Setosphaeria turcica, fungal mating and plant defence

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Cover: Top left, *S. turcica* conidiophore and conidia on maize leaf. Top right, *S. turcica* spores. Bottom, maize leaf with turcicum leaf blight symptoms.

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Abstract

The heterothallic ascomycete *Setosphaeria turcica* (anamorph: *Exserohlium turcicum*) causes turcicum leaf blight on maize and sorghum. A survey was undertaken in Uganda to examine the sorghum – *S. turcica* interaction in terms of disease severity and incidence, overall fungal population structure, and new resistant resources. Highest disease severities were recorded on caudatum accessions, whereas kafir genotypes were most resistant. Highly resistant sorghum accessions originating from a regional collection were found among the five local sorghum races. The disease was more severe in the most humid farmlands. Upon cross inoculation on maize differential lines, *S. turcica* isolates corresponding to race 0, 1, 2 and 3 were found, increasing the number of known races in Uganda. The two *S. turcica* mating type genes *MAT1-1* and *MAT1-2* were found in 20 of 23 districts sampled and in equal proportions on sorghum and maize indicating that sexual recombination is present in Uganda. Fungal mating types in Pezizomycotina are characterised by genes encoding either an HMG or α1 domain protein, occupying the same locus on corresponding chromosomes. We present sequence comparisons, phylogenetic analyses, and *in silico* predictions of secondary and tertiary structures, which support our hypothesis that the α1 domain is related to the HMG domain and share a common ancestor. We have also characterized a new conserved motif in α1 proteins of Pezizomycotina. This motif is immediately adjacent to and downstream of the α1 domain. The *S. turcica* genome contains 123 unique protein sequences not found in related fungi. These are of importance for plant cell wall degradation, ion-binding and transport. Genome comparisons of maize versus Brassica infecting fungi revealed 628 maize specific protein groups including a number of potential effectors.

Six NB-LRR encoding *St* genes residing in three pairs in one locus on chromosome 5 in sorghum were found to mediate resistance to *S. turcica*. The *St* gene homologs have all highly conserved sequences, and commonly reside as gene pairs in the grass genomes.

Keywords: Evolution, *Exserohlium turcicum*, Maize, Mating type, *R*-genes, Sorghum, turcicum leaf blight

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Dedication

To my family, those in the past, the present and the future…

'Imagination is more important than knowledge'
Albert Einstein

'Life moves pretty fast. If you don't stop and look around once in a while, you could miss it'
Ferris Bueller

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Ramathani, I., Biruma, M., Martin, T., Dixelius, C. and Okori, P.** (2011) Disease severity, incidence and races of *Setosphaeria turcica* on sorghum in Uganda. *Eur. J. Plant Pathol.* DOI 10.1007/s10658-011-9815-1.

II [§]**Martin, T.,** [§]**Lu, S-W., van Tilbeurgh, H., Ripoll, DR., Dixelius, C., Turgeon, GB. and Debuchy, R.** (2010) Tracing the origin of the fungal α1 domain places its ancestor in the HMG-box superfamily: implication for fungal mating-type evolution, *PLoS ONE*. DOI 10.1371/5:e15199.

III **Martin, T., Schwelm, A. and Dixelius, C**. Genome-wide comparative analysis reveals insight into maize fungal pathogens. (Manuscript).

IV **Martin, T., Biruma, M., Fridborg, I., Okori, P. and Dixelius, C.** (2011) A highly conserved NB-LRR encoding gene cluster effective against *Setosphaeria turcica* in sorghum. *BMC Plant Biol.* (Accepted pending revision).

Papers I-II are reproduced with the permission of the publishers.

The contribution of Tom Martin to the papers included in this thesis was as follows:

I Conducted all molecular work on maize derived *S. turcica* isolates. Contributed to writing and editing.

II Performed the experiments and analysed the data with SWL, HvT, DRR. Contributed to the writing and editing.

III Participated in planning of the project. Performed the bioinformatics analysis together with AS. Contributed to writing and editing.

IV TM carried out the majority of the lab-work. Made the bioinformatics analysis. Contributed to writing and editing.

Abbreviations

AFLP	Amplified fragment length polymorphisms
BMV	Brome mosaic virus
CC	Coiled coil
Cs	*Colletotrichum sublineolum*
HCT	Horizontal chromosome transfer
HGT	Horizontal gene transfer
HMG	High motility group
HR	Hypersensitive response
HT/Ht	*Helminthosporium turcicum*
LPS	Lipopolysaccharides
LRR	Leucine rich repeat
MAMP	Microbe-associated molecular patterns
MAT	Mating type
Mbp	Mega base pairs
NB	Nucleotide-binding site
NCLB	Northern corn leaf blight
PDS	Phytoene desaturase
PRR	Pattern recognition receptors
R	Resistance
ROS	Reactive oxygen species
SSA	Sub-Saharan Africa
St	*Setosphaeria turcica*
TIR	TOLL and human interleukin-1 receptor
TLB	Turcicum leaf blight
VIGS	Virus induced gene silencing

1 Introduction

The emergence of agriculture is one of the most fundamental transformations of the human-environment interaction. Evidence for agricultural activity appear in fossil records 7000-12000 years ago, spanning at least seven independent centres in the world (Richerson et al., 2001; Brown et al., 2008). With agriculture came diseases to humans, plants and animals and the earliest mention of plant diseases are in ancient Greek literature. Many significant human diseases are thought to have arisen concurrently with the advent of agriculture, creating transmission pathways between humans and animals (Pearce-Duvet, 2006). Emerging plant diseases are caused by pathogens that have increased in incidence, geographical- or host range; changed pathogenesis; or are newly recognised (Anderson et al., 2004). Superimposed, changes in cropping systems, climate, and human activities such as international trade significantly affect spread and crop losses incited by plant pathogens. Our history comprises of numerous examples of crop failures that have led to regional or national famine, emigration, political instability and huge economic losses.

This summary covers large research areas particularly within fungal genetics, plant-pathogen interactions and plant defence responses. The intention is not to fully cover all findings reported but to highlight present understanding and concepts. Thus, a number of important reviews and recent discoveries are referred to. Despite the strong conceptual framework for understanding plant-pathogen molecular interactions, much is based on few pathosystems and a range of fundamental, evolutionary and molecular questions remain to be answered.

1.1 Maize and sorghum cultivation in Sub-Saharan Africa

1.1.1 Origin

Maize (*Zea mays*) was domesticated in Mesoamerica between 6000-10000 years ago from teosinte, which translates to 'grain of the gods' and comprises a group of species in the genus *Zea* (Beadle, 1939; Bush et al., 1989; Doebley, 2004). Teosinte is similar in morphology to maize, however the female inflorescences differ considerably with teosinte producing a few kernels in a hard seed case compared to the many exposed kernels on maize. Sorghum (*Sorghum bicolor*) includes all wild sorghums and cultivated sorghum (*Sorghum bicolor* ssp. *bicolor*) and is indigenous to Africa and Asia and it is suggested that cultivated sorghum became domesticated in sub-Saharan Africa 5000-6000 years ago particularly in the Nile basin (DeWet and Huckabay, 1967; Kimber, 2000). The sorghum genus is noteworthy in that it includes one of the world´s most noxious weeds, Johnson grass (*S. halepense*), an interspecific hybrid of *S. bicolor* and *S. propinquum* (Holm et al., 1977). The latter contributing rhizomatousness, which together with seed dispersal by disarticulation of the mature inflorescence, contribute to the troublesome weedy traits of Johnson grass.

Domestication of maize and sorghum gave rise to landraces with seeds containing high levels of starch, typically 65-75% (Ai et al., 2011). This abundance of starch coupled with developments in agriculture provided a plentiful food supply allowing ancient civilisations to blossom for example, in pre-classic Mesoamerica (1250 BC to 250 AD) maize constituted 50% of the diet in the ancient Maya society (White and Schwarcz, 1989). Maize was so important for their survival the Maya worshiped a maize god and even believed that Man originated form maize. Maize arrived at the African continent during the 1600 century. It was shipped from Central America to the African west coast, together with cassava, beans and potato, and spread slowly, first in the costal areas and later in the inlands of Africa (McCann, 2006). Records claim that it was used as a garden vegetable crop in Ethiopian highland until the beginning of the twentieth century (McCann, 1995).

1.1.2 Economical importance of maize and sorghum

Today, maize and sorghum are the most important staple cereals for Sub-Saharan Africa (SSA) and constitute two of the top five cereal crops in the world along with wheat, rice and barley (FAO, 2011). In addition to a staple for human consumption, maize and sorghum are used for animal fodder and

recently, biofuel. On the African continent, maize and sorghum are grown on 28 and 27 million hectares, respectively, which constitutes approximately 20% and 60% of the world total. The world maize yield average is 5.0 tons per hectare (t/Ha) but the yield varies hugely with North America producing 9.6 t/Ha and Africa just 1.7 t/Ha. World average sorghum yields significantly are lower than maize with African yields averaging 0.9 t/Ha compared with 4.4 t/Ha in North America (FAO, 2011).

Despite the low yield, sorghum is used in an extensive range of cultivation systems because sorghum is generally tolerant to drought, high temperature stresses and low soil fertility (Doggett, 1988). These traits allow cultivation in arid and semi-arid areas with low or limited irrigation unsuitable for maize, strengthening food security in vulnerable regions. Low average yields of maize and sorghum in Africa compared to world averages are accredited to a multitude of factors including poor soils, low inputs (low yielding genotypes, fertilizers, agrochemicals, machinery etc), traditional farming practices and biotic constraints. Common pests and pathogens in SSA are stem bores (*Lepidoptera* spp.), sucking bugs (*Homoptera* and *Hemiptera* spp.), turcicum leaf blight (*Setosphaeria turcica*), grey leaf spot (*Cercospora zeae-maydis*, *Cercospora sorghi*), and maize streak virus disease (Ceballos et al., 1991; Adipala et al., 1993a; Nkonya et al., 1995; De Vries and Toenniessen, 2001; Pingali and Pandey, 2001; Tilahun et al., 2001). A combination of improved farming methods coupled with the greater availability of high yielding, pathogen resistant, maize and sorghum hybrid varieties is increasing yields in Africa. In this perspective, the International Maize and Wheat Improvement Centre (CIMMYT) and the International Crops and Reasearch Institute for the Semi-Arid Tropics (ICRISAT) breeding initiatives together with regional universities and research institutes, are important drivers in the crop improvement processes (Bantilan et al., 2004; Katema 2008; Olembo et al., 2010; www.cimmyt.org; www.icrisat.org).

1.1.3 Turcicum leaf blight (TLB)

The heterothallic ascomycete *Setosphaeria turcica* (Luttrell) Leonard & Suggs (anamorph: *Exserohlium turcicum*, former *Helminthosporium turcicum*) causes turcicum leaf blight (TLB) also know as northern corn leaf blight (NCLB) on maize, sorghum and related wild grasses (Hamid and Aragaki, 1974; Chiang et al., 1989). In Africa, the impact of TLB has historically been of minor importance. However, in 1988 in Uganda extensive yield losses were recorded on maize (Adipala et al., 1993a) and severe and sporadic outbreaks of TLB have since reappeared in East Africa (Ebiyau and Oryokot, 2001; Pratt and Gordon, 2006). Maize and sorghum share agro-ecologies and TLB epidemics

in SSA are now common (De Vries and Toenniessen, 2001). During epidemics, incidence of infection can reach 100% causing significant yield losses (Raymundo, 1981; Carson, 1995; Ramathani et al., 2011).

1.2 *Setosphaeria turcica*

1.2.1 Biology

S. turcica is a hemibiotrophic ascomycete fungus, living on live plant tissue before causing necrosis drawing nutrition from dead tissue. The pathogen thrives in temperatures ranging between 17-28°C and moderate to high humidity but can tolerate harsher conditions (White, 2000). *S. turcica* has been recorded in Europe, the Americas, Australasia, Asia and Africa however, to date, *S. turcica* has not been recorded in Sweden. Maize is expanding in area in Sweden and is expected to become a major crop for silage in near future (Roos et al., 2011). Thus, this has the potential of introducing to new disease problems such as TLB.

S. turcica primarily lives on leaf material spreading systemically through the plant however it will survive or over-winter on decaying plant material when conditions are favourable (Leach, 1977). Reproduction is almost entirely asexual producing conidia. Unequal distribution of mating types and gametic phase disequilibrium within tropical regions has been suggested to be the result of sexual reproduction, possibly while the fungus is residing in decaying plant material (Borchardt et al., 1998; Ramathani et al., 2011). Like many ascomycete plant pathogens, *S. turcica* is thought to spend the majority of its life as a haploid organism only becoming diploid for a brief stage during sexual recombination before undergoing meiosis to produce haploid ascospores (Moghaddam and Pataky, 1994). The sexual stage however has so far not been reported on any field material.

1.2.2 Origin

Based on DNA sequence information derived from 28S rDNA sequences, *Setosphaeria* species such as *S. turcica* are placed within the *Pleosporaceae* family (Kodsueb et al., 2006). *Pleosporaceae* contains many economically important pathogens of monocots including *Cochliobolu*s species such as *C. heterostrophus* (anamorph *Bipolaris maydis*) the casual agent of southern corn leaf blight on maize (Tatum, 1971), *Alternaria* species which attacks a vast number of crop species (Thomma, 2003) and *Pyrenophora* species like *P. teres* that causes barley net-spot blotch disease (Mathre, 1997).

The centre of origin for *S. turcica* has been suggested to be either Mesoamerica or East Africa through a co-evolution with either maize or

sorghum (Borchardt et al., 1998). In either case, a relationship with maize or sorghum would be complimented with wild grass relatives and may not be linked to the domestication of maize and sorghum, as shown in smut pathogens (Munkacsi et al., 2007).

1.2.3 Infection and spread

Turcicum leaf blight is primarily a foliar disease on maize and sorghum, and infection begins when an *S. turcica* spore lands on a susceptible plant leaf. *S. turcica* colonisation is dependant on specific climate conditions and usually begins after a dew period when moisture levels are high, temperature moderate and light levels are low. As the spore begins to germinate, hyphae then grow from the spore in a bipolar manner. Germination is dependant on light with constant light of 150 µmolE $m^{-2} \cdot sec^{-1}$ or blue light (465-480 nm) inhibiting germination by up to 85% when compared to normal light conditions (Levy and Cohen, 1983). The hyphae protruding from the spore grow along the leaf surface and then produce one or multiple appressoria (Figure 1). An infection peg develops from the appressorium, which penetrates directly through the cuticle and epidermis of the leaf and rarely through the stomata (Knox-Davis, 1974; Muiru et al., 2008).

After penetration is achieved the fungus continues to grow causing necrosis and tissue collapse. The mycelia grow through the leaf tissue and into the vascular system, spreading systemically throughout the plant (Muiru et al., 2008). The growth of mycelium in the xylem is copious, blocking the xylem and causing wilting due to water deficiency (Thakur et al., 1989). The developing lesions are cigar shaped and grey-brown in colour and typically 5-20 cm in length spreading longitudinally along the leaf. The infection is generally limited to the leaf material of the plant and does not cause damage to the seed directly. However, large and numerous leaf lesions are sufficient to cause wilting and a reduction in photosynthetic potential reducing yield (Raymondo and Hooker, 1981).

After the initial infection the fungus produces conidia on conidiophores that protrude directly out of the leaf surface and lesions (Knox-Davis and Dickson, 1960). Conidial dispersal is triggered by a reduction in humidity and is usually highest in the morning after a dew period and requires a minimum of 2h darkness as in constant light conidiophores are produced without conidia (Flaherty and Dunkle, 2005). Dispersal can involve rain splash or an electrostatic force that volleys the spore into the air to spread the spores over a larger distance (Leach, 1977). The conidial spread can cause secondary infections on the same plant or spread the infection to neighbouring plants or larger distances between fields.

In warm climates the broad host range of *S. turcica* including many wild grasses maintains high levels of inoculum, acting as a reservoir for the fungus allowing continuous re-infections (Harlapur et al., 2007). In cooler climates the fungus overwinters as mycelium, conidia or resting spores, chlamydospores, on stubble and decaying plant material allowing re-infection when conditions are favourable (Leach, 1977).

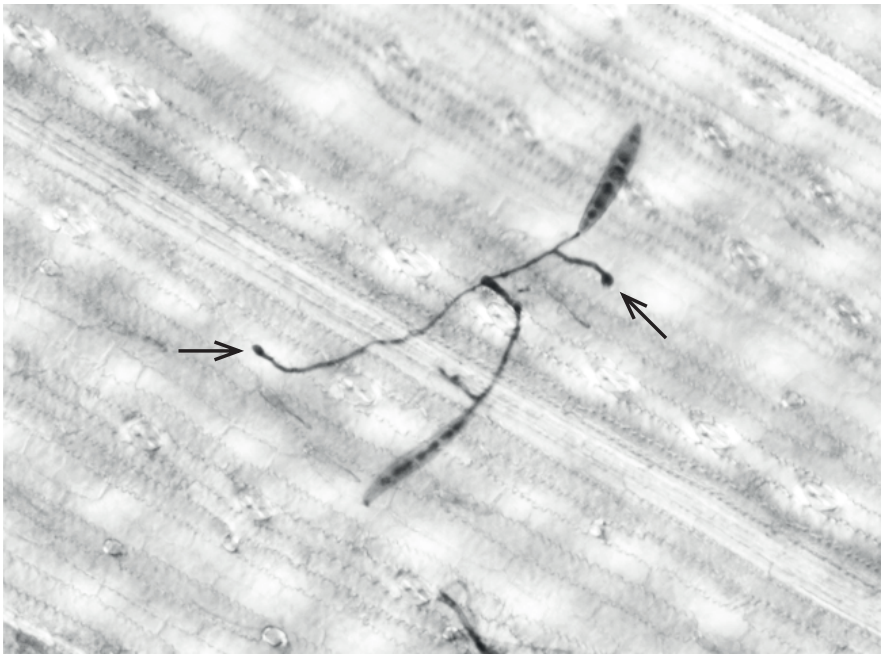


Figure 1. S. turcica spores on sorghum leaf surface. Mycelia can be seen growing out of the spore along the leaf surface. Arrows indicate appressoria.

1.2.4 Molecular data

Dissecting the molecular mechanisms underlying infection by *S. turcica* is critical to gaining a full understanding of the disease but also for successful resistance breeding. Many up-regulated genes associated with asexual conidial development under constant darkness have been identified such as homologs of monomeric GTP-binding and G-proteins, zinc binuclear cluster-type proteins, transcription factors and receptor proteins (Flaherty and Dunkle, 2005). In addition, germination of conidia and the formation of appressorium are regulated by a mitogen-activated protein kinase signal transduction pathway (Fan et al., 2007).

The pathogen facilitates penetration and colonisation with the production of a range of secondary metabolites and toxins. The *S. turcica* genome contains two genes encoding xylanase enzymes, which degrade arabinoxylan in the plant cell wall causing loss of integrity and aiding penetration (Degefu et al., 1997; Degefu et al*.,* 2004). Catalases have been detected from artificial cultures, where two particular isoforms showed implications in spore germination and early infection processes (Keissar et al., 2002). A multitude of toxins have been isolated from *S. turcica* (Robeson and Strobel, 1982; Zhang et al., 2007). One toxin, *Helminthosporium turcicum* (HT) toxin, has been identified as a lipophilic phytotoxin called Monocerin. Monocerin treated maize and sorghum plants cause inhibited chlorophyll synthesis, reduced root growth and lesions and necrosis on susceptible genotypes (Cuq et al., 1993; Bashan et al*.,* 1995; Zang et al., 2007). Furthermore, leaves punctured and treated with Monocerin develop necrosis that spreads throughout the vascular system suggesting that the Monocerin HT-toxin is not only involved in penetration but also in later stages of infection (Cuq et al., 1993).

1.3 Fungal genomics

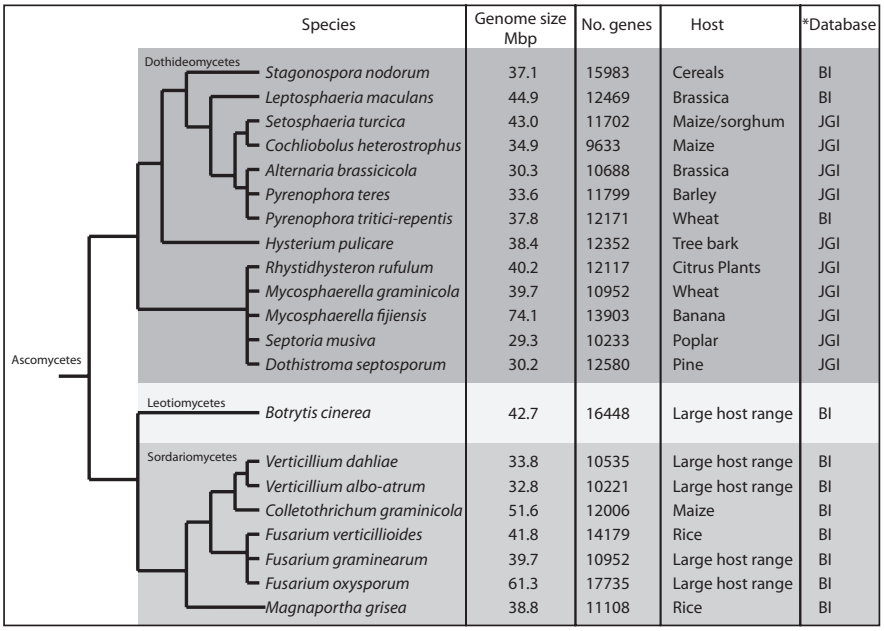
1.3.1 Genome sequencing

Developments in sequencing technologies and reductions in costs have resulted in the number of fungal genomes sequenced to expand rapidly (www.fungalgenomes.org). Fungi generally have small genomes with the majority between 20 and 50 Mbp, and low amounts of long repeats compared to other eukaryotes (Kullman et al., 2005; Karaoglu et al., 2005). Enormous quantities of data are becoming available to researchers providing an invaluable resource for diverse areas of research such as genomic comparisons, phylogenetics and effector biology. Fungal taxonomy has been previously based on morphology however, now groups of genes and genomes are used to produce more detailed resolution (Schoch et al., 2006; Zang et al., 2006). For example, data from early divergent fungi such as species from Zygomycota, Glomeromycota and Chytridiomycota is now becoming available (James et al., 2006).

1.3.2 *S. turcica* genome

In March 2011 the *S. turcica* genome v1.0 was completed using Roche (454), Sanger Fosmids, and shredded consensus from velvet assembled Illumina data (www.jgi.doe.gov). The *S. turcica* genome is 43 Mbp in size comprising 11,702 predicted gene models. The genome size is comparable to other plant pathogens currently sequenced with the exception of *Mycosphaerella fijiensis*

(74 Mbp), which has a complex genome containing eight dispensable chromosomes (Goodwin et al., 2011). The number of predicted genes models is also similar to that found in related fungal species (Figure 2). Culturing *S. turcica* on artificial media is possible and the fungus is amenable to genetic modifications using *Agrobacterium tumefaciens*-mediated transformation (Degefu and Hanif, 2003). Because genome data now is available it is anticipated that this knowledge will be widely exploited for functional studies.



Species	Genome size Mbp	No. genes	Host	*Database
Dothideomycetes				
Stagonospora nodorum	37.1	15983	Cereals	BI
Leptosphaeria maculans	44.9	12469	Brassica	BI
Setosphaeria turcica	43.0	11702	Maize/sorghum	JGI
Cochliobolus heterostrophus	34.9	9633	Maize	JGI
Alternaria brassicicola	30.3	10688	Brassica	JGI
Pyrenophora teres	33.6	11799	Barley	JGI
Pyrenophora tritici-repentis	37.8	12171	Wheat	BI
Hysterium pulicare	38.4	12352	Tree bark	JGI
Rhystidhysteron rufulum	40.2	12117	Citrus Plants	JGI
Mycosphaerella graminicola	39.7	10952	Wheat	JGI
Mycosphaerella fijiensis	74.1	13903	Banana	JGI
Septoria musiva	29.3	10233	Poplar	JGI
Dothistroma septosporum	30.2	12580	Pine	JGI
Leotiomycetes				
Botrytis cinerea	42.7	16448	Large host range	BI
Sordariomycetes				
Verticillium dahliae	33.8	10535	Large host range	BI
Verticillium albo-atrum	32.8	10221	Large host range	BI
Colletothrichum graminicola	51.6	12006	Maize	BI
Fusarium verticillioides	41.8	14179	Rice	BI
Fusarium graminearum	39.7	10952	Large host range	BI
Fusarium oxysporum	61.3	17735	Large host range	BI
Magnaportha grisea	38.8	11108	Rice	BI

Ascomycetes

* BI = www.broadinstitute.org JGI = www.jgi.doe.gov

Figure 2. A selection of fungal plant pathogens with genome sequence data available. Phylogeny based on trees presented in Schoch et al. (2006) and Zang et al. (2006). Genomic information was obtained from the Broad Institute (www.broadinstitute.org) and the Joint Genome Institute (www.jgi.doe.gov).

1.3.3 Comparative genomics

One area of particular interest is the comparison for environmentally and economically important fungal plant pathogens with non-pathogenic fungi, oomycete plant pathogens and plant pathogens with different infection strategies. This is being used to identify genes responsible and required for virulence (Soanes, 2007; Schirawski et al., 2010; Duplessis et al., 2011). Once the genome data is available, re-sequencing of different fungal strains is possible aiding research in functional genomics, population structure, mutation variation and pathogenicity among others (Schacherer et al., 2007; Montero et al., 2008; Timmermann et al., 2010; Nishant et al., 2010). The evolution of

pathogen-associated genes has produced anomalies in contention with the orthodox view of fungal evolution. One suggested explanation is horizontal transfer of genes (HGT), clusters or even chromosomes (HCT) between distantly related species (Mehrabi et al., 2010). Some toxin genes or chromosomes containing toxin genes are more similar to those in distantly related than closely related fungi, supporting the HGT/HCT theory (Inderbitzin et al., 2010). Evidence is also mounting for cross kingdom HGT between the morphologically similar but phylogenetically distinct oomycetes and fungi although the mechanisms are poorly understood (Richards et al., 2006). The possibility of transferring pathogenicity between species has the potential to create new pathogenic fungi, races and epidemics in a single instance. Further, gain of genes and loss of certain pathways like molybdopterin biosynthesis have recently been shown to play important roles for different lifestyle evolution on pathogens in Chromalveolata (Kemen et al., 2011). These, and similar analysis will have profound affect on our understanding of pathogenic organisms.

1.4 Mating in fungi

1.4.1 Sex and reproduction strategies

Sex is advantageous in fungi as it increases the efficiency of natural selection through recombination, diversification of the population and removal of deleterious mutations (Goddard et al., 2005). Ascomycete fungi reproduce sexually and/or asexually depending on lifestyle, environment and a number of other factors (Lee et al., 2010). For many plant pathogens it is beneficial to rapidly spread to all available host tissue to aid infection making asexual reproduction the most optimal method as millions of identical spores can be produced in a short period of time without the need to find a mate (Taylor et al., 1999). After infection is achieved, and if the conditions are favourable, the fungus may then switch to sexual reproduction. For many plant pathogenic fungi asexual reproduction has become the only strategy as the ability to reproduce sexually has been lost (Rossman and Palm-Hernandez, 2008). Interestingly, many fungi thought to be exclusively asexual have been found to maintain functional genes required for sexual recombination (Alby and Bennett, 2010). It has been hypothesised that they may have only recently lost the ability to reproduce sexually or, as shown in *Aspergillus fumigatus,* they may do so only once in thousands of generations (Taylor et al., 1999; Dyer and Paoletti, 2005).

1.4.2 Genetic control of mating

Sexual reproduction in Pezizomycetes is controlled by the mating type (*MAT1*) locus (Debuchy et al., 2010). In heterothallic fungi, the *MAT1* locus comprise of two mating types, termed *MAT1-1* and *MAT1-2,* with one of each mating type required for sexual reproduction (Figure 3). The *MAT1* locus contains dissimilar sequences between mating types and encodes *MAT* genes, which are numbered according to the system developed by Turgeon and Yoder (2000). The major genes in the *MAT1* locus are the alpha box domain (α1) containing gene *MAT1-1-1* found only in *MAT1-1* and the high motility group (MATA_HMG) domain-containing gene *MAT1-2-1* found in *MAT1-2* (Souza et al., 2003). The defining gene is the α1-containing gene *MAT1-1-1* as *MAT1-1* may contain genes with MATA_HMG domains but *MAT1-2* has no α1 containing genes. The α1 and MATA_HMG domain containing genes *MAT1-1-1* and *MAT1-2-1* code for proteins that are transcription factors that activate *MATα* and *MATa* specific genes respectively, causing developmental changes leading to mating.

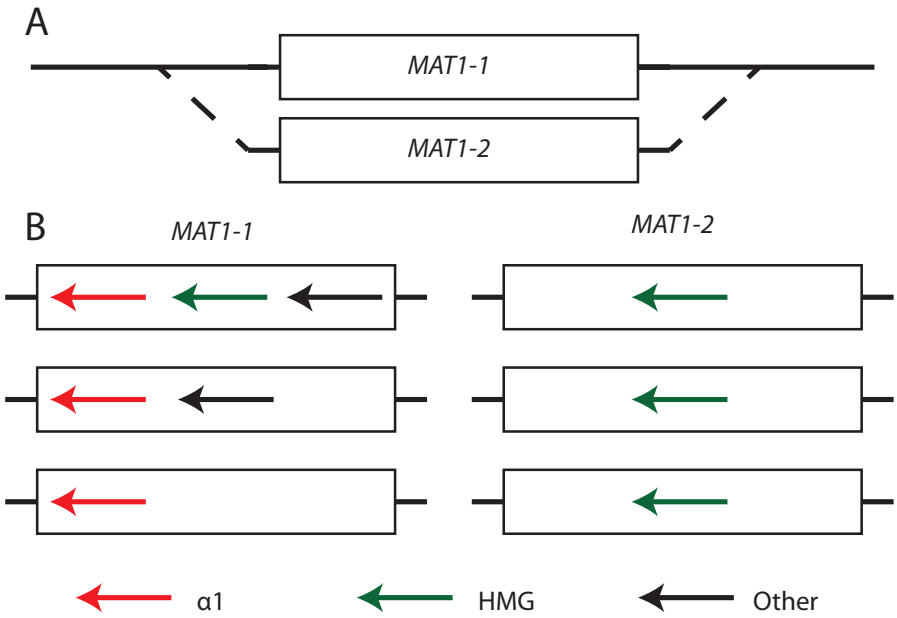


Figure 3. Schematic overview of mating type locus in Pezizomycetes. (A) The mating type locus can either contain *MAT1-1* or the *MAT1-2* genes. (B) Possible domains present in either *MAT1-1* or *MAT1-2*. For a more details see figure 7 (II).

The HMG domain is a small (~80 amino acids) domain that forms three alpha helices which produce a 'L' shape and binds the minor grove of DNA in

a non-specific manor (Read et al., 1993; Palasingam et al., 2009). During the binding with DNA, the side groups of specific amino acids on the internal surface of the protein intercalate with the DNA bases causing an ~80 degree bend required for transcription (Thomas and Travers, 2001; Klass et al., 2003). The fungal MATA_HMG domain binds DNA, facilitating the transcription of MATa specific genes including pheromone a-factor and the receptor for the opposite pheromone factor (Souza et al., 2003). The function of α1 domain has been determined as a transcriptional co-activator of mating pheromone α and the receptor for the opposite pheromone factor (Tsong et al., 2007; Debuchy et al., 2010). The α1 protein binds to a MADS-box transcription factor Mcm1, and is required before the complex can bind DNA and activate transcription of α specific genes (Hagen et al., 1993; Carr et al., 2004).

The MATA_HMG is a member of the ancient HMG superfamily, predating the split of plants, fungi and metazoa (Laudet et al., 1993; Griess et al., 1993; Fraser and Heitman, 2005). The α1 domain is found exclusively in ascomycetes and has a complex evolutionary history (Lee et al., 2010). As the MATA_HMG and α1 domain share the same locus and both have similar functions it has been hypothesised that they may share evolutionary history (Idnurm et al., 2008).

1.5 Sorghum taxonomy and genomics

1.5.1 Evolution of major crop plants

Monocots and eudicots diverged 150-300 million years ago (mya) and are fundamentally distinct in development and physiology (Figure 4). Eudicot include among others *Fabaceae*, *Solanaceae*, *Brassicaceae* and *Euphorbiaceae* families, which contain important crop species like pea, soybean, lentil, tomato, potato, rapeseed, cabbage and cassava. The monocots contain the most economically important staple crops in the world in the grass family (*Poaceae*) which is divided into three subfamilies; *Panicoideae,* containing sorghum, maize, sugarcane and millet, *Pooideae,* containing oats, barley and wheat, and *Ehrhartoideae*, containing rice. *Panicoideae* is believed to have diverged from other grasses 50-70 mya with *Ehrhartoideae*, and *Pooideae* diverging ~46 mya (Bolot et al., 2009).

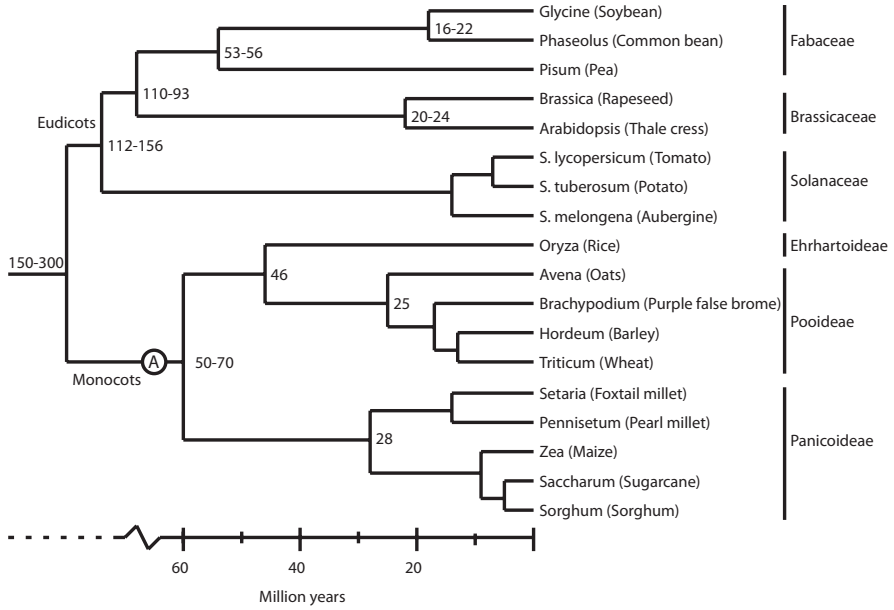


Figure 4. Phylogenetic relationships and divergence-time between crops (Bolot et al. 2009). All plant species descend from a single eukaryotic ancestor that acquired a photosynthetic endosymbiont. The estimated dates for evolutionary splits in the "green linage" in the "tree of life" is as following: green algae 1,200 mya, bryophytes (mosses) 450 mya, gymnosperms (trees) 250 mya, and for 150-300 mya, dicots (eudikots) and monocots (grasses) diverged from each other (Bowman et al., 2007). Dates are based on data by Yang et al. (1999) and Wang et al. (2008). Ⓐ Whole genome duplication event, ~70 mya (Salse et al., 2008; Wang et al., 2011).

Snowden (1936) made a thorough classification of sorghum species that has been reorganised several times. Present taxonomy place the genus *Sorghum*, subtribe *Sorghinae* in *Andropogoneae*. The genus *Sorghum* consists of 25 species, classified into five subgenera (Price et al., 2005). *S. bicolor* is considered to be the most primitive grain sorghum and the most ubiquitous breed, widely distributed in Africa and Asia, (Dahlberg, 2000; Kimber, 2000). Cultivated *S. bicolor* is further divided, based on morphology into five so-called races; bicolor, caudatum, guinea, durra, and kafir (Harlan and de Wet, 1972). However, several intermediate and spontaneous races are suggested that in some cases blurs present taxonomic classifications.

1.5.2 The sorghum genome

Sorghum is mainly a C_4, self-pollinating and diploid species (2n=2x=20) with a genome size of 730 Mbp. Plant genome size varies hugely between species and the sorghum genome is relatively small compared to other crop plants (Table 1). In 2009, Paterson and co-workers sequenced the entire sorghum genome revealing a genome content of 27,640 bona fide protein-coding genes from

34,496 gene models. Putative function has been designated to genes based on homology searches and conserved domain architecture allowing comparisons to be made with other genomes (www.phytozome.net). The sorghum genome contains 55% retrotransposons, which largely explain the relative net genome expansion compared to rice. The genome has experienced ancient duplications, today reflected as 57.8% collinear gene models compared to rice. Interestingly, as many as 9,503 orthologous gene families were found between sorghum, rice, Arabidopsis and Poplar genomes. In comparison, 5,337 gene families are shared between sequenced monocots and potato (The Potato Genome Sequencing Consortium 2011). Various tools are now under development for grass species like the integrative database GramineaeTFDB for putative transcription factors (Moshida et al., 2011). A consensus sorghum map with QTLs from a wide range of studies in order to exploit valuable markers for crop improvements have also been compiled (Mace and Jordan, 2011).

Table 1. *Selected plant species and their genome sizes.*

Species	Common name	~Mbp*
Arabidopsis thaliana	Thale cress	135
Brachypodium distachyon	Purple false brome	272
Oryza sativia	Rice	372
Setaria italic	Foxtail millet	406
Sorghum bicolor	Sorghum	698
Saccharum officinarum	Sugarcane	930
Brassica napus	Rapeseed	1130
Zea mays	Maize	2076
Pennisetum glaucum	Pearl millet	2450
Hordeum vulgare	Barley	5000
Avena sativa	Oats	11000
Triticum aestivum	Bread wheat	17000

*Mbp values derived from sequence data (www.phytozome.net; www.maizesequence.org; www.plantgdb.org).

In the *S. bicolor* genome 211 NB-LRR encoding R-proteins are present, which is approximately half the number found in rice and slightly more compared to Arabidopsis (Paterson et al., 2009). The number of NB-LRR encoding genes in the large maize genome draft is predicted to be 95 (Li et al., 2010), and in the small genome of the wild grass *Brachypodium distachyon* 178 (The International Brachypodium Initiative, 2010). Depending on search programs and threshold settings, slightly different *R*-gene numbers in each grass species are published (Li et al., 2010). The largest number of *R*-genes (62) is found on chromosome 5 (Paterson et al., 2009). The rice chromosome

11 is homologous to sorghum chromosome 5 and also contains a large number of *R*-genes (106) showing a conservation of *R*-genes in chromosome location (Wang et al., 2011). Sorghum chromosome 5 and 8 share an ancestor through a whole genome duplication event in the ancestor of grasses and show remarkable similarity to rice chromosome 11 and 12 (Salse et al., 2008; Wang et al 2011).

1.6 Plant resistance genes

Plants are constantly exposed to microbes. To be pathogenic, most microbes must access the plant interior, either by penetrating the leaf or root surface directly or by entering through wounds or natural openings such as stomata. Consequently, plants are under strong evolutionary pressure to maintain surveillance against pathogens. In the early 1990s the first plant resistant (*R*) genes were cloned and today we have approximately 100 identified *R*-genes including allelic variants correlated with a known pathogen. For details see (Hammond-Kosack and Jones, 1997; Hammond-Kosack and Parker, 2003; Nimchuck et al., 2003; Ravensdale et al., 2011). New sequencing technologies have resulted in important data on both plant hosts and pathogens leading to a deeper understanding of distribution of *R*-genes, evolutionary aspects of these genes and producing abundant information on pathogen genomes and presence of effector genes (Egan and Talbot, 2008; Van de Wouw and Howlett, 2011). Effectors in this instance are defined as secreted pathogen proteins and other molecules that modulate plant defence circuitry and enable parasitic colonization of plant tissue (Hogenhout et al., 2009). In particular, insights of the *Phytophthora* genomes and interactions with respective plant host have contributed to this area (Kamoun, 2007; De Wit et al., 2009; Thines and Kamoun, 2010).

We have learnt that plants have evolved two classes of immune receptors to detect non-self molecules. One class consists of membrane-resident pattern recognition receptors (PRRs) that detect microbe-associated molecular patterns (MAMPs). MAMPs are evolutionarily conserved structures that include components of fungal cell walls such as chitin (N-acetyl-chitooligo-saccharide oligomers), most likely lipopolysaccharides (LPS) from gram-negative bacteria, as well as short peptides derived from bacterial flagellin or the elongation factor EF-Tu (Zipfel and Felix, 2005; Altenback and Robatzek, 2007).

Plant resistance (R) proteins define a second mainly intracellular immune receptor class that have the capacity to detect directly or indirectly isolate-specific pathogen effectors, encoded by avirulence genes. Like PRR-triggered

immune responses, R protein-conditioned immunity is also linked to ROS accumulation and to defence gene activation, but differs both quantitatively and kinetically from the former, typically leading to host cell death at attempted invasion sites (Chisholm et al., 2006; Jones and Dangl, 2006; Maekawa et al., 2011).

Sequence comparisons among *R*-genes have revealed a remarkable conservation of structural features, despite the diversity of the pathogens with which their products, indirectly or directly, interact. Most commonly, resistance proteins are composed of a nucleotide-binding domains (NB) followed by a series of leucine repeats (LRRs). In contrast to animal NB-LRR proteins, plant R proteins usually have a different N-terminal domain. The N-terminal domain in plants may be a coiled coil (CC) sequence or a domain that shares sequence similarities with the *Drosophila* TOLL and human interleukin-1 receptor referred to as TIR. Intriguingly, plant and animal defence against microbes have striking similarities (Ronald and Beutler, 2010). R proteins can interact with effector proteins in various ways or become activated via other trigger proteins. R protein activation often has cell death or hypersensitive response (HR) as a final outcome, thus its activation has to be tightly regulated (Lukasik and Takken, 2009). It is further suggested that upstream interactions of R proteins involves conserved chaperon complexes for proper folding, accumulation and regulation (Shirazu, 2009). Effector recognition is thought to induce conformational changes in the R proteins, releasing inhibition and freeing NB-LRR domains to activate downstream signalling (Collier and Moffett, 2009). Concerning downstream signalling, events that link NB-LRR activation remains elusive and several studies suggest nuclear activity of some NB-LRRs to trigger proper responses. Besides nuclear localization, WRKY transcription factors seem to play crucial roles (Elmore et al., 2011). In an evolutionary perspective *R*-genes have been proposed to follow two distinct evolutionary patterns, i.e. fast or slow-evolving types (Vleeshouwers et al., 2011). Rapidly evolving *R*-genes are characterized by frequent sequence exchanges between paralogs, resulting in obscured allelic relationships. In contrast, slowly evolving *R*-genes are characterized by infrequent sequence exchange between paralogs, resulting in tractable relationships.

Plant pathogens differ markedly in the number of plant species they are able to colonize and cause disease on. A majority of plants are immune against most pathogens and the likelihood that a pathogen can infect two plant species in a habitat decreases with the phylogenetic distance between the plants (Gilbert and Webb, 2007). The non-host resistance phenomenon i.e. resistance response outside the host range of a given pathogen has received much attention (Kamoun, 2001; Mysore and Ryu, 2004; Nürnberger and Lipka, 2005).

Understanding nonhost responses on molecular levels is attractive since it could lead to more durable or broad-spectrum resistance to all strains/races of a pathogen species compared to specific *R*-genes. A handful of studies not least on responses in barley to various pathogens are presently given new insights in this area (Stein et al., 2006; Zellerhoff et al., 2010; Aghnoum and Niks, 2010). It has been hypothesised that the relative contribution of R proteins and PRR-triggered immunity to nonhost resistance changes as a function of phylogenetic divergence time between host and nonhost plants (Schulze-Lefert and Panstruga, 2011). Likewise, a pathogens host range expansion could be driven by variation in the effector repertoires, leading to reproductive isolation and subsequent pathogen speciation. Further experimental work is needed to provide sufficient data to test this intriguing concept.

2 Aims of the study

This thesis is one of several efforts in Sweden to strengthen North-South partnership in agricultural related bioscience research. The disease problems addressed are those prioritised by our partners in East Africa. The work has been tightly linked to the Eastern Africa Regional Programme and Research Network for Biotechnology, Biosafety and Biotechnology Policy Development (BIO-EARN), run 1999-2010, supported by the Swedish government via the Swedish International Development Cooperation Agency (Sida). Academic training of mutual interests have been one core component to establish a deeper understanding of near future problems involving food security, use of natural resources, climate change effects and several other global issues of importance. For more details on the BIO-EARN program, see www.bio-earn.org and its continuation http://bioinnovate-africa.org.

The emphasis of the work was to study the *Setosphaeria turcica* – sorghum pathosystem with some contributions on maize. The specific aims were to:

- Investigate the extent of TLB on sorghum in Uganda.
- Characterise the mating type genes in *S. turcica* and investigate fungal mating type evolution with emphasis on the α1 domain.
- Initiate work on *Setosphaeria turcica* genomics.
- Identify new resistance genes to *S. turcica* in sorghum to be used in breeding programs in Uganda.

3 Results and Discussion

3.1 *S. turcica*, disease and resistance in Uganda

3.1.1 Incidence and severity of TLB in Uganda

In collaboration with our colleagues at the Crop Science Department, Makerere University, a survey was undertaken in Uganda to examine the sorghum – *S. turcica* interaction in terms of disease severity and incidence, overall fungal population structure, and new resistant resources (**I**). The climate in Uganda varies from semi-arid in the north to tropical in south and central regions, which are ideally suited for *S. turcica* (White, 2000). Field trips and surveys in Uganda were carried out annually, starting 2006. On each occasion maize and sorghum leaf material was collected from different regions and disease incidence at field level recorded. TLB was found in every district sampled although severity varied between regions (**I**). Unsurprisingly the disease was most severe in humid farmlands where maize and sorghum are grown. The high susceptibility to *S. turcica* of the rather popular hybrid variety Epuripur (used mainly for brewing, can yield 1700kg/ha) is problematic. Improvement of Epuripur is of great importance to increase household income and resistance breeding and further advancements are now prioritised.

3.1.2 Resistance in sorghum accessions

Natural accessions and landraces of sorghum are important sources of new resistance to *S. turcica*. The race bicolor is the most commonly grown sorghum in East Africa and the incorporation in breeding programs of TLB resistant germplasm from kafir, guinea, caudatum, and durra races is seen as way to compliment important traits including resistance in bicolor germplasm. However, durra is rare in Eastern Africa.

A large collection of 196 sorghum accessions covering all five sorghum races was tested for resistance to *S. turcica* (**I**). A varying degree of resistance

was identified in all races and those identified are now implemented in regional breeding programs. Mapping populations are currently evaluated at Makerere University to enhance the knowledge on genetics. Qualitative resistance has been shown in maize to protect plants against specific races of *S. turcica* however this has never been successfully applied in Uganda despite earlier reports that only race 0 and 1 were present (Adipala, 1993b). Qualitative resistance is likely overcome and work on discovery of quantitative disease resistance in maize is on-going (Chung et al., 2011).

3.1.3 *S. turcica* races

During testing of 18 sorghum derived *S. turcica* isolates on maize differential lines, we found four *S. turcica* isolates identified as race 1, two as race 2, one race 0 and one race 3. The remaining 10 *S. turcica* isolates did not cause any disease symptoms on the maize lines assessed. Race proliferation has been reported in Africa, Asia and America in recent years. The genes responsible for race determination in *S. turcica* are poorly understood however the maize genotypes used to assess races contain single *R*-genes possibly indicating a gene-for gene system (Lim et al., 1974) but more complex segregating rations have been observed (Moghaddam and Pataky, 1994). Genes for toxins may determine race, and there is evidence that specific virulence to *Ht1* is conferred by a specific toxin (Zhang et al., 2007). That any fungal isolate from sorghum could cause disease on maize was not expected, however, it seems to have been known locally for some time. Surprisingly we also found that the expected susceptible A619 plants carrying no *Ht* genes were resistant to the isolates designated race 1, 2 or 3 (**I**). The progenitor of all races has been suggested to be race 0 and the emergence of new races is thought to have been driven by the use of the *Ht* genes in maize in USA (Ferguson and Carson, 2007). The new races we identified in Uganda where the *Ht* genes are not widely used in maize suggests that in Uganda, races other than 0 are not the product of *Ht* overexposure. Clearly this is a complicated pathosystem and far from fully understood and our results highlight the fact that any new breeding efforts and cultivation practices must take into consideration the notion that new races and sorghum isolates able to infect maize are present in Uganda.

3.1.4 Mating types

A small section of ~300 nucleotides of the highly conserved MAT_HMG DNA binding domain was previously identified using degenerate primers (Arie et al., 1997). We used a similar approach to align α1 containing sequences from related fungi, and sequenced a small section of ~250 nucleotides of the α1 domain from *S. turcica*. Armed with a small section from each idiomorph we

amplified flanking regions for both *MAT1-1* and *MAT1-2*. When sequence data for ~2500 nucleotides for each mating type was obtained we found that like most ascomycetes the *MAT1-1* and *MAT1-2* idiomorphs harbour identical flanking regions. Sequence data can be retrieved from Genbank no. GU997137.1 and GU997138.1. Previously the mating type of isolates was determined by crossing each isolate with an isolate of known mating type or using PCR primers designed to the HMG sequence (Furguson and Carson, 2004). The problem of using only one primer pair to determine mating type is that a negative result cannot confirm the presence of the one mating type meaning crossing with tester isolates is still required. Crossing *S. turcica* with tester isolates require the complex Sachs medium, is time consuming, and is not always successful (Hebert, 1971; Furguson and Carson, 2004), therefore a PCR based method is an excellent alternative. Using our sequence data we designed mating type specific primers for both mating types allowing large collections of isolates to be screened in a short time.

Using our mating type specific primers we tested two collections of *S. turcica* isolates, one from infected maize leaves and the other from infected sorghum. We found that the distribution of mating types in both collections were not significantly different from an even distribution (**I**). Furthermore, we found that there were many regions in Uganda where both mating types were present (20 of 29) on maize and/or sorghum meaning that the different mating types may be in direct contact with one another allowing mating to occur. Interestingly not all isolates tested were found to contain only *MAT1-1-1* or *MAT1-2-1* genes as a five maize derived isolates were found to contain both.

3.1.5 Searching for the perfect stage of *S. turcica*

The presence of the perfect stage on field level has never been reported in the literature however in Uganda we have been informed that it has been observed earlier. We wanted to clarify that issue and took on a specific field survey. The regions of Sotori, Iganga and Mbale were chosen as both mating type were present, the climate was optimal for *S. turcica*, and the crops were mature because it had been suggested that the sexual stage is likely to be present on dead leaf material or decaying leaf litter with advanced stages of the disease (Borchardt et al., 1998). We collected ~700 lower leaf samples from maize heavily infected with *S. turcica* in the later stage of the disease when large necrotic lesions were present. We failed to identify the presence of the sexual stage in any of our samples despite the large numbers of leaves surveyed. Likewise, a corresponding survey on sorghum materials did not show any evidence of a perfect stage. However, another pathogen, *Stenocarpella maydis*,

was common on our maize and sorghum samples. This pathogen is regarded as causing minor disease problems in East Africa but seems to be more frequent the last years. Our work on *S. turcica* raised a number of questions on basic fungal mating biology, which led us to the work on the origin of the fungal α1 domain (**II**).

3.2 Evolution of the fungal α1 domain

Whilst aligning *S. turcica* α1 and MATA_HMG sequences we noticed a small section of the α1 sequence that seemed to be highly conserved within related fungi. The section was ~40 amino acids in length and consistently had the same or similar amino acids in specific positions. This is not unexpected for related α1 sequences, however what was unexpected was that we found a similar ~40 amino acid region in the MATA_HMG domain. The MATA_HMG domain is a member of a long-standing gene family stretching back hundreds of millions of years (Laudet et al., 1993; Griess et al., 1993; Fraser and Heitman, 2005). The domains are found in plants, Oomycetes and many distantly related organisms and have roles in sex both Metazoa and Fungi.

It has been suggested that the α1 and MATA_HMG domain might share a common ancestor however no evidence had been put forward to support this theory (Idnurm et al., 2006). We called the ~40 amino acid region which showed similarities between the α1 and MATA_HMG the core region (**II**). The core region was also found to be conserved in the HMG domain of sex related genes in Metazoa and even to a lesser degree to the HMG domain of plants, oomycetes and other distantly related organisms. This core region and specifically the highly conserved positions revealed by the WebLogo tool, appear to be key components of the HMG domain. Using computer programs we predicted the secondary and tertiary structure of the α1 domain and found it was extremely similar to that of the HMG domain. Sequences that have low levels of exact sequence matches but have amino acids with similar properties can have remarkably similar tertiary structures. This is the case of the MATA_HMG and the α1 sequences with the predicted tertiary structures almost an exact match when overlaid (**II**). In collaboration with Robert Debuchy, Gillian Turgeon and co-workers we are involved in a project to determine the protein structure of the α1 domain coupled with genetic analysis of *Podospora anserina MAT* genes to support our earlier findings.

3.3 Fungal comparative genomics

Since the genome of *S. turcica* was released spring 2011, we have initiated a fungal genome comparative study in order to learn more on this pathogen (**III**). This work is in an early stage and will continue by co-workers after this thesis work. Early results looking at orthologous protein groups between *S. turcica* and closely related plant pathogens show 123 proteins unique to *S. turcica*. They are of predicted importance for plant cell wall degradation, ion-binding and transport, together with potential new types of effector candidates.

3.4 NB-LRR encoding genes in sorghum

3.4.1 Sorghum *R*-genes effective against *S. turcica*

During a germplasm screen for resistance to *S. turcica* in Uganda a number of valuable genotypes were identified. We chose to work with one of the most resistant types, GA06/18, and the susceptible cultivar (Sila) commonly grown in Uganda. In parallel, we used susceptible (A619) and resistant (A619Ht1) maize plants for the same purpose. We used cDNA-AFLP to identify novel or up-regulated gene fragments in the resistant plants compared to the susceptible (**IV**). From over 3000 transcript-derived fragments more than 150 were cloned and sequenced. The sequences included a number of stress related genes, pathogen associated genes and a maize putative resistance gene (*GRMZM2G005347*). This gene had the classic *R*-gene domain structure CC-NBS-LRR. The gene was found to be one of a pair in a single locus on chromosome 2. In sorghum, the *GRMZM2G005347* sequence was found to have 6 orthologous genes formed of three gene pairs in a single locus. We named those genes with resistance to *S. turcica* (*St*). We then looked for gene orthologs in other plant species. We found those to be highly conserved, with rice containing nine homologs in a single locus. We can only speculate that this conserved evolution and gene expansion reflects their importance. We also used the *St* gene sequences from sorghum to search the Arabidopsis genome for orthologs. We found that the *St* proteins had high e-values when using BLAST comparisons with similar Arabidopsis *R*-genes. We aligned a selection of known Arabidopsis resistance genes with the *St* genes and performed phylogenetic analysis. The Arabidopsis *RPM1* mediating resistance to *Pseudomonas syringae* isolates expressing the *avrRpml* or *avrB* genes (Grant et al., 1995) was found to be the closest gene to the *St* genes.

In a parallel study on sorghum anthracnose, we identified two loci on chromosome 9 containing NB-LRR genes mediating resistance to *Colletotrichum sublineolum* (Biruma et al., 2011). The Cs2 proteins clustering

closest to the St proteins, harbour a WD40 domain at their C-terminal ends, whereas the Cs1 pair is more distantly related (Figure 5).

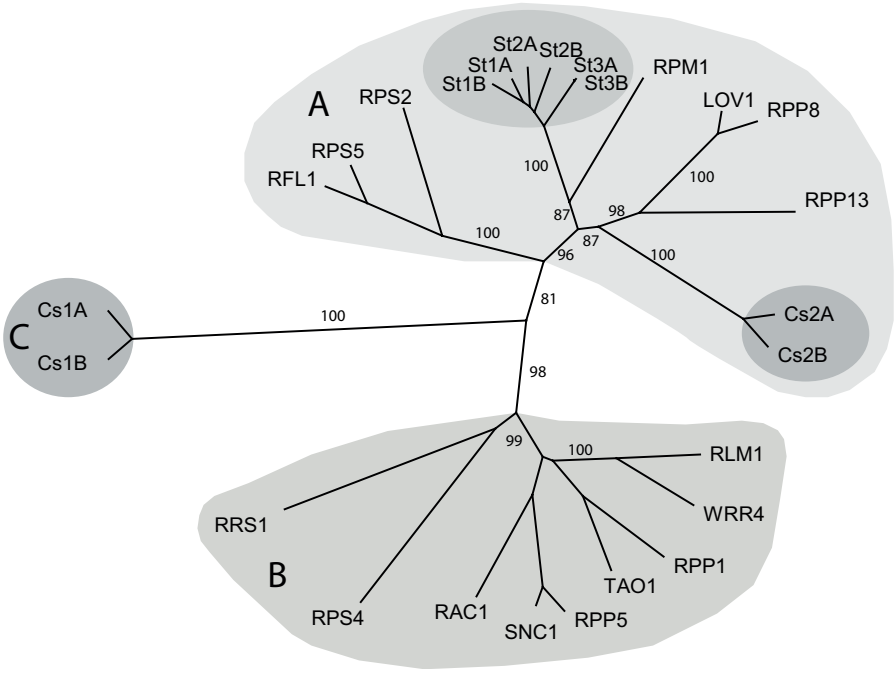


Figure 5. Unrooted maximum-likelihood phylogram inferred from nucleotide binding (NB) and leucine rich repeat (LRR) domains, of four resistance proteins to *C. sublineolum* (Cs) and six resistance proteins to *S. turcica* (St) in sorghum, compared with Arabidopsis NB-LRR resistance proteins with known function. LR-ELW values above 75% are shown. Labelling is as follows: (A) NB-LRR resistance proteins with NB-LRR proteins with a coiled-coil (CC) domain at the N-terminal end. (B) NB-LRR resistance proteins with a Toll/Interleukin-1 receptor (TIR) at the N-terminal end. (C) Two of the sorghum proteins did not clustering with any Arabidopsis protein candidate.

3.4.2 Virus induced gene silencing in sorghum

Complementation via transformation in sorghum is a laborious task therefore we used virus induced gene silencing (VIGs) to assess function of the *St*-genes. The Brome mosaic virus (BMV) had been previously used to silence genes in maize however it has never been reportedly used in sorghum (Ding et al., 2006). In the published study by Ding and co-workers an intermediate step after *in vitro* transcription involving barley was used in order to allow the virus to replicate before it was inoculated onto maize. We found that VIGs worked without this step through direct rub-inoculation of in vitro transcripts onto sorghum.

The transient nature of the VIGs procedure can be problematic, thus the system was first studied using a section of the *phytoene desaturase* (*PDS*) gene inserted to BMV p3 plasmid (Burch-Smith et al., 2006) enabling us to follow development of gene silencing more closely. Plants inoculated with the p3-PDS transcript had a white streaky phenotype indicative of PDS silencing. Silencing began in the second or third leaf above the inoculation site and continued to the third or fourth leaf to a lesser extent. Based on this experience, silencing of *St* genes was found to be successful. To determine if silencing was taking place the second leaf above the inoculation site RNA was collected and extracted. Real time qPCR confirmed a down-regulation of the target genes in most of the samples. We could conclude that the silencing of the *St* genes compromised resistance in the resistant sorghum GA06/18 genotype. Interestingly, the Sila variety used as a susceptible control also showed larger and more numerous lesions when the *St* genes were silenced compared to non-VIGS inoculated plants. Since inoculation with empty vector yielded no such response, we interpret the results as additional gene targeting in this particular plant material and not an effect of the virus infection.

3.5 Identifying resistant maize lines

We tested resistance and susceptibility of 13 CIMMYT maize lines from Kenya when challenged with two virulent maize *S. turcica* strains. One derived from a Ugandan maize leaf sample, MBRA14 and the other, a reference isolate from the USA, 18 (*MAT1-2*) kindly provided by M Carson, USDA-ARS Cereal Disease Lab, Univ. of Minnesota, USA. Inoculations of plants were carried out following the methods used in **II**. Two lines with stock no D61-3 and D61-4 showed a very high resistance response with no sign of spore colonization on the leaves. Two additional lines, D61-1 and D61-15 did respond with small chlorotic lesions but no sporulation was seen. The additional seven lines (D61-2, D61-8, D61-9, D61-12, D61-14, D61-20, D61-21) showed various degrees of susceptibility, and two lines (D61-7, D61-19) failed to germinate. No difference was observed between the two different fungal isolates used. This assessment was done 2008 and the result immediately transferred to the breeders in Kenya for implementation in their pedigrees.

3.6 Evaluating natural variation in Arabidopsis to *S. turcica*

Early in this thesis work we screened selected Arabidopsis accessions and mutant lines carrying mutations in known resistance genes in our collection

(>40 genotypes) against the highly virulent *S. turcica* isolate MBRA14 derived from infected maize leaves collected in Uganda. Four-week old Arabidopsis seedlings were wound-inoculated using 5ul of 75,000 spores/ml and incubated in 100% humidity for one week. Sorbo and An-1 showed mildly susceptible phenotype with small necrotic tissue around infection sites. RILs from a cross between Sorbo and the resistant Gy-0 have been previously produced (O'Neill et al., 2003). Further work on this material will be done elsewhere. Interestingly, the *lms1* mutant, in L*er* background (Bohman et al., 2004) was found to be very susceptible to *S. turcica*. *LMS1* codes for a lipid phosphate phosphatase localized to plastids (Oide et al., 2011). The *lms1* mutation involves sulfoquinovosyldiacylglycerol upon pathogen attack, resulting in over-activation of SA signalling, which collaborates with a yet unidentified signal to trigger the *lms1* disease susceptibility to both adapted and non-adapted pathogens.

4 Conclusions

These are the main conclusions from the work presented in this thesis:

- Mating type genes from *S. turcica* were cloned and sequenced and their distribution in 30 Ugandan districts on maize and sorghum mapped.

- *S. turcica* strains from sorghum can infect maize and new fungal races found.

- Resistance resources in Sorghum germplasm identified.

- The extant α1 box genes may originate from an ancestral HMG gene.

- Comparative genomics generated sets of candidate proteins of importance for virulence of two maize fungal pathogens.

- CC-NB-LRR encoding genes identified that confer resistance to *S. turcica*.

5 Future perspectives

This work started from near zero knowledge on the sorghum - *S. turcica* pathosystem and many difficulties and challenges have been overcome. Several already initiated studies will be completed in near future. The following areas however need further exploration:

It would be essential to collect *S. turcica* isolates from sorghum and test a larger set against maize lines to evaluate the reciprocal infection and generate deeper understanding of the cross-infection situation. It would be valuable to generate genetic data on the *St* genes using the mapping populations that are in progress and more information is needed on the actual gene function. To couple such knowledge with effectors in *S. turcica* is challenging. Genome analysis of *S. turcica* could hopefully reveal potential novel gene families of importance for virulence, generating a starting point for functional studies. The bulk of the sorghum gene pool, located in East Africa, is largely uncharacterised. Re-sequencing selected East African sorghum genotypes would give us valuable data on the genetic variation present in this important crop.

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