1	CTGTAGACTGCGTACC
	EcoR I 2	AATTGGTACGCAGTCTAC
	Mse I 1	GACGATGAGTCCTGAG
	Mse I 2	TACTCAGGACTCAT
Selective AFLP primers	*EcoR* I	AGACTGCGTACCAATTCNNN
	Mse I	GATGAGTCCTGAGTAANNN*

N = for *EcoR* I were AAC; AAG; ACA; ACT; ACC; ACG; AGC & AGG
N* = for *Mse* I were CAA; CAC; CAG; CAT; CTA; CTC; CTG & CTT

6.4.3 Electrophoretic Separation of Amplicons

After selective PCR amplification, the reactions were mixed with an equal volume (20 µl) of formamide dye and heated at 90℃ for 3min and ice-quenched. A 5% polyacrylamide gel with 0.4 mm spacers and sharkstooth comb was prepared (Sequi-Gen GT – Bio-Rad) and pre-

electrophoresed at 55 W until the gel temperature had reached 50℃. Three to five microliters of the reaction was loaded in wells that were rinsed with the 1x TBE running buffer. The gel was run at 55 W, 50℃ for 2.5 h or until the xylene cyanol dye was approximately two thirds down the length of the gel. After cooling, the gel was transferred to blotting paper, wrapped with cellophane and dried under vacuum at 80℃ for approximately 2 h. Excess paper was trimmed and the gel attached and exposed to X-ray film for at least three days.

Formamide Dye:	TBE-Puffer (5x):
98% (v/v) Formamide	500mM Tris-Borate
10 mM EDTA	10 mM EDTA (pH 7.9)
0.05% (w/v) Bromophenol Blue	in H_2O_{bid}
0.05% (w/v) Xylene Cyanol FF.	

6.4.4 Isolation and Cloning of Amplicons

Bands, identified on the autoradiograph as being differentially expressed, were lined up with markings on the film and the dehydrated gel and excised using a sharp scalpel. The gel pieces were placed in 50 µl 10 mM Tris-Cl (pH8.0) and incubated at 55℃ for 20 min to elute the DNA. Fragments were then re-amplified with 5 µl of the eluted DNA using the same PCR conditions and primer combinations used initially to generate the fragment. An aliquot of the PCR reaction was checked on a 1.5% agarose gel and a second used for cloning into pCR2.1 or pCRII vectors (Invitrogen) according to the manufacturer's instructions. Presence of the cloned fragment was confirmed by *EcoR* I digestion and three independent clones were selected for sequencing.

6.4.5 Sequence analysis

Sequence determination was by an automated sequencer through the services of ADIS at the MPI for Plant Breeding (Cologne). Primer sequences were removed and minor base calling errors corrected using the freeware program CHROMAS. All sequences were then analyzed for homology to all sequences non-redundant and EST databases using the WWW site of the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) running the blast programs Blastn, Blastx and tBlastx (for dbest) (Altschul *et al.,* 1990). For search of homologous nucleotide sequences, sequences were blasted against the *Ustilago* database http://mips.gsf.de/genre/ proj/ustilago/.

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Appendix

8.1 Sequence information of differentially expressed sequences identified by cDNA-AFLP

All relevant details pertaining to the identified fragments are tabulated and saved on a compact disc included with this thesis.

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