CHARACTERIZATION OF THE MAT LOCUS GENES IN THE FUNGAL PLANT PATHOGEN *Sclerotinia sclerotiorum* (Lib.) de Bary

By

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To my family

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bp	Base pair
C	Celsius
CWDE	Cell wall degrading enzymes
DAPI	4', 6-diamino-2-phenylindole
DNA	Deoxyribonucleic acid
EST	Expressed sequence tag
H3	Histone protein three
HMG	High mobility group
Kb	Kilobase
MAT	Mating type gene
mat	Mating type gene knockout
Mb	Megabase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
RNA	Ribonucleic acid
µg	Microgram
UTR	Untranslated region
UV	Ultra violet
WT	Wild type

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Sclerotinia sclerotiorum (Lib.) de Bary is an omnivorous, polyphagus, phytopathogenic fungus that relies on the completion of the sexual cycle to initiate new disease cycles. The sexual cycle is characterized by the development of apothecia that forcibly discharge ascospores for local and, under suitable conditions, long distance dissemination. A strategy for understanding the regulation of apothecial multicellular development was pursued through functional characterization of the mating type genes in *S. sclerotiorum*. These genes are hypothesized to encode master regulatory proteins required for aspects of sexual development ranging from fertilization through fertile fruiting body development. Experimentally, gene deletion strategies were performed to create loss-of-function mutants in the two conserved "core" mating type genes common to most ascomycete fungi as well for two lineage-specific genes found only in *S. sclerotiorum* and closely related fungi. The *mat1-1-1*, *mat1-1-5* and *mat1-2-1* mutants are able to form ascogonia but are blocked in all aspects of apothecia development. These mutants also exhibit defects in secondary sexual characters including lower numbers of spermatia and altered rates of mycelial growth. The *mat1-2-4* mutants

exhibited delayed apothecia production with altered disc morphogenesis and ascospore production. They too produce lower numbers of spermatia and exhibit a faster hyphal growth rate. All four *MAT* gene mutants showed alterations in the expression of putative pheromone precursor (*PPG-1*) and pheromone receptor (*PreA, PreB*) genes. Our findings demonstrate that *MAT* genes are involved in both sexual fertility, gene regulation and development in *S. sclerotiorum.*

CHAPTER 1
LITERATURE REVIEW

Introduction to Ascomycotal Life Cycle

Defining Characters

The phylum Ascomycota or sac fungi, of which *S. sclerotiorum* is a member, contains over 32,000 species and 3,400 genera (Kirk *et al*., 2001). The defining morphological characteristic of the group is the ascus, a sac-like structure formed during the sexual life cycle in which the non-motile sexual spores, ascospores, are formed. Ascomycetes may be saprotrophs, necrotrophic or biotrophic parasites of plants and animals, and mutualistic symbionts (Webster and Weber, 2007). Ascomycete environmental niches are equally as diverse ranging from the soil, above and below ground parts of plants, freshwater and saltwater environments (Webster and Weber, 2007).

The vegetative growth habits of the Ascomycota separates the members into two distinct subphyla: (1) the unicellular Saccharomycotina which reproduce by budding or fission and (2) the mycelial Pezizomycotina with septate hyphae (Moore, 1998; Pöggler *et al*., 2006; Webster and Weber, 2007). Some species in both groups have the ability to switch between the two states and are described as dimorphic (Moore, 1998; Webster and Weber, 2007).

A unifying feature of all members of the Ascomycota is the limitation of the dikaryotic state to specialized hyphae which form diploid cells that proceed directly into meiosis. The asexual spore or conidial states of Ascomycetes are highly variable ranging from complex multicellular spores and supporting tissues to simple fragmentation or intercalary modification of hyphae, to a complete lack of sporulation. Most conidial spores form on specialized hyphae called conidiophores and these asexual spores are more resilient than the hyphae state when in

a suspended state of growth (Adams, 1995; Ebbole, 1996). There are many different classifications of conidiogenesis filling a spectrum from blastic to thallic methods but most share some common themes (Cole, 1986). The conidia are formed from a conidiogenous cell and usually form a stalk called a conidiophore (Webster and Weber, 2007). Conidia have different morphological features such as germ pores and slits, ornamentations, and origination of spore walls (Read and Beckett, 1996). The condiophores may bundle together to form different types of conidioma. Synnemata or coremia are parallel bundles of which there are simple, compound or parallel, conidiophores may also develop from a cushion or stroma, and sporodochium is a cushion-like conidiomata bearing a layer of short conidiophores (Seifert, 1985; Webster and Weber, 2007). A relationship between the asexual spore and sexual spore production has been described in *A. nidulans* wherein an oxylipin driven mechanism helps regulate the two developmental pathways (Tsitsigiannis *et al*., 2004).

Individual hyphal cells of ascomycete fungi are generally haploid, multinucleate and homokaryotic, containing genetically identical nuclei. Vegetative hyphal fusion, governed by vegetative compatibility loci (Leslie, 1993; Saupe, 2000) or spontaneous mutations can give rise to heterokaryotic hyphae, containing genetically unique nuclei. Parasexual cycles are known to function in some ascomycete fungi which can generate genetic variation via mitotic recombination (Papa, 1973; Leslie, 1993). Ascomycota hyphae are delimited by cross walls known as septa which contain a single pore that allows cytoplasm and membrane-bound organelles to flow throughout the mycelia and use Woronin bodies, globose or hexagonal proteinaceous crystals, to block the pore (Moore, 1998; Webster and Weber, 2007). These Woronin bodies act as gate keepers of the septa and are unique to the Ascomycota.

Sex in the Ascomycota

Mycelial Ascomycetes typically form fruiting bodies called ascomata or ascocarps (Pöggler *et al*, 2006; Webster and Weber, 2007). These take on many different morphologies including the perithecium, a flask-like structure; the cleistothecium, a completely enclosed structure; the apothecium, a cup-like structure; and the pseudothecium ,forming locules containing the asci within an ascostroma (Webster and Weber, 2007). These structures develop from haploid hyphal tissue.

Meiosis in Ascomycetes occurs in the sac-like structure, the ascus, which is the namesake character only produced during the sexual life cycle and distinguishes the phylum. The sexual life cycle of Ascomycetes may be homothallic (self-compatible) where individual isolates are all of the same mating type or heterothallic (self-incompatible) where individual isolates exists as one of two mating types. Heterothallic and some forms of homothallic reproduction are preceded by plasmogamy, the fusion of two different gametangia, and has three different forms: gametangio-gametangiogamy specifying fusion between an antheridium and an ascogonium, gameto-gametangiogamy specifying fusion between a spermatium and an ascogonium, and somatogamy specifying fusion between undifferentiated hyphae (Webster and Weber, 2007). A third form of reproduction known as pseudohomothallism or secondary homothallism gives the appearance of self-compatible but occurs when two nuclei of differing mating types are incorporated in ascospores and resulting hyphae (Debuchy and Turgeon, 2006; Whittle and Johannesson, 2011). Regardless of mechanism, the first step of sexual reproduction in mycelial fungi occurs when compatible nuclei are combined in the same cell.

In gameto-gametangiogamy and gametangio-gametangiogamy fusion, the gametangia are morphologically distinct and in some species the female ascogonium is surrounded by sterile hyphae to form a pre-fruiting body which then bears a specialized receptive hypha called the trichogyne (Pöggler *et al*., 2006). The male gamete may be formed on the mycelium or in specialized structures called spermogonia and defined as either uninucleate spermatia, microconidia, or multinucleate macroconidia. The female and male gametangia then fuse and a fertilizing nucleus from the male gametangia migrates to the ascogonium and this female organ undergoes a series of mitotic divisions creating a multi-nucleate aggregate of cells (Bistis, 1981; Debuchy and Turgeon, 2006; Pöggler *et al*., 2006).

Following fertilization nuclei of opposite mating types migrate and form heterokaryons and ultimately a dikaryon within the ascogenous hyphae maintaining a 1:1 ratio of parental nuclei (Debuchy and Turgeon, 2006; Pöggler *et al*., 2006). The tip of the ascogenous hyphae differentiates into a crozier made up of two uninucleate and one dikaryotic cell where the nuclei fuse and meiosis immediately follows (Saupe, 2000; Debuchy and Turgeon, 2006; Pöggler *et al*., 2006). The cell elongates forming the ascus and a post-meiotic mitotic division occurs after which the ascospores are delineated within the ascus (Saupe, 2000; Debuchy and Turgeon, 2006; Pöggler *et al*., 2006).

The post meiotic divisions determine the number of ascospores within the ascus and varies in a species-specific manner yet the most common number of ascospores is eight (Read and Beckett, 1996; Raju and Perkins, 2000; Pöggler *et al*., 2006; Webster and Weber, 2007). The timing of this process varies but the steps are mainly conserved throughout the Ascomycetes (Read and Beckett, 1996). Ascospores between species and sometimes within a

single ascus may be morphologically distinct having different sizes and shapes (Read and Beckett, 1996). At the tip of the ascus there may be a cap-like structure called an operculum, these are operculate fungi, conversely inoperculate fungi have a pore and make up the majority of the Ascomycetes (Read and Beckett, 1996) including *S. sclerotiorum*. The ascus is surrounded by a protective layer of filamentous tissue which includes paraphyses, that grows from different vectors along the ascus; this sterile tissue makes up the hamathecium (Read and Beckett, 1996). The asci are arranged scattered or in a defined pattern in the area of the fruiting structure called the hymenium. The four major types of fruiting bodies described before act as a platform from which ascospores are forcibly discharged or not (as in the cleitothecial/protunicate species).

The release of the ascospores is either passive or active in nature with a polarized ascus shape correlating to the active mechanism (Read and Beckett, 1996). The polarized shape allows for hydrostatic pressure buildup by solute production and water uptake necessary to forcibly discharge spores through the apical pore (Read and Beckett, 1996; Fischer *et al*., 2004). The release of spores may be simultaneous among asci of a fruiting body or singular (Meredith, 1973). Passive release relies on other mechanisms such as desiccation, wind, rain, dew (Meredith, 1973; Read and Beckett, 1996; Fisher *et al*., 2004). Ascospores delivered to a suitable environment germinate forming hypahe and restart the life cycle of the fungus.

Introduction to *Sclerotinia sclerotiorum* (Lib.) de Bary

Sclerotinia sclerotiorum (Lib.) de Bary is an omnivorous, necrotrophic fungal pathogen that affects a wide range of plants. More than 60 names have been used to refer to diseases caused by this fungal pathogen (Purdy, 1979), with many of the more common names including rot and/or mold in the description reflecting the symptoms created. *S. sclerotiorum* has a wide host range of over 400 crops and a global distribution of which contain many important agricultural crops: soybeans, canola, sunflowers, , lettuce, potatoes, tomatoes and (Boland and Hall, 1994). Based on estimated yield losses from 1996 through 2009, it was estimated that Sclerotinia stem rot in USA soybeans caused yield losses of 10 million bushels (270 million kg) in seven of the 14 years. (Wrather and Koenning 2009, Koenning and Wrather 2010, Peltier *et al*., 2012). In Germany potato yield loss was as high as 30% in some areas (Quentin, 2004) and canola yield losses vary from 11.1 – 75% across the world (Morrall and Dueck, 1982; Shivpuri and Ghasolia, 2009; Peltier *et al*., 2012). According to the reports of the National Sclerotinia Initiative in 2005, collective annual losses in the United States caused by *S. sclerotiorum* for canola, soybeans, dry beans, sunflowers and pulse crops have been as high as 250-280 million US dollars in a single year (Anonymous, 2005a; Anonymous, 2005b), making it one of the most devastating and important plant pathogens of agriculture crops.

Taxonomy

The Sclerotiniaceae is a family of fungi within the order Helotiales in the phylum Ascomycota. The *Sclerotiniaceae* family is defined by stromatal-forming inoperculate discomycetes that produce stipitate apothecia containing ellipsoid ascospores with globose spermatia and produce tuberoid sclerotia that do not incorporate host tissue within the

sclerotial medulla, develop an apothecial ectal excipulum composed of globose cells and lack a disseminative conidial state (Whetzel, 1945; Kohn, 1979; Bolton *et al*., 2005). This family has been modified multiple times currently including 33 genera. The Sclerotinia genus contains three economically important plant pathogens *S. sclerotiorum*, *S. minor* Jagger and *S. trifoliorum* Eriks (Dumont and Korf, 1971; Korf 1973; Holst-Jensen *et al.*, 1997; Willetts, 1997). The earliest description of *S. sclerotiorum* came from Libert (1837), who placed it within the Pizizomycetes as *Peziza sclerotiorum*. Subsequently, the taxonomic placement of this species has been revised several times (Wakefield, 1924). The current name, *Sclerotinia sclerotiorum* (Lib.) de Bary which was first used by Heinrich Anton de Bary in 1884 was accepted as the conserved name in 1979 (Kohn, 1979) and approved by the International Botanical Congress in 1981.

Defining Characters

S. sclerotiorum produces simple, septate hyphae containing membrane bound vesicles, mitochondria, lipid bodies, rough and smooth endoplasmic reticulum and ribosomes (Maxwell *et al*., 1972). Hyphae are hyaline, septate, branched, multinucleate, and look white to tan in culture and *in planta* (Bolton *et al*., 2006). Vegetative hyphae are haploid and the number of nuclei vary per cell (Maxwell *et al*., 1972; Willets and Wong, 1979). Spermatia produced in chains are uninucleate from phialides on branched microconidiophores (Willets and Wong, 1979). Spermatia have been observed on aerial mycelia in culture, on the surface of sclerotia, and on the apothecial hymenium (Le Tourneau, 1970; Kohn, 1979). The function of these spermatia in *S. sclerotiorum* has not been confirmed as the species is homothallic and fertilization by spermatia has not been resolved (Kohn, 1979).

Sclerotia

S. sclerotiorum produces a resting phase stroma described as a sclerotium and may remain dormant and viable for years in soil which adds to the difficulty of controlling this plant pathogen (Adams and Ayers, 1979, Bourdot *et al.*, 2001). Sclerotial development has been defined to encompass three stages: 1) Initiation (aggregation of hyphae to form a white mass called a sclerotial initial); 2) Development (hyphal growth and further aggregation to increase size); and 3) Maturation (surface delimitation, melanin deposition in peripheral rind cells and internal consolidation). Sclerotia development involves a complex collaboration of environmental and nutritional factors but generally sclerotia are produced after a nutritionally competent mycelial growth encounters a physically limiting environment (Christias and Lockwood, 1973; Le Tourneau, 1979; Willets and Wong, 1980; Hausner and Reid, 1999; Rollins and Dickman, 2001; Erental *et al.*, 2008; Liang *et al*., 2010).

Sclerotia are hyphal aggregates that are composed of two distinct layers, the medulla or inner mass, and the rind which is darkened by melanin and protects from microbial degradation in many fungi (Bell and Wheeler, 1986; Willetts, 1997; Henson *et al*., 1999). The medulla is imbedded in a fibrillar matrix composed of carbohydrates and proteins (Le Tourneau, 1979). This is a character of the Sclerotiniaceae although the shape and size of the sclerotia varies and even within the species depending on the plant host, nutritional conditions and physical environment (Bolton *et al*., 2006). It is an over-wintering structure used to survive harsh environmental conditions for up to 8 years in soil and to disperse the forcably discharged ascospores from the apothecia produced from sclerotia (Adams and Ayers, 1979; Willetts and Wong, 1980). Hyphae may also emerge from the sclerotia depending on the conditions and

directly infect host tissue specifically lettuce, carrots and sunflower (Le Tourneau, 1979; Bardin and Huang, 2001).

Sexual Fruiting Body Production

Prior to carpogenic apothecia development, non-propagative spermatia are formed from the hyphae associated with sclerotia and then hypothesized fertilization occurs (Kohn, 1979). Apothecial development is not time dependent but relies on an external signal or signal combinations of soil temperature, moisture and sclerotial stratification (Morrall, 1977; Willets and Wong, 1980; Mylchreest and Wheeler, 1987; Bardin and Huang, 2001). Apothecial development begins with an etiolated stipe which is photoresponsive, growing towards or away from different spectrums of light when present, and then proceeds to form an expanding circular concave receptacle (3-6 mm diameter) containing the hymenial layer which is tan in color (Thanning and Nilsson, 2000; Bolton *et al.*, 2006). The stipe initials may form in complete dark, the development of the apothecial cup is light dependent which directs the development of the apothecia above ground when the sclerotia are under soil or debris (Willets and Wong, 1980; Thanning and Nilsson, 2000). Normal apothecia production is triggered in wavelengths of light between 276 and 319 nm and the stipe phototropism was observed under light wavelengths not responsible for apothecial development indicating two photosystems (Thanning and Nilsson, 2000). Multiple apothecia may develop from a single sclerotium. Histological studies by Kosasih and Willets (1975) separated the disc into four parts: 1) ectal exipulum 2) medullary excipulum 3) hymenium 4) subhumenium. The hymenial layer contains rows of asci which are cylindrical sac-like zygote cells that contain eight hyaline ellipsoid binucleate ascospores (4-6 x 9-14 µm) per ascus (Kohn 1979).

Ascospores are formed from selfing (Codron, 1974) or outcrossing by heterokaryon formation and recombination (Ford *et al*., 1995) which are forcibly discharged under optimum conditions for more than 10 days in the field at a rate of 1600 spores/h (Clarkson *et al*., 2003). Often *S. sclerotiorum* releases ascospores in mass by puffing when disturbed by changes in relative humidity or physical interactions (Hartill and Underhill, 1976). These ascospores may be distributed over long distances but the vast majority of them land in the field from where they were produced (Li *et al*., 1994; Wegulo *et al*., 2000). If landing on dead or dying tissue, the ascospores will germinate producing infectious hyphae which may colonize the rest of the healthy plant completing the life cycle.

Epidemiology, Host Range and Control of *S. sclerotiorum*

The ability to survive for long periods of time in a dormant stage and then disperse ascospores over long distances makes the sexual cycle a key factor for the epidemiology of *S. sclerotiorum* (Fig 1-1). When soils are shaded, moist and cool (4 –16°C), sclerotia within the top five centimeters of the soil profile can germinate to produce apothecia (Adams and Ayers 1979, Grau and Hartman 1999, Wu and Subbarao 2008). Infection is also favored by cool to moderately warm temperatures and a higher moisture content (Workneh and Yang, 2000).

A single apothecium may produce 2.3×10^8 ascospores and multiple apothecia may be produced from a single sclerotium (Schwartz and Steadman, 1978). Infection by *S. sclerotiorum* occurs typically after a saprophytic growth stage, the initial infection is commonly by the landing of ascospores, which have a sticky mucilage, on the above ground senescent or dead plant tissue of the host (Bolton *et al*., 2006; Grau and Hartman 1999). A white mycelia and compound appressoria are produced from the initial colonization and are able to penetrate the

cuticle of a healthy host plant using cell wall degrading enzymes, the toxic metabolite oxalic acid and mechanical force via appressoria unless a natural opening or wound is present (Lumsden and Dow, 1973; Lumsden, 1979).

Host cells are killed and the middle lamella destroyed in advance of fungal colonization (de Bary, 1884). Anton de Bary (1884) was also the first to describe the oxalic acid production of *S. sclerotiorum* and determined that it solely was not responsible for symptom development, but other hypothesized factors were also required. Since then a multitude of enzymatic activities produced by *S. sclerotiorum* during infection have been characterized. Pectinolytic enzymes, polygalacturonases, endopolygalacturonases, exopolygalacturonases, proteases, cellulases, and glucoamylases are all produced by *S. sclerotiorum* during infection (Riou *et al*., 1991; Alghisi and Favaron, 1995; Fraissinet and Fevre, 1996; Poussereau *et al*., 2001; Bolton *et al*., 2006). Oxalic acid production is increased during infection which creates a low pH environment (Bateman, 1964). Bateman also showed that treatment with oxalic acid alone caused injury and host tissue bleaching while treatment with oxalic acid and an extracted enzyme mixture produced tissue damage and hypocotyl collapse. This study determined that oxalic acid and polygalacturonase activity functioned synergistically to cause disease symptoms.

Later a dynamic among oxalic acid production, environment pH acidification, multiple enzyme activities, enzyme gene regulation, carbon food source and ambient pH-signal transduction was shown to play a significant role in *Sclerotinia* disease development and plant responses respectively (Vega *et al.*, 1970; Marciano *et al*., 1983; Godoy *et al.*, 1990; Dutton and Evans, 1996; Cessna *et al*., 2000; Rollins, 2003; Guimareaes and Stoltz, 2004). *S. sclerotiorum* is also able to secret several different molecular forms or isozymes of polygalacturonases that

exhibit similar enzymatic activities which target the plant cell wall component pectin and has been hypothesized to allow for *S. sclerotiorum's* large host range (Bolton *et al*., 2006). Similar enzymatic arsenals are found in the closely related plant pathogen *Botrytis cinerea* where five endopolygalaturonases were recently found in this fungus that display different biochemical properties and necrotizing activity on different hosts (Kars *et al*., 2005, Bolton *et al*., 2006). Altered pH levels also provides a favorable environment for sclerotia development (Maxwell and Lumsden, 1970; Rollins and Dickman, 2001). Necrotrophic colonization of host tissues is followed by a saprotrophic growth and the development of sclerotia to complete the life cycle.

Three mechanisms by which oxalic acid aids in pathogenicity having been proposed (Bolton *et al*., 2006): 1) Provide a favorable acidic pH environment for optimum CWDE activities (Favaron *et al*., 2004) or pH-regulated genes necessary for the pathogenesis (Rollins, 2003; Kim *et al*., 2007) 2) Suppress oxidative burst initiating the plant defense response (Cessna *et al*., 2000) 3) Facilitate hyphal penetration by guard cell deregulation to induce stomatal opening (Guimarães and Stotz, 2004). Oxalic acid appears to be a primary physiological determinant of pathogenicity and shows an important regulatory role as well. Full virulence of *S. sclerotiorum* relies on the correct regulation of genes and timing in response to the ambient pH and nutrient source. Current evidence for this is that *Pac1* activating mutants can constitutively accumulate oxalic acid under both low and high pH but exhibit an attenuated virulence phenotype (Kim *et al*., 2007).

Disease Management

Sclerotinia disease management has a variety of means: Chemical and biological control, transgenic and naturally derived resistance, and agricultural practices. Chemical control has been successful with Remedier Phenylthiourea, Difenoconazole, Actigard, Flint, Rovral, Switch, Topsin, Botran and Quadris (Kuckarek, 2003; Gengotti *et al*., 2011). Biological control strategies involve *Coniothyrium minitans* and *Sporidesmium sclerotivorum*, two mycoparasites which degrade sclerotia (Ayers and Adams, 1991) and antagonistic *Pseudomonas spp*. (DF-41 and PA-23) has shown success in greenhouse and field plots (del Rio *et al*., 2002; Li *et al*., 2006; Partridge *et al*., 2006). Biological control agents which have been approved for use on *S. sclerotiorum* and *S. minor* include: Intercept WG and Contans WG. Variations and alterations to plant canopy morphology has shown to decrease disease severity as well as agricultural practices such as: field flooding, crop rotation, control of alternate weedy hosts, removal of crop residues post-harvest, deep plowing of fields, maintaining a well-spaced plant density, avoiding excessive irrigation and selecting crop varieties with open canopies that may decrease disease incidence (Moore, 1949; Steadman, 1979; Savchuk and Fernando, 2004; Jurke and Fernando, 2008; Pohronezny and Purdy, 1981).

Partial inherited resistance to *Sclerotinia* diseases has been observed in a few agricultural crops but complete inherited resistance has not been observed in any crop to date (Grau *et al*., 1982; Boland and Hall, 1987; Nelson *et al*., 1991; Kim and Diers, 2000; Rousseau *et al*., 2004; Diers *et al*. 2006; Yin *et al*., 2010). Evaluation of this resistance has proven difficult and dependent heavily on environmental conditions (Rousseau *et al*., 2004; Diers *et al*., 2006; Bastien *et al*, 2012; Ebrahimi *et al*., 2013). Transgenic resistance focusing on the degradation of

oxalic acid by oxalate oxidase and other enzymes has been successful in sunflower, soybean and peanut (Donaldson, *et al*. 2001; Hu *et al*., 2003; Kersarwani *et al*., 2000; Livingstone *et al*., 2005; Cunha *et al*., 2010). The introduction of lipid transfer proteins and polygalacturonase inhibitor proteins has also shown to increase resistance to *S. sclerotiorum* infection (Bashi *et al*., 2013; Fan *et al.*, 2013).

To date there is no completely successful method of control for *S. sclerotiorum* and most of the genetically derived methods of control center around the production of oxalate and interruption of the life cycle at various points. There are a large number of targets both from chemicals produced by the pathogen and genes regulating the life cycle which may be exploited in the future for a more comprehensive and successful mechanism of control.

Genome Sequence

The genome of *S. sclerotiorum* was released in 2005. The project was carried out by the Broad Institute for Biomedical Research in partnership with academic labs at the University of Florida (Dr. Jeffrey A. Rollins), the University of Nebraska (Dr. Martin B. Dickman) and the University of Toronto (Dr. Linda M. Kohn) (*S. sclerotiorum* Sequencing Project: Broad Institute of Harvard and MIT. http://www.broad.mit.edu). Genome sequencing information has been used to promote identification of genes for genetic and functional studies as well as serve as a template for comparative genomics. Sequence information may also aid in the identification of potential anti-fungal targets and facilitate the elucidation of signal transduction pathways.

Whole genome shotgun sequencing provided an average of 8X coverage. It is estimated that the *S. sclerotiorum* genome is 38Mb in size with 14,522 genes where 41.3% of the genome contains coding sequence. Automated calling of genome sequence data was released in late

2005 and was done by analyzing the genome sequence data via FGENESH and GeneID programs combined with the analysis of expressed sequence tags (ESTs). Analysis of genome data revealed that there is ~1 gene for every 2.6 kb of sequence with an average intron length of 140 bp. The shortest intron designated was 24 bp and the longest was 1494 bp. The average exon length was estimated at 389 bp with the longest being 17,212 bp and the shortest as 1 bp (*S. sclerotiorum* Sequencing Project. Broad Institute of Harvard and MIT. http://www.broad.mit.edu).

Ascomycota Mating Type Genes (*MAT*)

In fungi mating type is used to define individuals that are compatible for mating and was discovered and defined by Blakeslee (1904) in *Rhizopus*. There are two states defined by the mating type system: homothalism is the state of being self-fertile, and heterothallism is the state of being self-infertile. The mating type genes at loci of heterothallic species appear not to be evolutionarily related and the term idiomorph rather than allele has been put forth to describe the very dissimilar sequences at the opposite mating type loci (Metzenberg and Glass, 1990; Souza *et al*., 2003; Debuchy and Turgeon, 2006). These mating type genes encode transcription factors in mycelial ascomycetes (Souza *et al*., 2003). Ascomycetes have a bipolar system with two mating type determinants and one mating type locus.

The core mating type genes in filamentous Ascomycetes share functional sequences that encode high mobility group (HMG) and α-box proteins, both of which are transcription factors (Turgeon *et al*., 1993; Debuchy and Turgeon, 2006). The MAT1-1 idiomorph is defined by the *MAT1-1-1* gene that encodes the α-box protein, and MAT1-2 idiomorph is defined by the

MAT1-2-1 gene which encodes the HMG protein. These two genes make up the conserved genes of the two mating types in the filamentous Ascomycetes, subsequent novel genes found at each locus are labeled sequentially, e.g., *MAT1-1-2* encoding a HPG protein and *MAT1-1-3* encoding for another HMG- protein at the *MAT1-1* locus in *Neurospora crassa* (Ferreira *et al*., 1996). In heterothallic species one isolate will carry only one of the *MAT* idiomorphs and a sexually compatible isolate will carry the other, specifically one *MAT1-1-1* (alpha 1) and one *MAT1-2-1* (HMG box) gene (Debuchy and Turgeon, 2006; Dubuchy *et al*., 2010). In homothallic species there is only one version of the MAT locus often referred to as a fused idiomorph in that it usually carries *MAT1-1 and Mat1-2* genes. The genus *Cochliobolus* contains both homothallic and heterothallic species and exchanging genes within these loci may convert one mating type system for another (Lu *et al*., 2011).

S. sclerotiorum is homothallic where the MAT1-1 and MAT1-2 idiomorphs are fused together in one locus (Amselem *et al*., 2011). There are four genes present at the *MAT* locus: *MAT1-1-1*, *MAT1-1-5*, *MAT1-2-1*, and *MAT1-2-4* (Amselem *et al*., 2011). *Botrytis cinerea*, a related member of the Sclerotiniaceae has the same two novel genes *MAT1-1-5* and *MAT1-2-4* with the first being present in other Leotiomycetes and the latter being found exclusively in *B. cinerea* and *S. sclerotiorum* (Amselem *et al*., 2011). *B cinerea* has these genes arranged in a heterothallic MAT arrangement where *MAT1-1* isolates have the *MAT1-1-1* and *MAT1-1-5* genes and *MAT1-2* isolates have *MAT1-2-1* and *MAT1-2-4* (Amselem *et al*., 2011). In preliminary reports of *B. cinerea MAT* gene functions, knockouts of the *MAT1-1-5* ORF are unable to develop an apothecial disc in the dikaryon (van Kan *et al*., 2010). In mutants of *B. cinerea* where the *MAT1-2-4* or *MAT1-1-5* gene is deleted and crossed with the wild type

isolate of the opposite mating type, stipe formation is successful but disc differentiation is interrupted (Terhem *et al*., 2011.)

Mating type switching from heterothallism to homothallism and homothallism to heterothallism is a rare occurrence in the filamentous Ascomycetes but a few instances of switching have been described (Mathieson, 1952; Uhm and Juji, 1983; Leslie et al., 1986; Faretra and Pollastro, 1996; Samuels and Lodge, 1996; Harrington and McNew, 1997). Recently, *S. sclerotiorum* has been shown to have a 3.6kb region which is inverted between meiotic generations and correlates with changes in *MAT* gene expression (Chitrampalam *et al.*, 2013). The authors of this study speculate that a similar inversion region may be involved in mating type switching in the filamentous ascomycetes *Chromocrea spinulosa*, *Sclerotinia trifoliorum* and in certain *Ceratocystis* species. The role of the *MAT* genes includes regulation of pheromone and pheromone receptor production that are involved in mating partner recognition, transcription factor production, heterokaryon incompatibility and possibly nuclear pairing post-fertilization (Bistis, 1981; Zickler *et al*., 1995; Arnaise *et al*., 1997; Debuchy, 1999; Shiu and Glass, 2000; Bobrowicz *et al*., 2002; Glass and Kaneko, 2003; Debuchy and Turgeon, 2006; Bidard *et al*., 2011; Whittle and Johannesson, 2011). Phenotypes associated with mating type switching involving ascospore size and appearance have been described in *S. trifoliorum* and *Chromocrea spinulosa* where large ascospores are self-fertile homothallic and smaller ascospores were self-sterile heterothallic but in this case the switching does not reverse (Harrington and McNew, 1997).

Genetic Approach to Studying *MAT* Gene Loci

The *MAT* locus in Ascomycetes is often associated with conserved flanking regions, specifically the *APN2* (DNA Lyase) and *SLA2* (Cytoskeleton Assembly Control) genes. Targeting these genes and exploring the intervening regions has proven effective in identifying *MAT* loci for individual species (Turgeon *et al*., 1993; Pöggler and Kuck, 2001; Casselton, 2002; Rydholm *et al*., 2007). Gene deletion or gene interruption has also been proven successful in determining the roles of the genes at the *MAT* locus giving detectable phenotypes focusing around various parts of the fungal life cycle as previously mentioned (Bistis, 1981; Zickler *et al*., 1995; Arnaise *et al*., 1997; Debuchy, 1999; Shiu and Glass, 2000; Bobrowicz *et al*., 2002; Glass and Kaneko, 2003; Pöggler *et al*., 2010; Bidard *et al*., 2011). A study of the interactions of the *MAT* gene mutants and their regulation of pheromone and pheromone receptor genes was done in *F. graminearum* (Zheng *et al*., 2013) and blast searches of the *S. sclerotiorum* genome for the genes characterized was performed. Three genes were identified: SS1G_04155.3 the putative pheromone precursor gene 1 (*PPG-1*), SS1G_10310.3 the putative pheromone receptor protein B (*PreB*) and SS1G_07464.3 the putative pheromone receptor protein A (*PreA*) and included for genetic expression analysis.

The functions of the genes found at the *MAT* locus of *S. sclerotiorum* have not been characterized to date. This study seeks to understand the function of these genes by creating loss of function mutants. Four genes have been selected for complete gene knockout: *MAT1-1-5* (SS1G_04003.3), *MAT1-1-1* (SS1G_04004.3), *MAT1-2-4* (SS1G_04005.3) and *MAT1-2-1* (SS1G_04006.3) (Fig 1-2). These genes have been described by Amselem *et al*., (2011) where they were shown to have high sequence similarity but different structural organization as in *B.*

cinerea. My hypothesis is that through gene deletion and phenotypic characterization, functions involving sexual fruiting body development and other aspects of the sexual life cycle of *S. sclerotiorum* will be defined for the mating type genes.

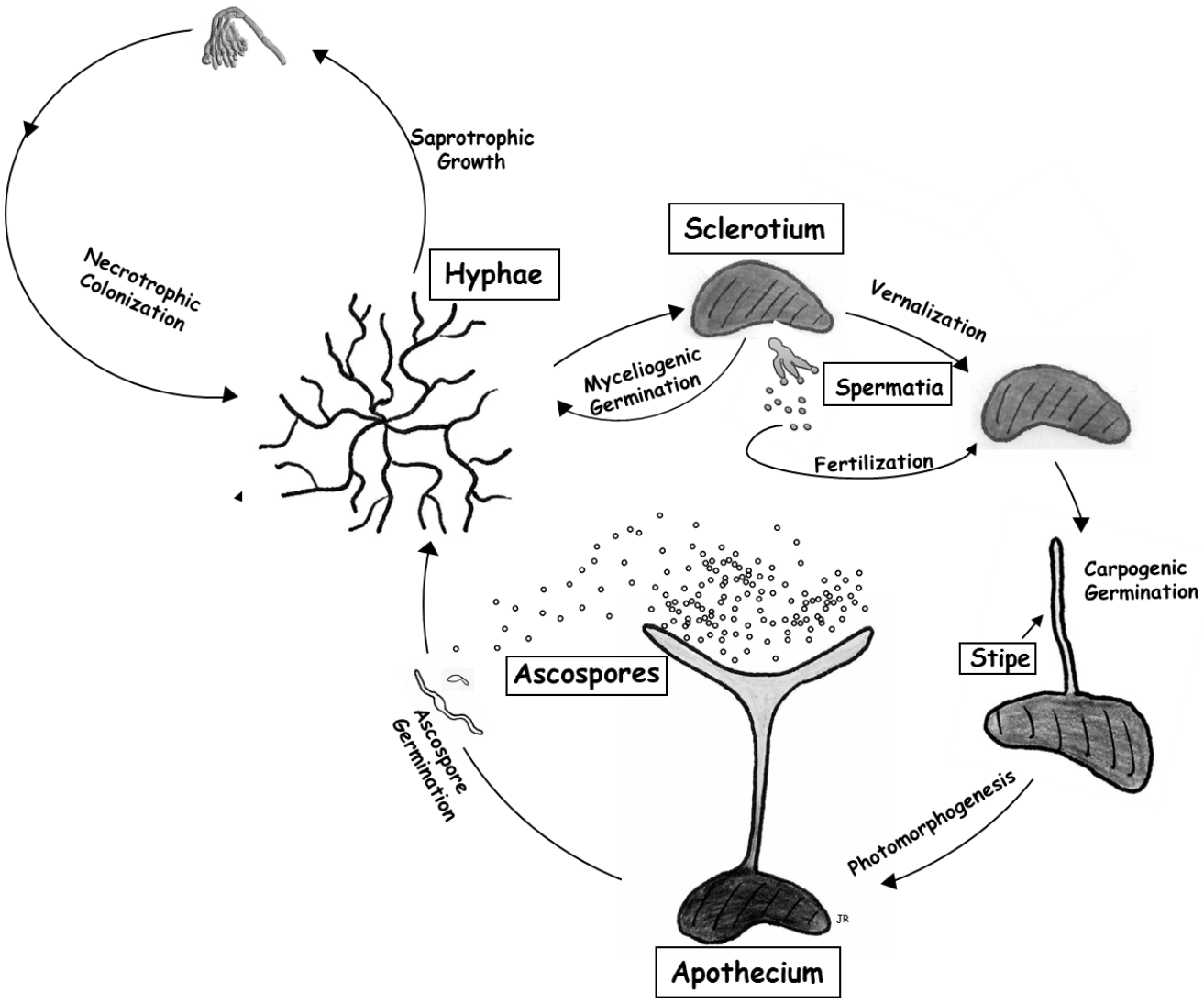


Figure 1-1. The life cycle of *S. sclerotiorum*.

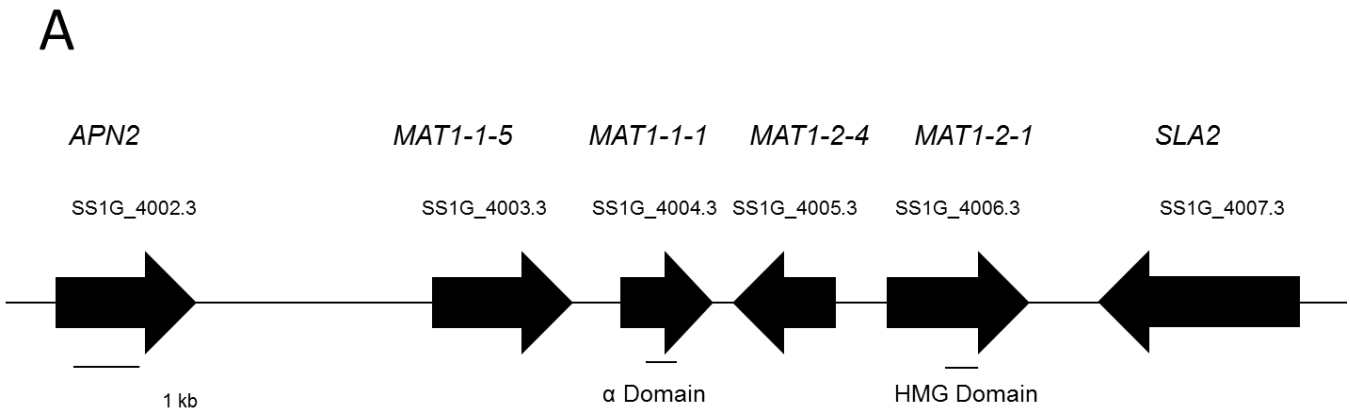


Figure 1-2. The *MAT* locus of *S. sclerotiorum*. The orientation of all four *MAT* locus genes, location of the alpha and HMG domains and their Broad Institute *S. sclerotiorum* database gene calls.

CHAPTER 2

CHARACTERIZATION OF THE *MAT* LOCUS GENES IN THE FUNGAL PLANT PATHOGEN *Sclerotinia sclerotiorum* (Lib.) de Bary

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is an omnivorous, polyphagus, phytopathogenic fungus that has a wide host range of over 400 plant species (Boland and Hall, 1994). Included within the host range are many important agricultural crops: soybeans, canola, sunflowers, lettuce, potatoes, tomatoes and peanuts. The disease cycle relies on the completion of the sexual cycle characterized by the development of apothecia that forcibly discharge ascospores for local and, under suitable conditions, long distance dissemination. A single apothecia may produce in excess of 2×10^8 ascospores and multiple apothecia may be produced from a single sclerotium (Schwartz and Steadman, 1978). Infection by *S. sclerotiorum* occurs typically after a saprophytic stage, the initial infection is commonly achieved by the landing of ascospores, which have a sticky mucilage, on above ground senescent or dead plant tissue of the host (Grau and Hartman 1999; Bolton *et al*., 2006). A white mycelium is produced from the germinated ascospores, commonly on flower blossoms, and hyphae are able to penetrate the cuticle of the host plant mediated by cell wall degrading enzymes, oxalic acid and mechanical force via compound appressoria (Lumsden and Dow, 1973; Lumsden, 1979). The lack of asexual conidia spores and the dependence on ascospores for initiating disease makes the sexual cycle of *S. sclerotiorum* an enticing target for disease control.

In Ascomycetes the role of *MAT* genes include regulation of pheromone and pheromone receptors required for mating partner recognition, heterokaryon incompatibility, sexual fruiting body development, and possibly nuclear pairing post-fertilization (Bistis, 1981; Zickler *et al*., 1995; Arnaise *et al*., 1997; Debuchy, 1999; Shiu and Glass, 2000; Bobrowicz *et al*., 2002; Glass

and Kaneko, 2003; Bidard *et al*., 2011; Whittle and Johannesson, 2011). These wide ranging roles make them major regulators of the sexual life cycle. The core mating type genes in filamentous Ascomycetes share functional sequences that encode the high mobility group (HMG) and α-box protein transcription factors (Turgeon *et al*., 1993).

Ascomycetes mating systems may be homothallic which allows for self-compatibility but also the potential for outcrossing, or heterothallic requiring obligate outcrossing (Debuchy *et al*., 2010). In addition to the two core *MAT* genes, subsequent genes found at MAT loci are lineage specific with conservation observed at the family and class levels (Debuchy *et al*., 2010). In heterothallic species one isolate will carries an idiomorph of the MAT locus and a sexually compatible isolate carries the opposite MAT locus, specifically one *MAT1-1-1* (alpha 1) and one *MAT1-2-1* (HMG) gene (Dubuchy *et al*., 2010). In homothallic species there is only one version of the locus that usually carries both core *MAT* genes.

In the Helotiales only a few species have had their *MAT* locus characterized: *Pyrenopeziza brassicae* (Singh and Ashby, 1998), *Tapesia yalludae* (Singh *et al*., 1999), *Rhynchosporium secalis* (Foster and Fitt, 2004), *S. sclerotiorum*, and *B. cinerea* (Amselem *et al*., 2011). The two core *MAT* genes (*MAT1-1*-1 and *MAT1-2*-1) are present in all species. The lineage-specific gene *MAT1-1-3* is present in *R. secalis* and *MAT1-1-4* is present in *P. brassicae*. *S. sclerotiorum* and *B.* cinerea share the *Mat1-1-5* and the *MAT1-2-4* genes. These lineage-specific genes have not been described from any other species to date. Little is known about the function of the core or lineage-*specific* MAT genes within the Helotiales as characterized gene mutants have not been published.

S. sclerotiorum is homothallic with fused *MAT1-1* and *MAT1-2* idiomorphs containing *MAT1-1-1*, *MAT1-1-5*, *MAT1-2-1*, and *MAT1-2-4* (Amselem *et al*., 2011). The heterothallic MAT locus of *B. cinerea has an identical gene content but is organized as two idiomorphs. MAT1-1* isolates contain the *MAT1-1-1* and *MAT1-1-5* genes and *MAT1-2* isolates have *MAT1-2-1* and *MAT1-2-4* genes (Amselem *et al*., 2011). In preliminary reports, *B. cinerea k*nockout mutants of the *MAT1-1-5* ORF are unable to expand the apothecial disc structure in the dikaryon (van Kan *et al*., 2010) and *MAT1-2-4* gene deletion mutants crossed with a wild type *MAT1-1* isolate, is able to form stipes but disc differentiation is interrupted (Terhem *et al*., 2011.)

In this study each of the four MAT locus genes of *S. sclerotiorum* were targeted for functional analysis to test the hypothesis that *MAT* genes effect multiple aspects of the sexual life cycle. Phenotypic consequences of these mutants in the context of the entire lifecycle were characterized. Each of the four MAT genes was found to function in both the sexual and the vegetative portions of the life cycle. Evidence that MAT gene products function cooperatively is evidenced by altered expression of the *MAT* genes in independent mutants and the altered regulation of putative pheromone and pheromone receptors.

Materials and Methods

S. sclerotiorum wild-type isolate 1980 was used for the generation of complete gene knockouts by homologous recombination and was maintained on potato dextrose agar (PDA) (Difco, Mi., USA) at room temperature. Gene deletion mutants were maintained on PDA with 100µg/ml hygromycin at room temperature. All strains were propagated by mass hyphal tip transfer. Liquid shake cultures in YPsucrose medium (4g/L yeast extract (Difco, Detroit, MI), 15 g/l sucrose, 1g/L K_2HPO_4 and 0.5 g/L $MgSO_4$ pH 6.5) were grown for mycelial harvest and later

DNA extraction. Mutants and wild type strains were grown on potato plates (15g/L agar, two large mashed potatoes, 500ml H_20 20 cm diameter plates) until mature sclerotia were produced. These sclerotia were used for apothecia induction as described below and harvested for cytological analysis at developmental stages 3 and 5 described in Li, 2008.

Apothecia were induced from surface sterilized (0.5% bleach solution), dried, mature sclerotia which were frozen at -20 degrees C for 24 h and thawed to room temperature for 24 hours three times in a sterile plastic tube. They were grown in water-saturated vermiculite and maintained under constant light conditions in a Percival growth chamber (Boone, IA, USA) at 15˚C using cool florescent lighting (40 µmoles/m^2/s). Apothecia were harvested at every stage defined in Figure 2-1 for tissue dissection and RNA extraction.

Nucleic Acid Extraction and Tissue Manipulations

Mycelia from liquid shake cultures were flash frozen in liquid nitrogen, lyophilized and stored at -80 degrees C. Lyophilized mycelia were used to isolate genomic DNA in a modified procedure from Yelton *et al*. (1984), a phenol-chloroform 24:1 extraction step was added to the procedure and Lithium Chloride precipitation was removed in order to increase the quality of the DNA extracted. RNA was extracted from lyophilized mycelia using Trizol reagent (Gibco BRL, Rockville, MD) according to manufacturer's instructions for Northern blot analysis. RNA electrophoresis was conducted as previously described (Rollins and Dickman, 2001). For quantitative PCR analysis, RNA was extracted from lyophilized mycelia using the RNeasy Plant Mini Kit and treated with the RNase-free DNase kit according to manufacturer's instructions (QIAGEN, Hilden, Germany).

Escherichia coli strain DH5α was used to propagate all plasmids in this study. Plasmid isolations, agarose gel electrophoresis, DNA restriction digests, ligation reactions, and transformations of *E. coli* were conducted using standard laboratory procedures (Sambrook and Russell, 2001). For Southern hybridization analyses, digested genomic DNA was transferred to MagnaGraph Nylon Membrane (Micron Separations Inc. Westborough, MA) by downward alkaline transfer (Chomczynski, 1992) then UV cross-linked. For Northern hybridization analyses, RNA's were transferred to MagnaGraph Nylon membrane by standard procedures (Ausubel *et al*., 1991). RNA and DNA hybridization analyses was carried out with 10 µg of nucleic acids at high stringency as defined by Ausubel *et al*. (1991). Probes for all hybridizations were made using the DIG High Prime DNA Labeling and Detection Kit (Roche) according to manufacturer's specifications on a Biometra Thermocycler (Biometra, Germany). Northern and Southern hybridization procedures were performed to the specifications for the DIG labeling kit.

Gene Knockout Design and Execution

Complete gene knockout constructs were designed for each *MAT* gene using a split marker technique (Fairhead *et al*., 1996; Fu *et al*., 2006). This strategy is described for each gene with restriction enzymes used in Figures 2-2 through 2-5. A plasmid with ~1 kb of the 5' untranslated region (UTR) and 3' UTR of each gene was constructed with an intervening hygromycin resistance cassette as described by Jurick and Rollins (2007) (Table 6-1). The 5' UTR reverse (R1) primer for each construct had an added *Asc*I restriction enzyme sequence at the 3' end and the 3' UTR forward (F1) primer had the *Asc*I restriction enzyme sequence added to the 5' end to facilitate hygromycin resistance sequence ligation. Primer pairs for the amplification

of the partial hygromycin cassette (~2/3 total) and complete amplification of the respective UTR for split marker recombination were designed and are detailed in Table 2.1. The hygromycin cassette was a derivative of pCSN43 (Staben *et al*., 1989) in the PGEM-HPH plasmid (Hutchens, 2005) and maintained in DH5α and isolated using the Wizard Prep SV Minipreps DNA Purification System (Promega, WI, USA). All primers used were made by Integrated DNA Technology (Coralville, IA, USA). Transformations were performed in wild type protoplasts using PEG mediated induction by the method described in Rollins (2003). Successful replacement of the coding sequences with the hygromycin resistance cassette (gene knockouts by homologous recombination) were confirmed by Southern hybridization and PCR analysis utilizing probes of the UTR sequence or coding sequence of each gene (Table 6-2).

Quantitative Reverse Transcription Polymerase Chain Reaction

Quantitative reverse transcription PCR (qRT-PCR) analysis was performed to measure the affect of gene mutation on select gene expression at various points of the life cycle. Actively growing mycelia, enlarging sclerotia (stage 3) and pigmented sclerotia (stage 5) were chosen for analysis by measuring expression of the four *MAT*, one putative pheromone, and two putative pheromone receptor genes for each tissue type of all four *mat* gene mutants.

One microgram of total RNA was used to synthesize cDNA for each sample using the ProtoScript II First Strand cDNA Synthesis Kit by oligo DT priming according to manufacturer's specifications (New England Biolabs, MA, USA). One microliter of the reaction was used for subsequent PCR analysis. Each sample was checked for DNA contamination by PCR with histone H3 coding sequence (*H3*; GenBank accession XM_001589836) primers and a wild-type DNA sample prior to quantitative reverse transcription PCR (qRT-PCR) analysis. Quantitative

RT-PCR was performed using the iQ SYBR Green Supermix #170-8880 kit from BIO RAD (CA, USA) according to manufacturer's specifications. qRT-PCR reactions were performed on a BIO RAD CFX Connect RT System using the CFX managing program for design and later analysis (BIO RAD, CA, USA). qRT-PCR was programed to run for 30 seconds at 95˚C, then 44 cycles of (30 seconds 95˚C, 40 seconds 55˚C), and finally a melting curve analysis consisting of 5 seconds at 55˚C then 30 seconds at 95˚C. The primers in Table 6-3 were used for PCR reactions. All primers were designed to anneal optimally at 55 ˚C and amplify a target sequence between 200-250 bp. The data was analyzed with the CFX managing software using the comparative C_T method described in Schmittegen and Livak (2008) where the internal control (*H3*) was calculated to have no difference in gene expression between control and test samples by a t-test (0.95). Wild type gene expression data with three technical replications was compared to absolute zero with *H3* gene expression as a reference sample and two negative controls. The ΔΔCq method of normalized expression was selected to measure the gene expression data of each sample using the *H3* gene expression as a reference (BIO RAD CFX Manager, BIO RAD, CA, USA). A two way ANOVA of the fold changes was performed utilizing Prism 6 software (Graphpad Software, Inc.) to determine the significance of the changes, fold changes with a p-value less than 0.05 or less than three-fold difference in gene expression were considered statistically significant for this study. The p-value was an adjusted p-value calculated by the Prism 6 software as defined by Wright, 1992.

Tissue Fixation, Embedding, and Sectioning for Microscopic Observations

Sclerotia and apothecia were harvested at defined stages of development for mutant and wild type strains, fixed and embedded for sectioning using the method described in Kladnik

et al. (2004). Embedded samples were sectioned (3µm) using a rotary microtome HM 325 (Richard-Allan Scientific, MI, USA) and heat fixed onto ProbeOne Plus microscope slides (Fisher Scientific, USA). Samples to be analyzed were de-waxed using Histoclear (National Diagnostics, GA, USA) and rehydrated in an ethanol series where they were stained in methylene blue and mounted in cytoseal (Rochard-Allan Scientific, MI, USA). All slides and microscopic material was observed on a Leica DMR microscope (Leica Microsystems, Germany) using the SPOT Digital Camera and software (SPOT Microsystems, MI, USA). Thin sections of sclerotia and apothecia from mutant strains were compared to the wild type for phenotypic analysis. Squash slides were also made of mutant apothecia and analysis of apothecia structures, ascus morphology and ascospore production was compared with wild type.

Macroscopic Observations

Pathogenicity comparisons were completed using a detached-leaf assay with tomatoes in a sterile moist chamber (Rubbermaid Tupperware, moistened paper towels, constant cool fluorescent light, room temperature) where they were inoculated with a 3 mm plug of actively growing mycelia on PDA and then covered in Tupperware under constant light conditions at room temperature where they were documented with digital photography daily. This experiment was repeated three times and there was no visible difference between wild type and the mutant abilities to infect and cause disease symptoms. Apothecia and other macroscopic material were digitally photographed with a Canon EOS T2i camera at multiple focal planes and combined using the CombineZM software program and edited for publication using Photoshop CS3 software (Adobe, CA, USA).

Growth rates of the mutants were analyzed with race tubes made from modified 25 ml sterile plastic pipettes with PDA media (Park and Lee, 2004). Three independent gene knockout strains of *mat1-1-1*, *mat1-2-4* and *mat1-2-1* plus two independent knockouts of *mat1-1-5* were analyzed with three replications each and grown under constant temperature and moisture with 12 hours light (cool fluorescent light) and 12 hours dark in a growth chamber until they reached the end of the tube. Measurements of each day's growth were used to compare the mutants and wild type isolates using a two sample independent t-test with a significance of greater than 0.95.

Results

Gene Knockouts

Replacement of each of the four *S. sclerotiorum MAT* gene coding sequences with a hygromycin resistance marker was carried out using a split marker strategy. A probe in the coding region and the UTR of each gene in conjunction with PCR amplifications were used to confirm gene deletion and replacement of the coding sequences. With each UTR probe, restriction fragment sizes were detected by Southern hybridization and compared with sizes predicted from the genome sequence for each gene (Fig, 2.2B, 2.3B. 2.4B. 2.5B). Heterokaryotic transformants produced both wild type and homologous recombinant bands. Transformants positive for homologous integration were taken through a process of genetic purification involving hyphal tipping and, when required, carpogenic germination to produce apothecia for single ascospore purification. Following purification, PCR amplification of coding sequences from each *MAT* gene produced a positive amplicon of predicted size in the wild type but failed to amplify the respective gene sequence in the corresponding mutant strain

indicative of their homokaryotic gene deletion status (Figs. 2.2B, 2.3B. 2.4B. 2.5B). Multiple gene knockout strains for all four *MAT* locus genes were created: *MAT1-1-5* (two independent knockouts, Fig 2-2); *MAT1-1-1* (three independent knockouts, Fig 2-3); *MAT1-2-4* (three independent knockouts, Fig 2-4) and *MAT1-2-1* (three independent knockouts, Fig 2-5). Ectopic integration of transforming DNA was evidenced by multiple hybridizing bands of sizes inconsistent with homologous recombination and were observed in 80 to 90% of transformants across all transformations (data not shown). These ectopic and heterokaryotic transformants acted as wild type in every assayed phenotype (data not shown).

Mycelial Growth

Gene deletion mutants of each *MAT* gene were assayed across all major stages of growth and development to determine the phenotypic consequences of *MAT* gene loss of function throughout the lifecycle. Mutants were grown on PDA media and microscopic examination revealed no differences between wild type and mutant hyphae size or branching patterns (data not shown). Growth rates were measured using race tube assays and a significant difference between the wild type and all mutants was measured ($p<0.01$). *mat1-1-5* (20.3 mm/day) and *mat1-2-4* (20.6 mm/day) grew at a faster rate than wild type and *mat1-1-1* (19.8 mm/day) and *mat1-2-1* (19.4 mm/day) grew at a significantly slower rate than wild type (20.2 mm/day) (Fig 2-6). Mycelial compatibility was tested for all *MAT* gene mutants and no discernible changes were observed between pairs of mutants or between mutant and wild type pairings (data not shown).

Pathogenicity

Pathogenicity trials using detached tomato leaves were performed for each mutant and compared against wild type for general symptom development and for changes in the level of virulence. There was no visible difference between the mutant and wild type strains in the timing or rate of disease symptom development (Fig 2-7).

Sclerotial Development

Each mutant produced sclerotia with rind, cortex and medulla layers visibly indistinguishable from wild type. Average sclerotium mass (Fig 2-8), total number of sclerotia produced, and spermatia production (Fig 2-9) were calculated for each mutant. The average individual sclerotium mass appeared to vary from wild type in the *mat1-2-4* mutant exhibiting an increase in mass while the other *MAT* locus gene mutants exhibited a decrease when compared to wild type. Despite the apparent qualitative differences, variation in the samples proved to be too large to be statistically significant ($p>0.20$). Spermatia production was also affected in all four mutants with a significant decrease in production compared to wild type ($p<0.015$) and no significant difference ($p<0.05$) among the four *MAT* locus gene mutants. Thin sections for microscopic observation were made of the sclerotia on a weekly basis from the stage of maturation through wild type apothecia development. At week two of sclerotial incubation for apothecia induction, all mutants formed multiple ascogonia within their sclerotia with numbers, morphology and developmental timing indistinguishable from the wild type (Fig 2-10).

Apothecia Development

Sclerotia from each mutant and the wild type were assayed for apothecium development under standard conditions of light, and controlled moisture and temperature. After a period of 4-6 weeks (35.7 days on average), wild type apothecia began to develop. The *mat1-2-4* mutant began producing apothecia an average of 151.3 days after the wild type controls. The *mat1-1-5*, *mat1-1-1* and *mat1-2-1* mutants never exhibited signs of carpogenic germination or apothecia development following 40 weeks of incubation and assaying greater than 500 to 1400 sclerotia per mutant over 4-6 experiments (Fig 2-11). The majority of apothecia produced by *mat1-2-4* were arrested at the initial stage of stipe development. Of 258 total apothecia observed, only 42 (16%) proceeded beyond stage one (undifferentiated stipes) of apothecia development. Of the developmentally arrested apothecia, stipes developed in bundles of one (54), two (72), three (68) and four (22) and did not develop beyond the point of undifferentiated, arrested stipe emergence (Fig 2-12). Over the course of all experiments, the number of apothecia produced per sclerotium was calculated at 0.03 for the *mat1-2-4* mutant and 0.47 for the wild type.

The apothecia of mat1-2-4 mutants developing beyond stage 1 showed a wide variety of aberrant disc morphologies. This phenotype ranged from the development of long tubular apothecia (Fig 2-12) with uniform undifferentiated hyphae throughout the length of the stipe and failing to form an apical depression indicative of apothecial disc initiation. Other apothecia had a long tubular morphology with a partial apical depression that originated early but never fully expanded (Fig 2-13, Fig 2-15). The final morphological class included stipes producing partially expanded crenulated discs (Fig 2-14, Fig 2-15). Apothecia in this class produced viable

ascospores and tissue layers including the ectal excipulum, the medullary excipulum, subhymenium and hymenium with microanatomies as in wild type. Several examples of aberrant apothecial morphologies were selected for thin section and ascospore analysis.

Fresh apothecia from the *mat1-2-4* mutant were imaged with differential interference contrast and brightfield microscopy for ascus and ascospore formation. Of the 100 observations generated from the various preparations, an ascus exhibiting the wild type number of eight ascospores was never observed. Variations of two, four and six ascospores were observed and commonly, when six ascospores were found, two were distorted and shriveled (Fig 2-16). Ascospores exhibiting wild-type morphology were examined following DAPI staining and contained two nuclei as observed in the wild type (data not shown). Ascospores plated on PDA and later transferred to PDA with 100µg/ml hygromycin were viable, stably expressed hygromycin resistance and recapitulated the phenotypes observed with the progenitor *mat1-2-4* mutant.

Gene Expression

Quantitative reverse transcription PCR (qRT-PCR) analysis was performed to measure the affect of gene mutation on select gene expression at various points of the life cycle. Actively growing mycelia, enlarging sclerotia (stage 3) and pigmented sclerotia (stage 5) were chosen for analysis by measuring expression of the four *MAT*, one putative pheromone, and two putative pheromone receptor genes for each tissue type of all four *mat* gene mutants.

***MAT* Gene Expression**

During vegetative mycelial growth, overall MAT gene expression increased when compared to wild type with one exception. The *mat1-1-5* mutant showed a significant increase

in *MAT 1-2-4* (+3.39) and *MAT1-2-1* (+5.05) gene expression. The *mat1-1-1* mutant showed a decrease in *MAT1-1-5* (-4.38) gene expression and an increase in *MAT1-2-1* (+3.07) gene expression. The *mat1-2-4* mutant displayed an increase in *MAT 1-2-1* (+3.07) gene expression. The *mat1-2-1* mutant showed an increase in *MAT1-2-4* (+41.79) gene expression (Fig 2-17).

During stage 3 of sclerotial development, a trend of decreased or no change in *MAT 1-1-5* and *MAT 1-1-5* gene expression and an increase or no change in *MAT 1-2-4* and *MAT 1-2-1* gene expression was observed. The *mat 1-1-5* mutant exhibited no significant alterations in *MAT* gene expression (Fig 2-18). The *mat 1-1-1* mutant showed very large decreases in *MAT 1-1-5* (-11.07) and *MAT 1-2-4* (-8.16) gene expression. The *mat 1-2-4* mutant showed a large decrease in *MAT 1-1-5* (-8.14) and a large increase in *MAT 1-2-1* (+5.38) gene expression. The *mat 1-2-1* mutant showed an increase in *MAT 1-1-1* (+3.32) and a very large increase in *MAT 1-2-4* (+24.42) gene expression. There was one observed difference in the trends described for the *mat 1-1-1* mutants where *MAT 1-2-4* gene expression decreases, an effect also seen with the putative pheromone and pheromone receptor gene expression (see below).

During stage 5 sclerotial development an overall trend of no change or a decrease in *MAT* gene expression was observed with only one exception. The *mat 1-1-5* mutant showed an increase in *MAT 1-2-1* (+3.41) gene expression (Fig 2-19). The *mat 1-1-1* mutant showed a very large decrease in *MAT 1-2-4* (-21.93) and an increase in *MAT 1-2-1* (+3.55) gene expression. The *mat 1-2-4* mutant showed a very large decrease in *MAT 1-1-5* (-31.67) gene expression. The *mat 1-2-1* mutant showed a small decrease in *MAT 1-1-1* (-3.02) and a very large increase in *MAT 1-2-4* (+22.14) gene expression. The one exception in the trend was the *mat 1-2-1* mutant and the significant increase in *MAT 1-2-4* gene expression.

Putative Pheromone and Pheromone Receptor Gene Expression

In mycelial tissue, putative pheromone and pheromone receptor gene expression was increased or showed no change with only one exception. The *mat 1-1-5* mutant showed an increase in *Ppg-1* (+5.91), *PreA* (+8.86) and *PreB* (+3.06) gene expression (Fig 2-20). The *mat 1-1-1* mutant showed a large increase in *Ppg-1* (+5.64) and *PreA* (+20.28) and a large decrease in *PreB* (-6.16) gene expression. The *mat 1-2-4* mutant likewise showed a large increase in *Ppg-1* (+5.1) and *PreA* (+11.07) gene expression. The *mat 1-2-1* mutant showed a large increase in *Ppg-1* (+5.72) and *PreB* (+5.3) gene expression. The one exception to the trend was in the *mat 1-1-1* mutant where *PreB* gene expression was decreased, this coincides with other early trends of down regulation within the *mat 1-1-1* mutant for other *MAT* genes.

In stage 3 sclerotial tissue there was an increase or no change in putative pheromone and pheromone receptor gene expression in all mutants except for mat 1-1-1 where all three genes exhibit decreased gene expression. The *mat 1-1-5* showed no significant change in the putative pheromone and pheromone receptor gene expression (Fig 2-20). The *mat 1-1-1* mutant showed a significant decrease in *Ppg-1* (-22.09), *PreA* (-20.65) and *PreB* (-4.5) gene expression. The *mat 1-2-4* mutant showed a significant increase in *PreB* (+6.39) gene expression. The *mat 1-2-1* mutant showed no significant changes in the putative pheromone and pheromone receptor gene expression. The extreme decreases of gene expression in the *MAT* genes and the putative pheromone and pheromone receptor genes shown in the *mat 1-1-1* gene mutant supports an early role for regulation of other *MAT* locus and pheromone genes.

In stage 5 sclerotial tissue all putative pheromone-related genes in all mutants showed a drastic decrease of more than 80 fold when compared to wild type gene expression except for

the *mat 1-2-4* mutant with the *PreB* (-4.37) gene (Fig 2-21). Fold decreases below -2000 fold of wild type levels were interpreted as no gene expression (Table 2-4). The smallest decrease in *PreB* for the *mat 1-2-4* mutant may support a role for *PreB* in the pre-fertilization or fertilization events necessary for apothecia development as the *mat 1-2-4* mutant was the only *MAT* gene mutant to show apothecia development.

Discussion

Many *MAT* loci genes have been described and used for epidemiological identification of isolates; yet, the number of studies characterizing the function of *MAT* genes in respective species are few (Debuchy and Turgeon, 2006). This study has shown that the *MAT* locus of *S. sclerotiorum* has functions throughout the lifecycle. The *mat1-1-5* gene mutant displayed a complete lack of apothecia development although successfully produced ascogonia with the timing and frequency of wild type. This mutant, and all of the *mat* gene mutants, had a significant decrease in the production of spermatia compared to wild type. These two characters indicate that *MAT1-1-5* is involved in pre-fertilization or fertilization events which when mutated prevented differentiation leading to stipe development from the ascogonium. When performing homology searches with the protein sequence of the *MAT1-1-5* gene, the only other fungal species containing a significant match is *B. cinerea* which has been reported in a preliminary study to require this ortholog in matings with MAT1-2 idiomorph strains for apothecial disc development (van Kan *et al*., 2010). The most significant alteration noted in gene expression of *mat 1-1-5* gene mutants are in the putative pheromone and pheromone receptors which supports the involvement in pre-fertilization and fertilization events. In vegetative mycelial tissue there is an elevation of gene expression for all three pheromone-

related genes similar to what has been observed in the *F. graminearum MAT* genes (Zheng *et al*., 2013). In stage 3 sclerotial and stage 5 sclerotial tissue we see a dramatic down regulation of all three putative pheromone-related genes analyzed with the most significant decrease of *Ppg-1* where the transcript is virtually undetectable.

Functional characterization other *MAT* locus gene mutants located proximal to the *MAT 1-1-1* gene has revealed a varied set of phenotypes in other fungal species. Altered regulation of pheromone and pheromone receptors in *Sordaria macrospora* and arrested sexual development at the point of protoperithecia formation in *SmtA-2* (*mat1-1-2)* gene mutants adjacent to the core *MAT1-1-1* gene (Klix *et al*., 2010). It is interesting to note that the *mat1-1-2* gene of *S. macrospora* does not have an HMG domain but rather a PPF domain which plays a role in *N. crassa* fertility and, in *P. anserina*, blocks protoperithecia production despite lack of evidence for DNA-binding (Klix *et al*., 2010). These examples show that mating type genes other than the core *MAT* genes may have significant morphological phenotypes and alter gene expression without demonstrated transcription factor activity. In *S. sclerotiorum* there is no evidence of co-regulation between the proximal *MAT 1-1-5* and *MAT 1-1-1* genes. This is evident as only a slight decrease of *MAT 1-1-1* (-1.81 to -2.07 fold) gene expression in the *mat 1-1-5* gene mutant. This finding supports independent roles in apothecial development for these two genes.

The findings presented here demonstrate that loss of function mutants of the core mating type genes, *mat1-1-1* and *mat1-2-1*, completely blocked apothecia development despite producing ascogonia in week two of sclerotia incubation. Both genes encode well documented transcriptional regulators. This phenotype is found in other Ascomycetes but is not universal.

Deletion or altered gene expression of either of the core *MAT* genes may result in infertility (*N. crassa, C. heterostrophus*) and/or inability to form sexual structures (*N. crassa, P. anserina, C. heterostrophus, G. zea, B. cinerea*) (Debuchy and Turgeon, 2006; van Kan *et al*., 20122; Zheng *et al*., 2013). Indirectly regulated products of the core *MAT* genes include pheromone and pheromone receptors genes responsible for the attraction of the male and female gametangia in Ascomycetes which when mutated arrest sexual development prior to fruiting body development (Mayrhrofer *et al*., 2005; Debuchy and Turgeon, 2006; Pöggler *et al*., 2006). The homothallic Ascomycete *Giberella zeae* differs slightly in that the ability to form sexual structures (perithecia) is not impeded with core *MAT* gene mutations but the perithecia show altered morphology and lack of ascospores (Desjardins *et al*., 2004). These mutants can be made to reproduce heterothallically by the removal of the *MAT1-1-1* or *MAT1-2-1* gene and crossing with a strain containing the other gene (Lee *et al*., 2003). The *SmtA-1* (*MAT1-1-1*) and *SmtA-3* (*MAT1-1-3*) genes in *S. macrospora* show no role in fruiting body development (Klix *et al*., 2010); however, the *Smta-1* (*MAT1-2-1*) gene in *S. macrospora* is essential to fruiting body development and is arrested after ascogonium and protoperithecia development (Pöggler *et al*., 2006). This range of phenotypes makes the characterization of each individual *MAT* gene a necessary step to understanding each respective *MAT* locus and thus sexual regulation of that species. As seen here, *MAT 1-1-5, MAT 1-1-1 and MAT 1-2-1* are essential for regulating early stages of sexual compatibility and *MAT*1-*2-4*, late stages of sexual compatibility including apothecium morphogenesis and ascospores development.

The *MAT* locus of *S. sclerotiorum* undergoes a 250-bp inversion described by Chitrampalam *et al*. (2013) resulting in truncation of the *MAT 1-1-1* coding sequence. Strains

carrying the inverted form of the *MAT* locus remain fertile and develop apothecia indistinguishable from non-inverted strains. In this study I simultaneously confirmed the occurrence of the *MAT* locus inversion and observed both the inverted and non-inverted strain apothecium production. Upon further analysis of the MAT1-1-1 alpha protein domain encoding sequences it was established that the three essential alpha helices which make up the ancestral form of the alpha box (Martin *et al*., 2010) are retained in the inversion strains (Fig A-4). The retention of the ancestral alpha box, the development of apothecia in both inverted and non-inverted strains, and the lack of development of apothecia in the *mat 1-1-1* gene mutant suggests that the truncation of the alpha-1 protein at the inverted locus does not effect its function.

All *MAT* gene mutations altered gene expression of other *MAT* locus genes. The most significant change that occurred in the *mat1-2-1* mutant was that *MAT1-2-4* gene transcription levels increased across vegetative hypha and sclerotial development stages (+41.79, +24.42, +22.14) above wild type and far above any of the other genes analyzed. The *mat1-1-1* mutant showed a significant decrease in *MAT1-2-4* gene transcription beginning in stage 3 sclerotia (-8.16) and continuing to stage 5 sclerotia (-21.93). As the homologous replacement events were designed to not alter the promoter sequences of adjacent mating type genes, the changes in gene expression are interpreted to result from the loss of regulatory interactions among the MAT proteins. *MAT1-2-4* is flanked by the core *MAT* locus genes so this still may be a form of structural regulation or direct gene product interaction similar to the cis-regulation seen in *N. crassa* (Ferreira *et al*., 1997).

There is a decrease (-11.07) in *MAT 1-1-5* gene expression in the *mat 1-1-1* gene mutants that appears in stage 3 sclerotial tissue but subsides in stage five sclerotial tissue (-1.53). The *mat 1-1-1* gene mutants also showed the earliest change in the putative pheromone and pheromone receptor gene expression in stage 3 sclerotial tissue. In this tissue most of the other *MAT* gene mutants show elevated putative pheromone gene expression and the *mat 1-1-1* gene mutants have begun to down regulate all three putative pheromone genes. By stage five *mat 1-1-1* gene mutants show the most severe down-regulation of all the *MAT* locus mutants in all three putative pheromone genes. The *mat 1-2-1* gene mutant showed increases in all putative pheromone genes until stage 5 sclerotia where it showed a decrease in all putative pheromone genes. The *MAT 1-1-1* gene appears to be involved in regulatory events of both the other *MAT* genes and the three putative pheromone and pheromone receptor genes before the other three *MAT* locus genes. By stage five all four *MAT* locus genes are highly active in regulation of either other *MAT* genes or the three putative pheromone and pheromone receptor genes analyzed.

The *mat1-2-4* gene mutant was the only mutant to retain apothecia production although they were unable to form fully expanded discs and the majority of stipes did not proceed beyond stage 1 of apothecial development. The production of apothecia was also extremely delayed, ranging from 49 to 196 days beyond the time of wild type apothecia initiation. Following carpogenic germination, the mutation appears to affect the timing of apical disc depression and disc expansion consistent with a defect in interpreting signaling processes such as light reception or endogenous spatial orientation signals required for proper patterning of tissue development. A subset of apothecia from this mutant displayed tubular

forms in which disc expansion appears to initiate early but manifests as an expansion parallel to the stipe axis rather than horizontal. Tissue differentiation reminiscent of early disc development appears in microscopic sections of these tissues. A more readily observed phenotype in the *mat1-2*-4 mutants was apothecia with malformed discs of crenulated morphology or completely folded in upon themselves. These malformations occurred very early in the process of disc expansion and were never corrected as the apothecial disc matured. These observations would be consistent with a hypothesis that that the signals to widen and invaginate the stipe apex to form a fully developed apothecia are present but not coordinated well and perhaps the signals for such morphological development are being interpreted through secondary sub-optimal pathways as a result of the primary pathway being interrupted.

A distortion in the formation of ascospores of *mat1-2-4* mutants was also observed such that a single ascus with a full set of eight ascospores was never found. Sets of two, four, and six were common. When six ascospores were present, two of the ascospores were shriveled and much smaller than the wild type ascospores. A similar observation has been made in the homothallic Ascomycete *Coniochaeta tetraspora* where a non-random disintegration of sets of two ascospores, four in total, result in four-spored asci and is hypothesized that similar to other fungi where *MAT* locus genes are involved in nuclear pairing and an epigenetic trigger may be signaling ascospore loss (Raju and Perkins, 2000). The disrupted arrangement of the *MAT* locus is *N. crassa MATA-2* mutants followed with ectopic complementation, restored sexual development up to the point of ascospore formation but no ascospores are produced (Ferriera *et al*., 1997). The arrangement of the *MAT* locus has also been shown to have an effect on cytoskeleton stability and arrangement. Yeast homokaryotes expressing *MATa/MATa* showed

decreased abilities to undergo cytokenisis, chromosomal pairing and microtubule formation when compared to a heterokaryote expressing *MATα/MATα* which exhibited increased abilities to do the same (Steinberg-Neifach and Eshel, 2002). Cytoskeletal disruptions have also been found to disrupt fruiting body development in *Aspergillus nidulans* (Pöggler *et al*., 2006). This leads me to propose three hypotheses for the disrupted ascospore formation in *mat1-2-4* gene mutants: 1) a regulatory pathway disruption involving the *MAT* locus itself has led to a deficiency in ascospore formation, 2) improper nuclear pairing due to cytoskeletal instability and nuclear pair recognition errors lead to a deficiency in ascospore formation, or 3) programed death of ascospores from meiotic progeny which do not recognize each other at the nuclear level. The hypothesis that MAT1-2-4 regulates fundamental aspects of cytoskeleton function could most parsimoniously account for the gross morphological phenotypes and well as the distorted ascospores numbers and could be tested in the future with specific cytoskeletal mutants or chemical treatments known to disrupt cytoskeleton functions.

The effect of the *mat 1-2-4* gene deletion on *MAT 1-1-1* and *MAT 1-2-1* gene expression were not as severe as the effects of the *mat 1-1-1* and *mat 1-2-1* gene deletions on *MAT 1-2-4* gene expression. This supports a hypothesis that *MAT 1-2-4* may be regulated by *MAT 1-1-1* and *MAT 1-2-1*. In stage five sclerotia of *mat 1-2-1* mutants *MAT 1-2-4* gene transcription is up-regulated (+23.14) this would indicate that it is down-regulated in the wild type at the earliest stages of apothecia development but required later for proper apothecial disk development as seen in the *mat 1-2-4* mutants apothecial phenotypes. The largest *MAT* gene expression effect of the *mat1-2-4* mutant was on the *MAT1-1-5* gene beginning in sclerotial stage 3 tissue (-8.14) and continuing in stage 5 sclerotial growth (-31.67 fold). This may indicate that *MAT1-2*-4 is an

upstream regulator of MAT1-*1-5* in sclerotial tissue but does not impede the function of *Mat1-1-5* to the level that affects apothecium development.

Sexual and non-sexual developmental phenotypes involving pheromone and pheromone receptor gene double knockouts and individual pheromone gene knockouts have been well documented (Pöggler and Kůck, 2001; Seo *et al*., 2004; Mayrhofer et al., 2005; Lee et al., 2008; Zheng et al., 2013). The alteration of putative pheromone production in the *mat 1-2-4* gene mutant was seen as an increase in transcript levels in mycelial tissue and stage 3 sclerotial tissue and then a decrease in stage 5 sclerotial tissue. The *PreB* (-4.37) gene expression was the least down-regulated putative pheromone gene observed and may support a role in the pre-fertilization and fertilization events that allowed the *mat 1-2-4* gene mutant to develop apothecia. In the other three *MAT* gene mutants *PreB* is down-regulated from -129.3 to -1075 fold relative to wild type which may prevent them from fertilizing successfully or may play a role in developing elongated stipes and expanded discs later. The *Ppg-1* and *PreA* genes down-regulation may also support a role in the phenotypes observed in the stages of development after a mature sclerotia are formed in *S. sclerotiorum*.

Gene knockouts of the *Ppg-1*, *Ppg-2*, *Pre1* (*PreA*) and *Pre2* (*PreB*) genes in *G. zeae* were analyzed for expression and function (Lee *et al*., 2008). *Ppg-2,* which was not found in *S. sclerotiorum*, was not found to be expressed in any *G. zeae* tissue analyzed. This short (25 AA) protein that is not well conserved among Ascomycete species (Lee *et al*., 2008). *Pre2*/*Ppg-1* were found to enhance but not be essential for selfing and outcrossing while *Ppg-2/Pre1* had no discernible role (Lee *et al*., 2008). This lack of function or possible redundant function for *Ppg-2* may have led to the eventual loss of the sequence completely in *S. sclerotiorum*.

This study has shown the *MAT* locus is involved in a broad range of developmental processes including regulation of sexual development, sexual structures, mycelial growth (*MAT1-1-1* and *MAT 1-2-1*), and *MAT* locus and putative pheromone and pheromone receptor gene regulation. Further study into the roles of the putative pheromone and pheromone receptors by functional analysis and the characterization of other genes regulated by the MAT locus will provide a more complete understanding to the sexual regulation of *S. sclerotiorum* and the roles of endogenous and exogenous signaling in coordinating complex fruiting body development.

Table 2-1. Primers used for UTR amplification and cloning.

Primer Name	Primer Sequence
MAT1-1-5 5' UTR F1	AGG TCT GCC GTC TAG ATC AT
MAT1-1-5 5' UTR R1	AGG CGC GCC TGT TAC GAG GTT TGC CGT CT
MAT1-1-5 3' UTR F1	AGG CGC GCC ACC GTT TAA GGG AAA TCC AG
MAT1-1-5 3' UTR R1	ACG TCC AAA TCT GTC AAG GT
MAT1-1-1 5' UTR F1	CGC CAG GAA CGA AAT GCG AAA GAA
MAT1-1-1 5' UTR R1	AGG CGC GCC TAT GAA TTG ACA GAG CGC CGA GGA
MAT1-1-1 3' UTR F1	GGC GCG CCA GGC ATT GTC CTG TTC CCA CGA TA
MAT1-1-1 3' UTR R1	TCC AAG ACG ACA ACT CCA CAA CCA
MAT1-2-4 5' UTR F1	AGG AAA GCT GAT GGA AGA GGT GGT
MAT1-2-4 5' UTR R1	AGG CGC GCC TCT CAA GCG GTA TGA TCC CAC CAA
MAT1-2-4 3' UTR F1	GGC GCG CCC GGC AAG CTT GAT GCT TAA CAG GT
MAT1-2-4 3' UTR R1	TAC GAG ACA AAT CGG GTG GGT TGA
MAT1-2-1 5' UTR F1	AGG GAG GAG ATG GGA CAT AGT TCT
MAT1-2-1 5' UTR R1	AGG CGC GCC TTG GTG GGA TCA TAC CGC TTG AGA
MAT1-2-1 3' UTR F1	GGC GCG CCT TGG TGC GAA CAG CAA TTA CGA GC
MAT1-2-1 3' UTR R1	AAG AAC AAC CGA TGG ACT GAG GGT
HY Split Marker	AAA TTG CCG TCA ACC AAG CTC TGA TAG
YG Split Marker	TTT CAG CTT CGA TGT AGG AGG GCG

Table 2-2. Primers used for generation of the probes in Southern blot analysis of the *MAT* locus genes in *S. sclerotiorum.*

Primer Name	Primer Sequence `5-3`
MAT1-1-5 Coding Probe F1	ATA TTC GCC CCT GCG CCC TT
MAT1-1-5 Coding Probe R1	TGG ATT CTC CTG CCG TCT GA
MAT1-1-5 3' UTR Probe F1	GTT AGC CGA CTC CAG CCA CA
MAT1-1-5 3' UTR Probe R1	GAG CCT CAA CCC ACC CGA TT
MAT1-1-1 Coding Probe F2	TTG CTC CAC CTC CCA AGC CA
MAT1-1-1 Coding Probe R2	AGA GAT ATC GCC AGG AAC AT
MAT1-1-1 3' UTR Probe F1	ACA CTC CCC AGT ATG GAT
MAT1-1-1 3' UTR Probe R1	ACG AGG AAG CCT GAT GCG TA
MAT1-2-4 Coding Probe F2	AGC CGA TTT GGG GCT GGT GA
MAT1-2-4 Coding Probe R2	AAA GGG AGG AGA TGG GAC AT
MAT1-2-1 Coding Probe F1	TCA ACC CAT GGT GTG AAC TA
MAT1-2-1 Coding Probe R3	AAG CCT GCG ACG GCT AAC AT
MAT1-2-1 3' UTR Probe F1	GGC GCG CCT TGG TGC GAA CAG CAA TTA CGA GC
MAT1-2-1 3' UTR Probe R1	AAG AAC AAC CGA TGG ACT GAG GGT

Table 2-3. Primers designed for QPCR analysis.

Primer Name	*Primer Sequence `5-3`*
MAT 1-1-5 QPCR Primer F1	CGG TGA AAT TGA GGG TGG TA
MAT 1-1-5 QPCR Primer R1	GGC ATG AAA AGG ATC ACG TC
MAT 1-1-1 QPCR Primer F1	TTG GAT CCA TCG ACT TTT CC
MAT 1-1-1 QPCR Primer R1	CCG CAT ACT TCG TGG GTA AT
MAT 1-2-4 QPCR Primer F1	AAA GGG AGG AGA TGG GAC AT
MAT 1-2-4 QPCR Primer R1	AGC CGA TTT GGG GCT GGT GA
MAT 1-2-1 QPCR Primer F1	TCA ACC CAT GGT GTG AAC TA
MAT 1-2-1 QPCR Primer R1	AAG CCT GCG ACG GCT AAC AT
PPG-1 QPCR Primer F1	CAA ACG CAA TTG CTC TCG CT
PPG-1 QPCR Primer R1	CCG CAC CAT GCC TCA GCA TT
PreA QPCR Primer F1	AAC CGC TGG TCC GGC CAT AT
PreA QPCR Primer R1	AAG TGC TCA TCA GGT GTG GT
PreB QPCR Primer F1	TCT CTT CGC GCT CAA TAC CC
PreB QPCR Primer R1	CTC CGT ACT ATC TCG CGG AA

Table 2-4. Composite of the gene expression comparisons in the *MAT* locus mutants.

		Mycelia		*Stage 3 Sclerotia*		*Stage 5 Sclerotia*	
Transcript	Mutant	Fold Change	Standard Deviation	Fold Change	Standard Deviation	Fold Change	Standard Deviation
MAT 1-1-1	*mat1-1-5*	-2.07	1.61	-1.84	0.86	-1.81	0.2
MAT 1-2-4	*mat1-1-5*	3.39	2.04	-1.64	0.56	1.12	0.35
MAT 1-2-1	*mat1-1-5*	5.05	1.37	1.13	0.81	3.41	0.35
Ppg-1	*mat1-1-5*	5.91	1.24	-1.21	0.52	-9630.09	4.4
PreA	*mat1-1-5*	8.86	0.59	1.13	0.64	-895.57	1.35
PreB	*mat1-1-5*	3.06	0.97	2.1	2.47	-1074.91	3.05
MAT 1-1-5	*mat1-1-1*	-4.38	1.06	-11.17	1	-1.53	0.24
MAT 1-2-4	*mat1-1-1*	-2.06	1.6	-8.16	0.75	-21.93	1.77
MAT 1-2-1	*mat1-1-1*	3.38	1.06	-1.08	0.97	3.55	0.35
Ppg-1	*mat1-1-1*	5.6 4	0.83	-22.09	0.84	-14197.34	3.22
PreA	*mat1-1-1*	20.28	1.27	-20.65	0.51	-2836.7	1.69
PreB	*mat1-1-1*	-6.16	3.49	-4.5	0.51	-925.02	1.35
MAT 1-1-5	*mat1-2-4*	1.96	2.34	-8.14	3.43	-31.67	0.77
MAT 1-1-1	*mat1-2-4*	1.09	1.58	-1.28	1.41	-2.64	1.04
MAT 1-2-1	*mat1-2-4*	3.07	1.72	5.38	2.59	1.66	0.44
Ppg-1	*mat1-2-4*	5.1	0.81	2.52	0.37	-482.15	4.57
PreA	*mat1-2-4*	11.07	1.11	2.35	0.71	-80.08	3.6
PreB	*mat1-2-4*	-1.56	0.78	6.39	1.14	-4.37	2.78
MAT 1-1-5	*mat1-2-1*	-2.74	2	-2.32	1	-1.1	0.35
MAT 1-1-1	*mat1-2-1*	1.02	1.93	3.32	0.41	-3.02	1.3
MAT 1-2-4	*mat1-2-1*	41.79	1.93	24.42	0.23	22.14	0.34
Ppg-1	*mat1-2-1*	5.72	2.21	2.67	1.48	-954.32	1.99
PreA	*mat1-2-1*	2.57	0.86	1.43	0.44	-94.68	1.46
PreB	*mat1-2-1*	5.3	0.83	2.56	0.32	-129.34	2.93

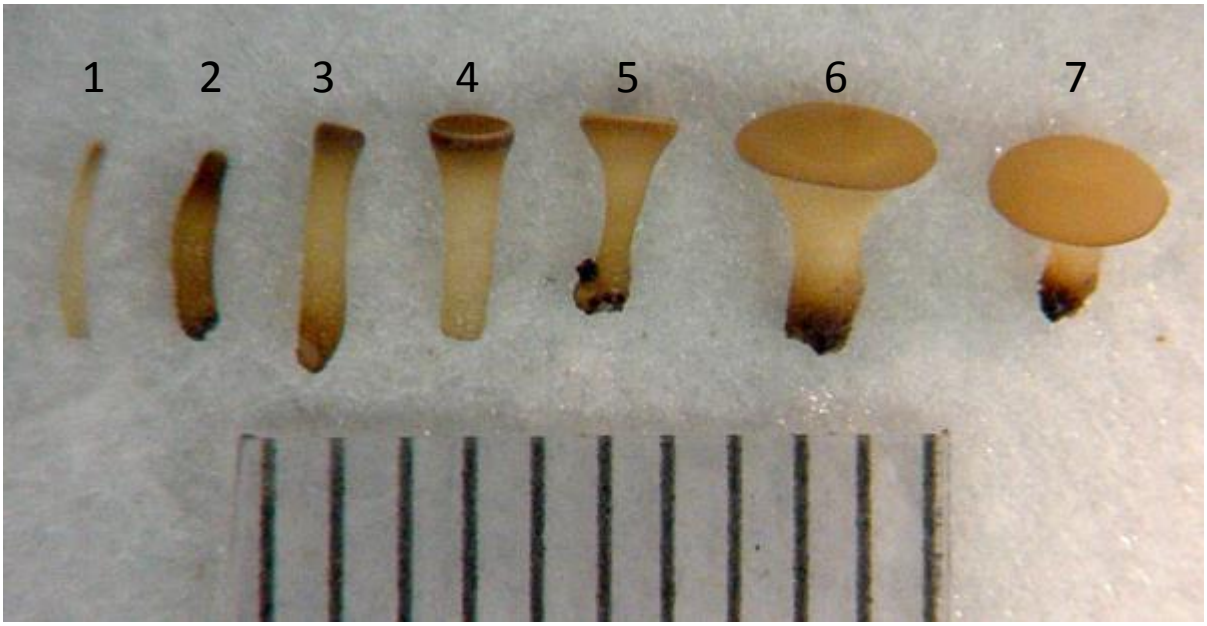
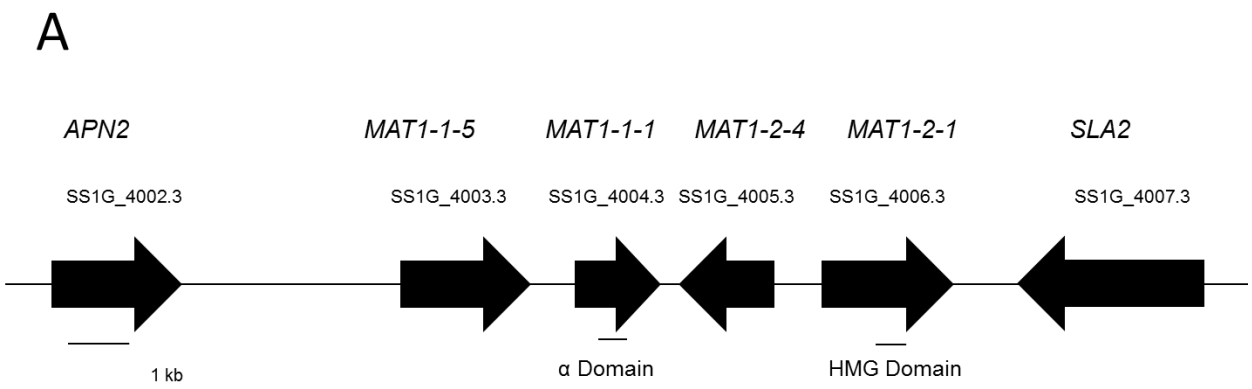
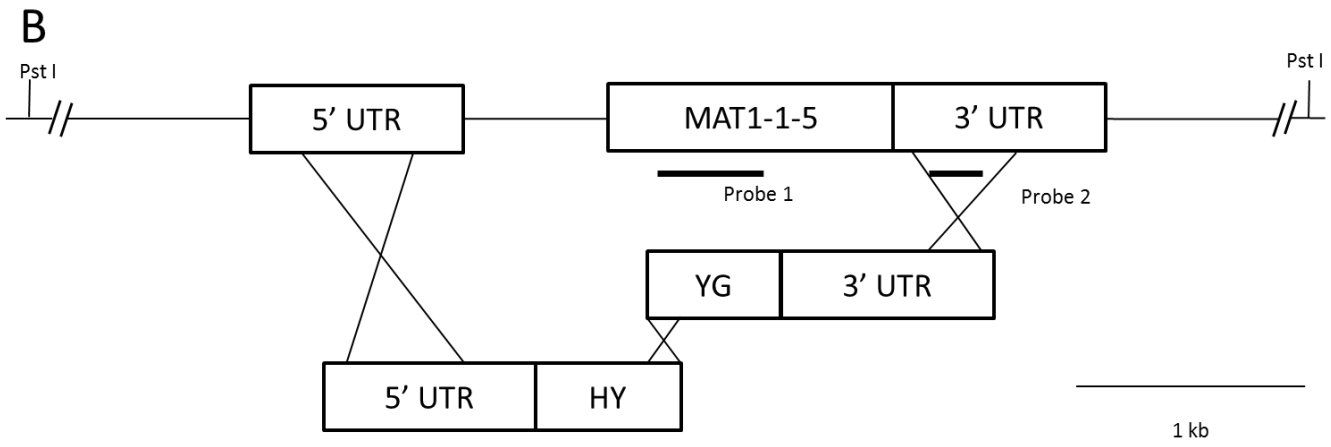


Figure 2-1. The apothecial stages of *S. sclerotiorum*. Apothecia are numbered sequentially and defined by easily measurable morphological changes: Stage 1 (undifferentiated stipe elongation); Stage 2 (concave stipe tip), Stage 3 (deepening of the stipe tip); Stage 4 (widening of the stipe), Stage 5 (disc expansion), Stage 6 (outturning of the disc) and Stage 7 (mature apothecia with no further expansion).


A
APN2
MAT1-1-5
MAT1-1-1
MAT1-2-4
MAT1-2-1
SLA2
SS1G_4002.3
SS1G_4003.3
SS1G_4004.3
SS1G_4005.3
SS1G_4006.3
SS1G_4007.3
1 kb
α Domain
HMG Domain


B
Pst I
Pst I
5' UTR
MAT1-1-5
3' UTR
Probe 1
Probe 2
YG
3' UTR
5' UTR
HY
1 kb

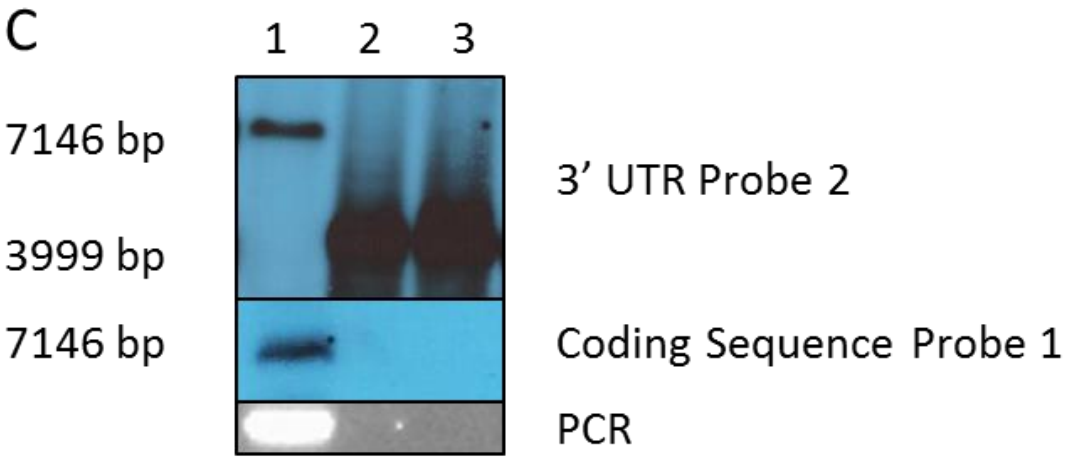
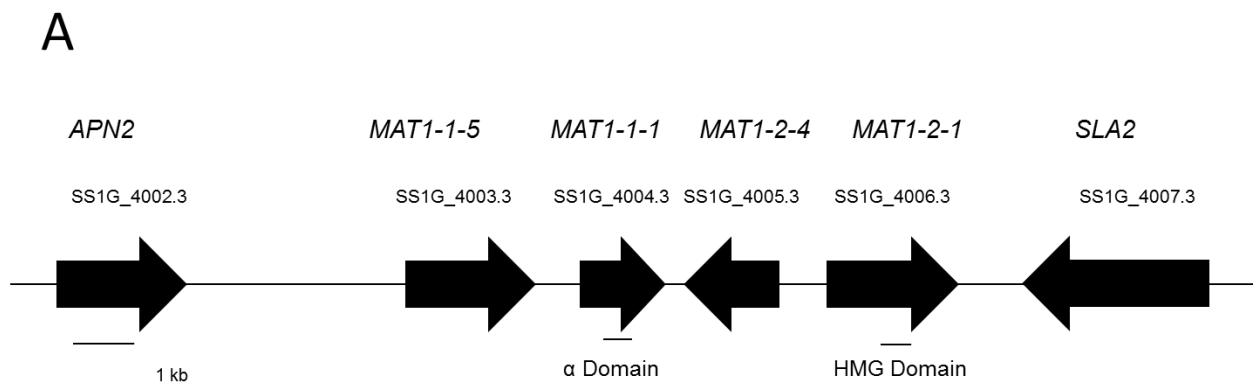
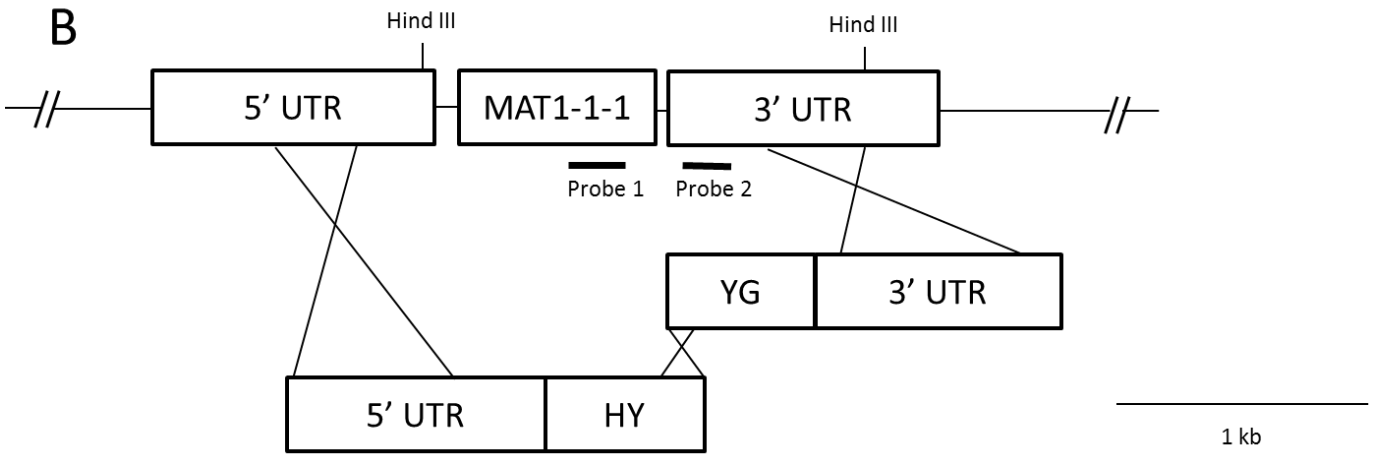

C
1
2
3
7146 bp
3999 bp
7146 bp
3' UTR Probe 2
Coding Sequence Probe 1
PCR

Figure 2-2. The split marker strategy for *MAT1-1-5* gene replacement. A) The *MAT* locus of *S. sclerotiorum*. B) Graphic representation of the split marker strategy. C) Genetic confirmation of the homologous recombinations. All homologous recombinations required along with the probes designed for identification of successful gene knockout (B). Genomic DNA was isolated from wild type (C, 1) and each transformant (C, 2-3) and digested with Pst I which would produce either a 7146 wild-type bp band during Southern analysis or a 4999 bp homologous recombination band when utilizing the 3' UTR probe (C). The transformants were also analyzed by PCR utilizing the coding sequence probe primers (C).


A
APN2
MAT1-1-5
MAT1-1-1
MAT1-2-4
MAT1-2-1
SLA2
SS1G_4002.3
SS1G_4003.3
SS1G_4004.3
SS1G_4005.3
SS1G_4006.3
SS1G_4007.3
1 kb
α Domain
HMG Domain


B
Hind III
Hind III
5' UTR
MAT1-1-1
3' UTR
Probe 1
Probe 2
YG
3' UTR
5' UTR
HY
1 kb

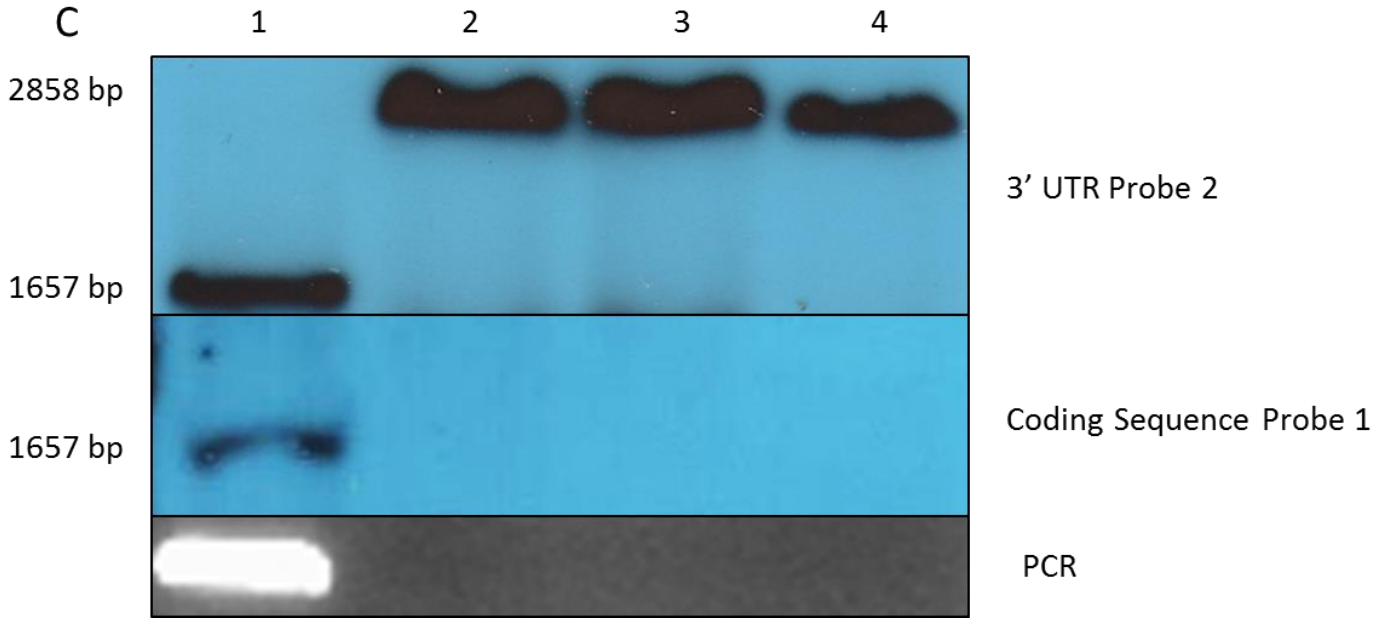

C
1
2
3
4
2858 bp
1657 bp
1657 bp
3' UTR Probe 2
Coding Sequence Probe 1
PCR

Figure 2-3. The split marker strategy for *MAT1-1-1* gene replacement. A) The *MAT* locus of *S. sclerotiorum*. B) Graphic representation of the split marker strategy. C) Genetic confirmation of the homologous recombinations. All homologous recombinations required along with the probes designed for identification of successful gene knockout (B). Genomic DNA was isolated from wild type (C, 1) and each transformant (C, 2-4) and digested with Hind III which would produce either a 1657 wild-type bp band during Southern analysis or a 2858 bp homologous recombination band when utilizing the 3' UTR probe (C). The transformants were also analyzed by PCR utilizing the coding sequence probe primers (C).

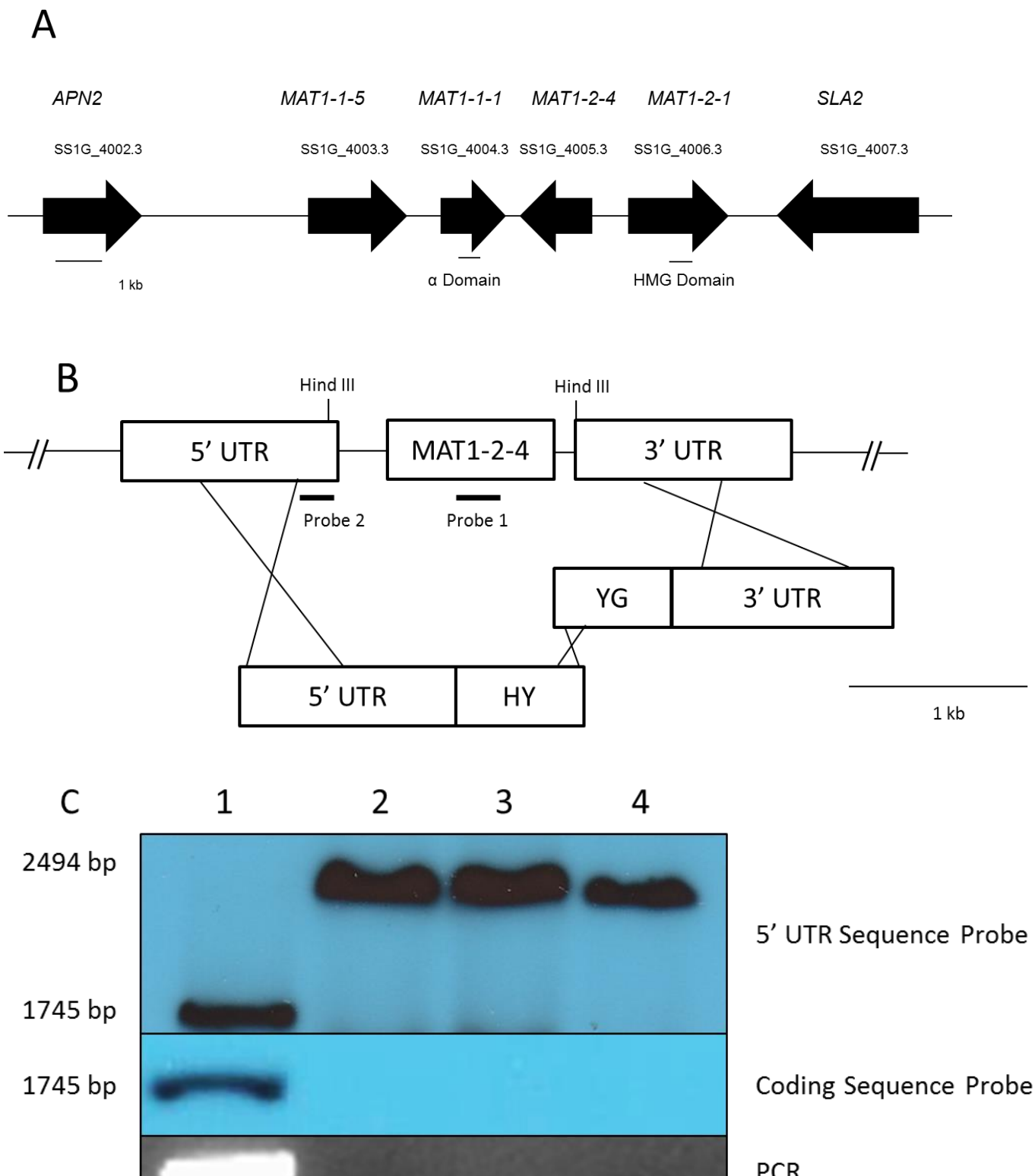

A
APN2
MAT1-1-5
MAT1-1-1
MAT1-2-4
MAT1-2-1
SLA2
SS1G_4002.3
SS1G_4003.3
SS1G_4004.3
SS1G_4005.3
SS1G_4006.3
SS1G_4007.3
1 kb
α Domain
HMG Domain
B
Hind III
Hind III
5' UTR
MAT1-2-4
3' UTR
Probe 2
Probe 1
YG
3' UTR
5' UTR
HY
1 kb
C
1
2
3
4
2494 bp
1745 bp
1745 bp
5' UTR Sequence Probe
Coding Sequence Probe 1
PCR

Figure 2-4. The split marker strategy for *MAT1-2-4* gene replacement. A) The *MAT* locus of *S. sclerotiorum*. B) Graphic representation of the split marker strategy. C) Genetic confirmation of the homologous recombinations. All homologous recombinations required along with the probes designed for identification of successful gene knockout (B). Genomic DNA was isolated from wild type (C, 1) and each transformant (C, 2-4) and digested with Hind III which would produce either a 1745 wild-type bp band during Southern analysis or a 2494 bp homologous recombination band when utilizing the 5' UTR probe (C). The transformants were also analyzed by PCR utilizing the coding sequence probe primers (C).

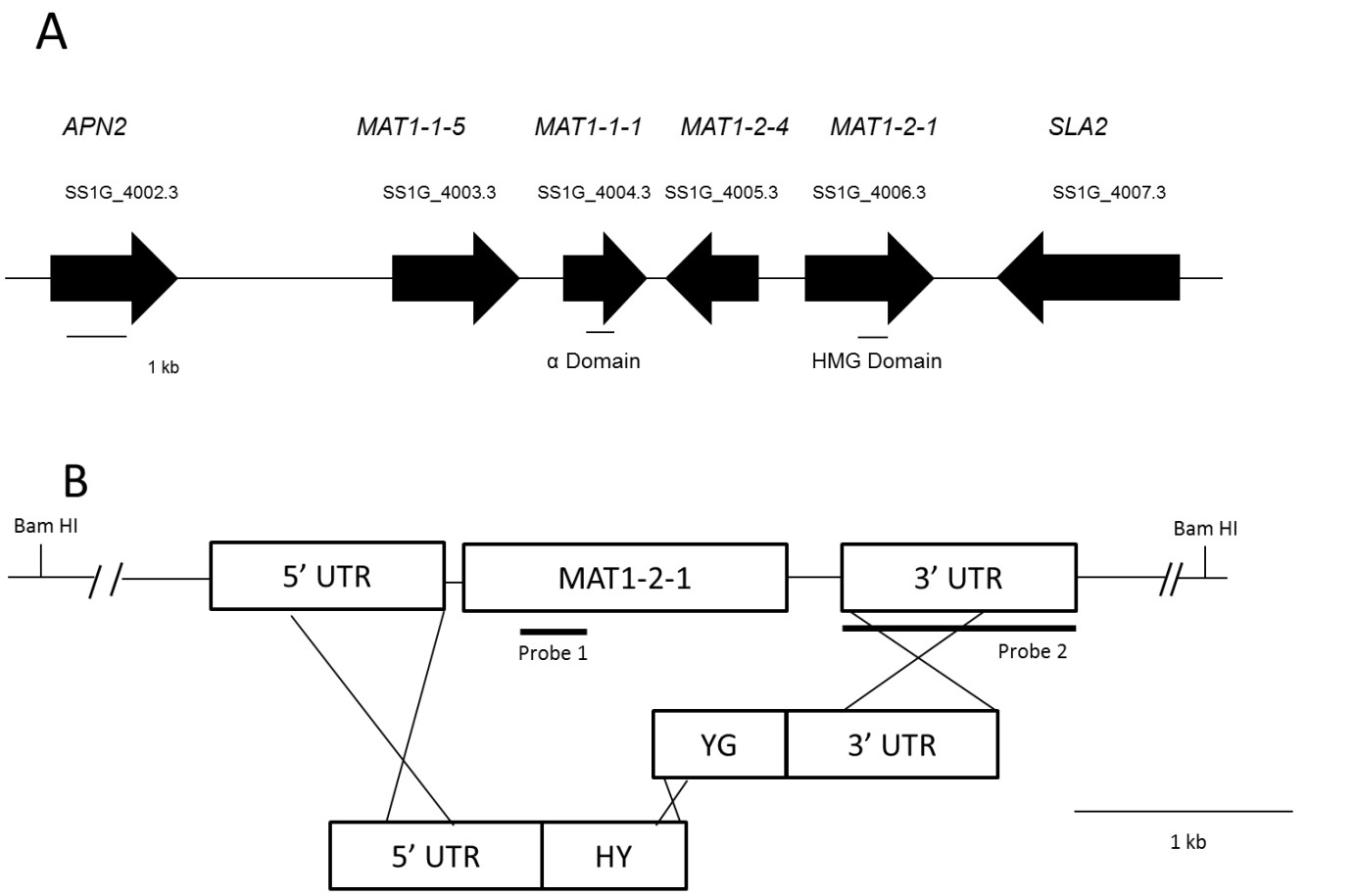

A
APN2
MAT1-1-5
MAT1-1-1
MAT1-2-4
MAT1-2-1
SLA2
SS1G_4002.3
SS1G_4003.3
SS1G_4004.3
SS1G_4005.3
SS1G_4006.3
SS1G_4007.3
1 kb
α Domain
HMG Domain
B
Bam HI
Bam HI
5' UTR
MAT1-2-1
3' UTR
Probe 1
Probe 2
YG
3' UTR
5' UTR
HY
1 kb
C
1
2
3
4
5840 bp
7759 bp
7759 bp
3' UTR Sequence Probe 2
Coding Sequence Probe 1
PCR

Figure 2-5. The split marker strategy for *MAT1-2-1* gene replacement. A) The *MAT* locus of *S. sclerotiorum*. B) Graphic representation of the split marker strategy. C) Genetic confirmation of the homologous recombinations. All homologous recombinations required along with the probes designed for identification of successful gene knockout (B). Genomic DNA was isolated from wild type (C, 1) and each transformant (C, 2-4) and digested with Bam HI which would produce either a 7759 wild-type bp band during Southern analysis or a 5840 bp homologous recombination band when utilizing the 3' UTR probe (C). The transformants were also analyzed by PCR utilizing the coding sequence probe primers (C).

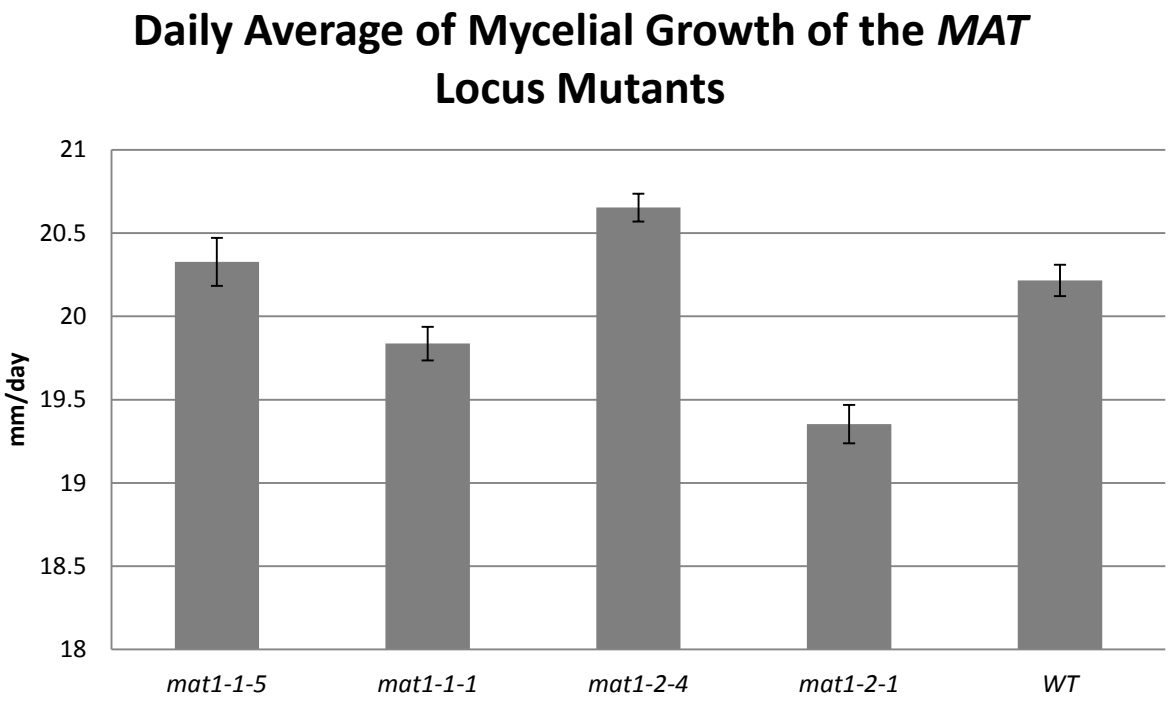


Figure 2-6. The average daily mycelial growth of the *MAT* locus mutants. All transformants were grown under 12hr light and 12 hr dark cycles in race tubes. Daily measurements of nine technical replications of each mutant were made and compared against wild type growth and showing one standard deviation. Growth was analyzed with a 2 sample t test and all mutants exhibited statistically significant growth different from wild type and each other ($p<0.01$).

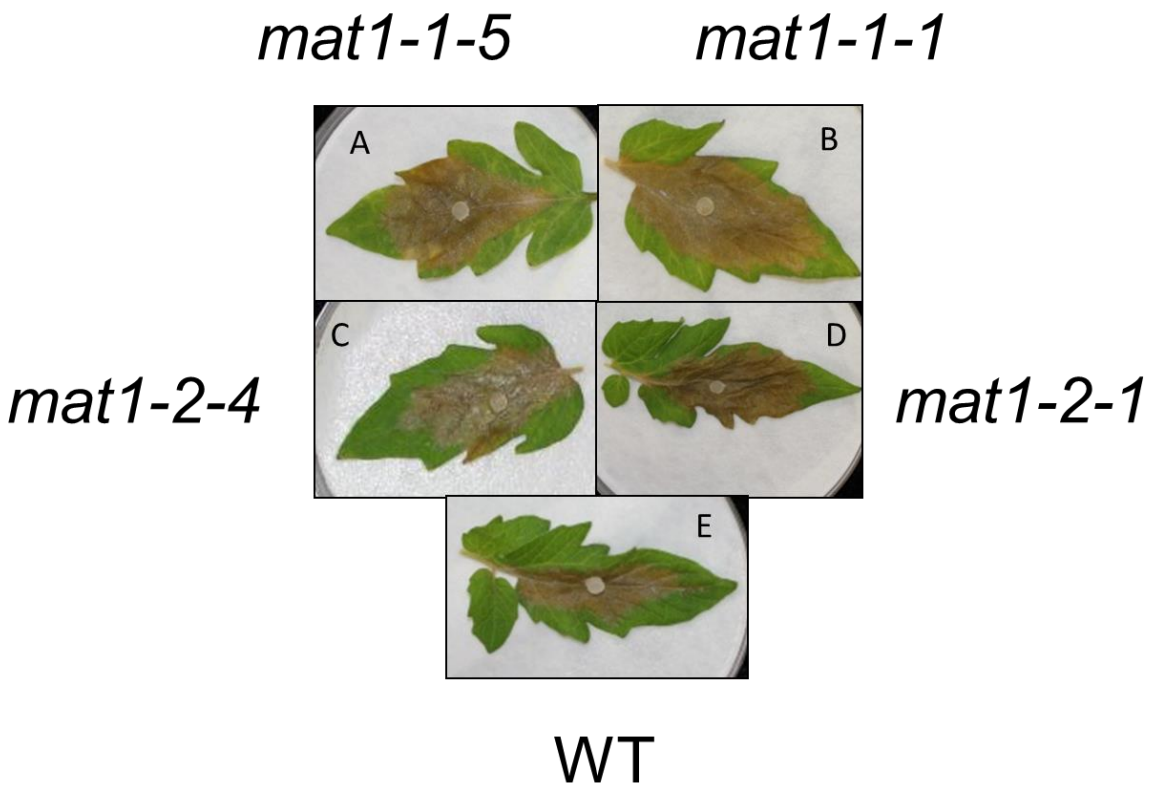


Figure 2-7. Pathogenicity assays for *MAT* locus mutants. A) *mat1-1-5* assay. B) *mat1-1-1* assay. C) *mat1-2-4* assay. D) *mat1-2-1* assay. E) WT assay. Detached tomato leaf assays were performed and inoculated with a 3mm plug of actively growing mycelia from a PDA plate. There was no differences in ability to infect and cause disease symptoms.

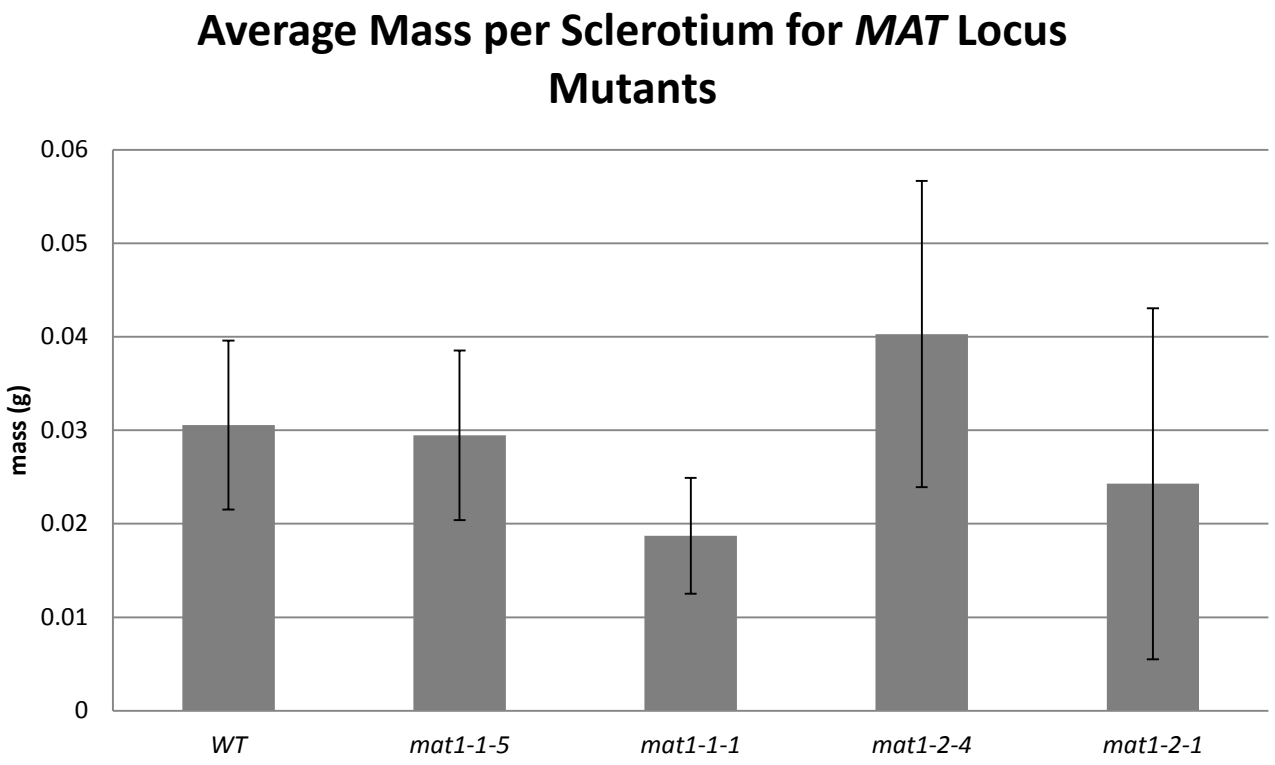


Figure 2-8. Average mass per sclerotium for *MAT* locus mutants. The average mass for sclerotium was determined by sample statistic from ten sclerotia grown in 20 cm potato plate cultures and shown with one standard deviation. *mat1-2-4* showed a higher average than wild type sclerotial mass with the remaining *MAT* locus mutants showing a decrease in average sclerotial mass however this was not found to be statistically significance due to the high variance of all the sclerotia sampled ($p>0.20$).

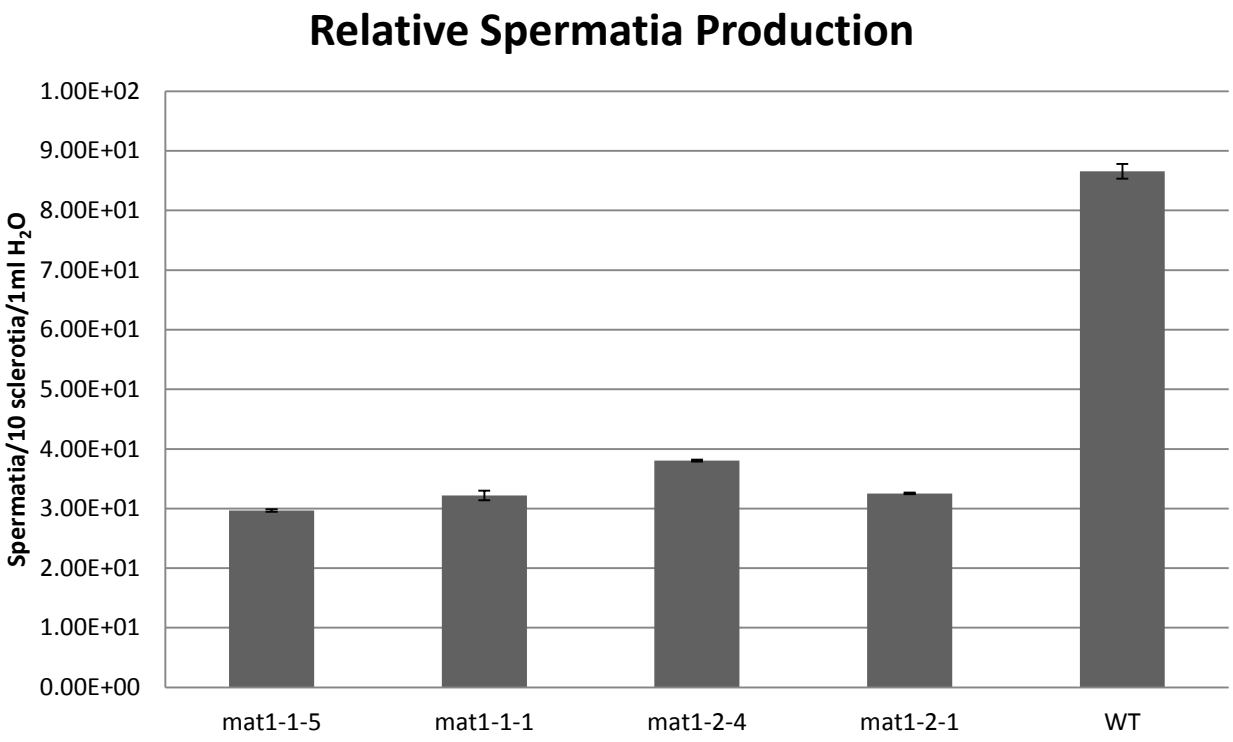


Figure 2-9. Relative spermatia production for *MAT* locus mutants. The average number of spermatia produced was calculated for 10 sclerotia from each mutant and wild type into 1 ml of water for three replications and shown with one standard deviation. All mutants showed significantly decreased spermatia production in relation to wild type ($p<0.015$) however little difference between each other ($p<0.05$).

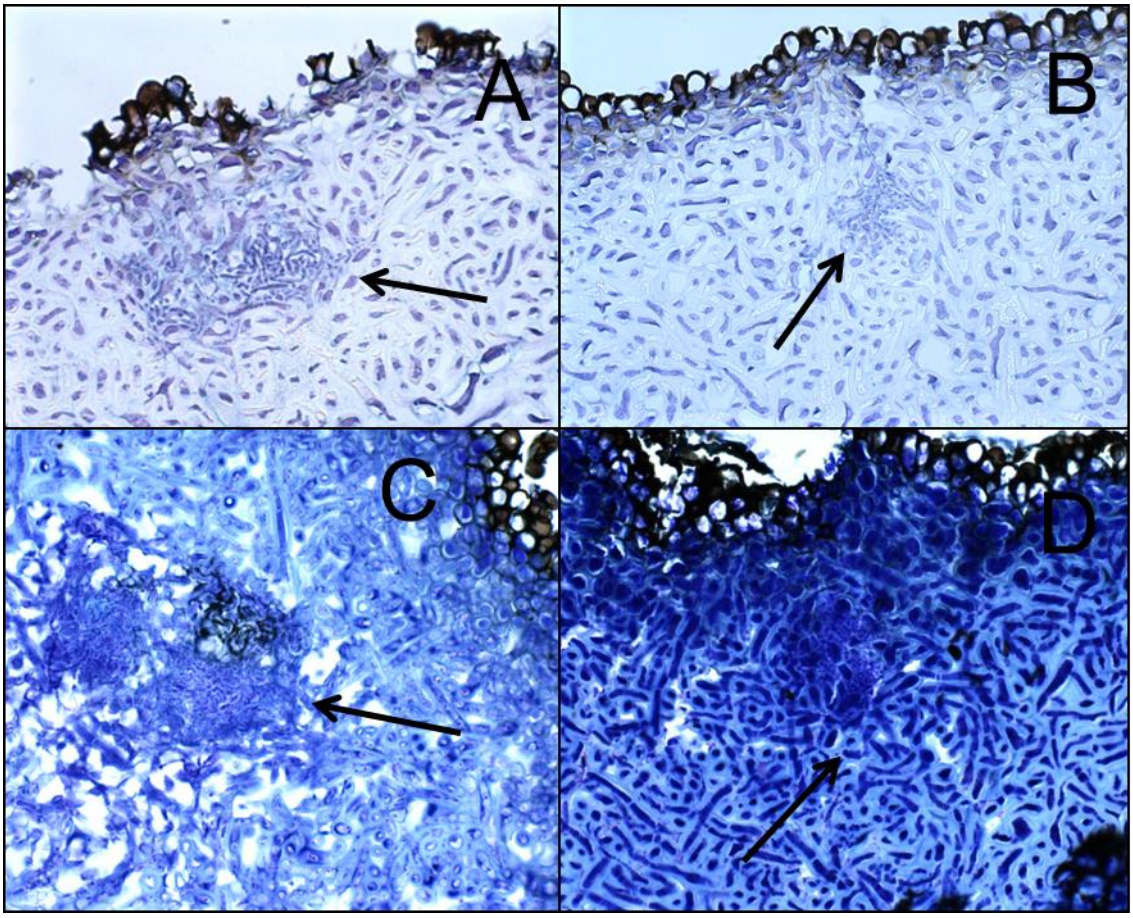


Figure 2-10. Sclerotial cross sections for *MAT* locus mutants. A) *mat1-1-5* sclerotial cross section. B) *mat1-1-1* sclerotial cross section. C) *mat1-2-4* sclerotial cross section. D) *mat1-2-1* sclerotial cross section. Cross sections stained with methylene blue of the mutant sclerotia with ascogonium indicated by arrows. No differences in timing or morphology of ascogonium development were observed among mutants and the wild type.

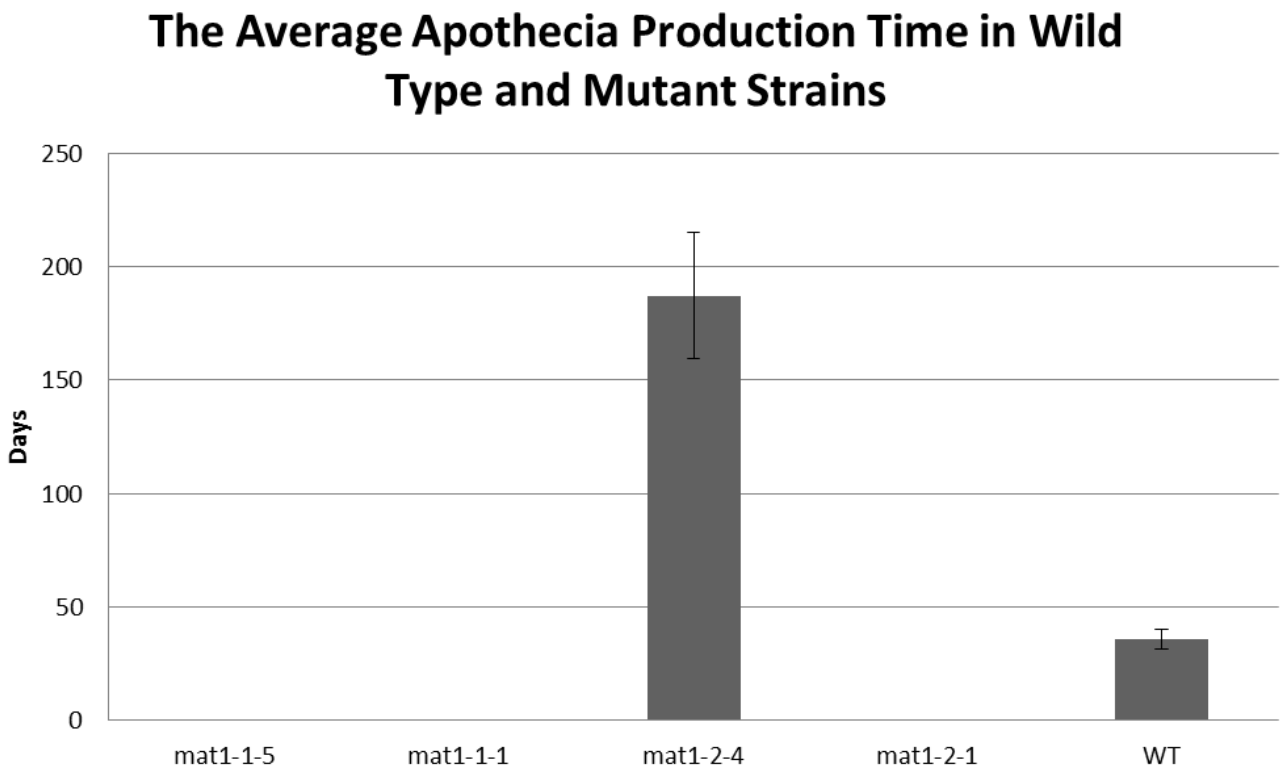


Figure 2-11. The average apothecia production time in wild type and mutant strains. Apothecia were grown in a growth chamber under constant cool white lights, moisture and temperature. The time each apothecium was first detected was recorded and the average incubation time to first detection for *mat1-2-4* (187.1 days) was found to be significantly longer ($p<0.025$) than wild type (35.7 days) incubation and shown with one standard deviation. The other *MAT* locus mutants never exhibited any signs of apothecia production.

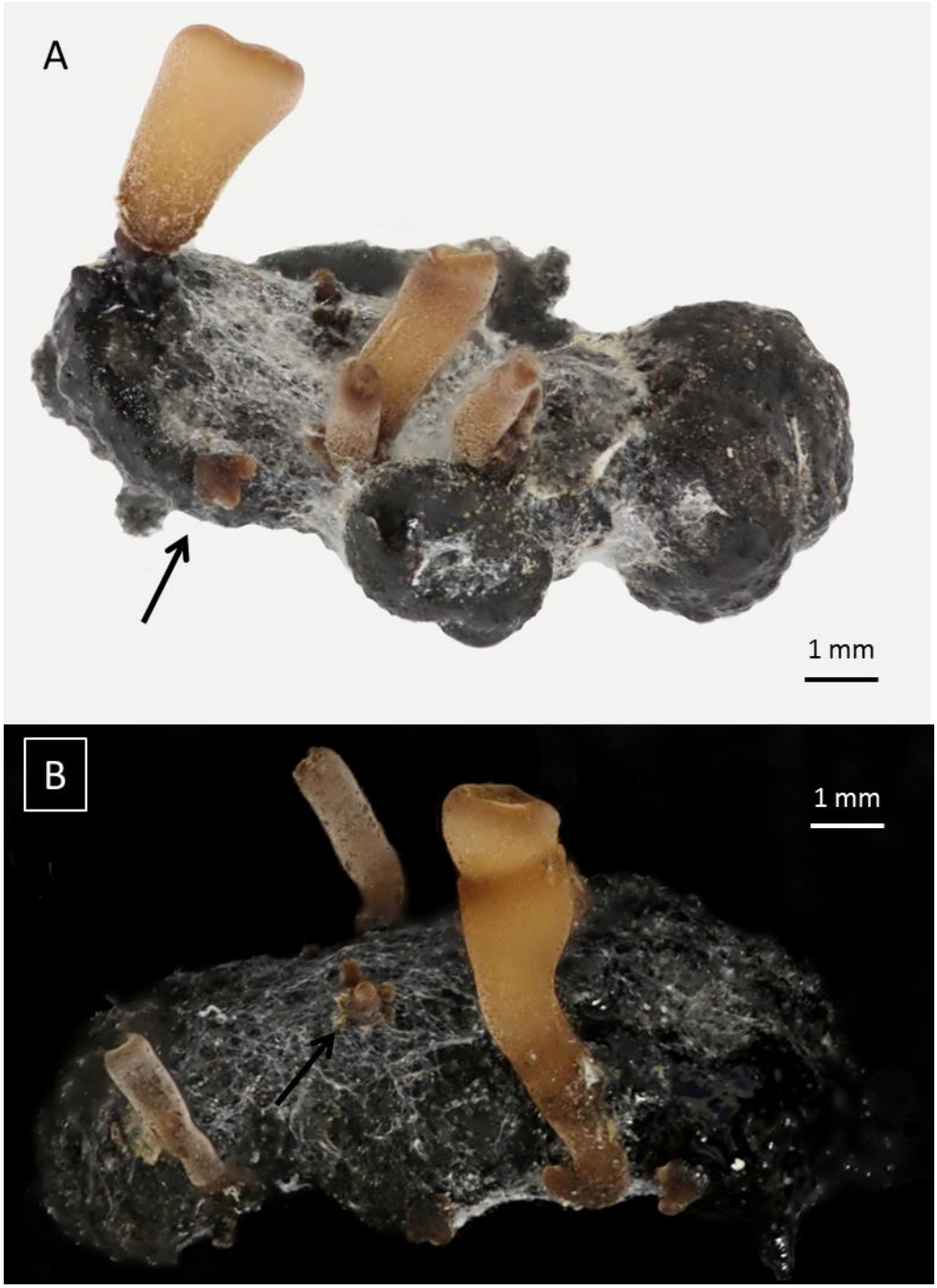


Figure 2-12. Tubular *mat1-2-4* apothecia and arrested stipes. A) *mat1-2-4* sclerotium and apothecia. B) Additional *mat1-2-4* sclerotium and apothecia. *mat1-2-4* apothecia showed a variety of phenotypes ranging from arrested stipe initials in bundles of two (A, arrowed) and three (B, arrowed) to long tubular stipes with no disc widening (A,B) and long tubular stipes with arrested disc widening.

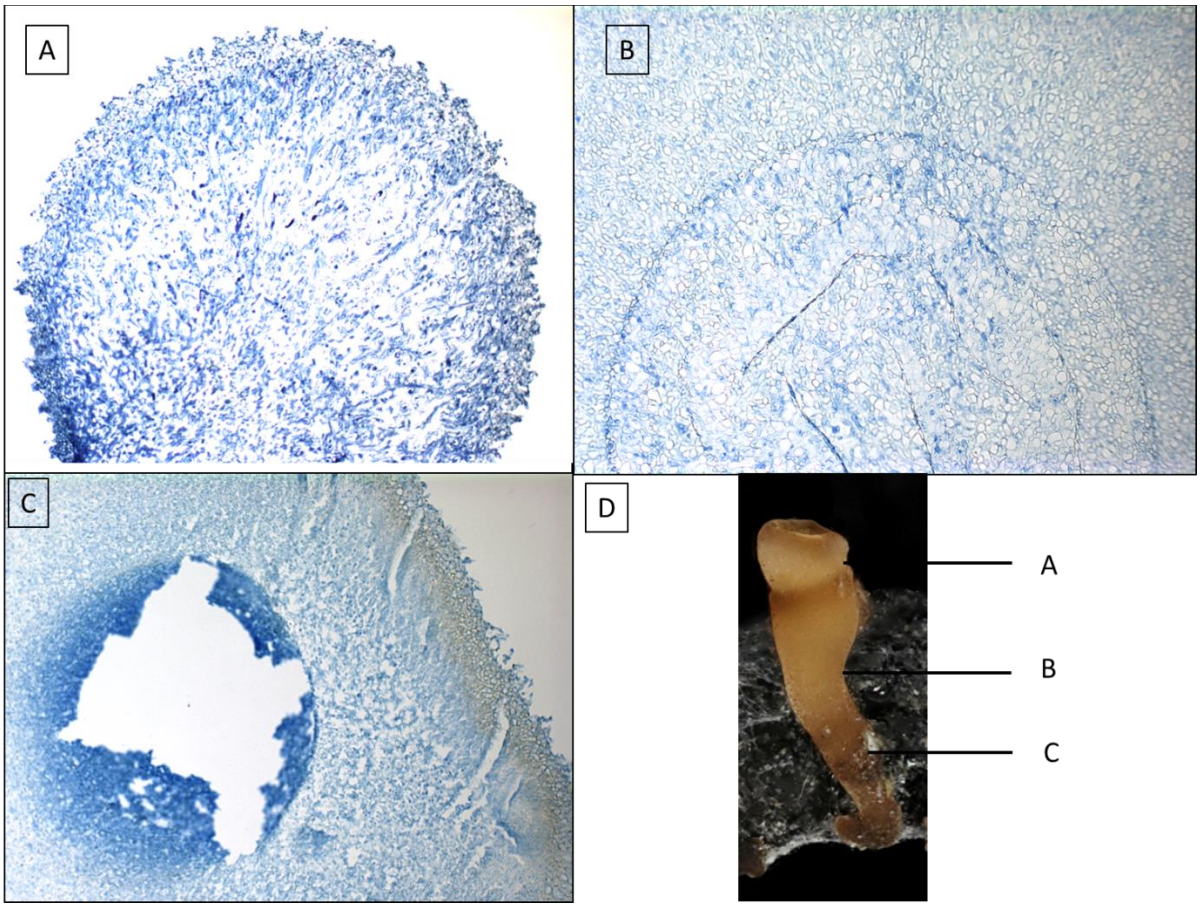


Figure 2-13. Cross sections of tubular *mat1-2-4* apothecia. A) Cross section of point A in panel D. B) Cross section of point B in panel D. C) Cross section of point C in panel D. D) *mat1-2-4* apothecia from figure 2-16 labeled for cross sections. The *mat1-2-4* apothecia showed partial disc invagination showing the uniform tissue type (A) throughout the tubular portion of the stipe and the beginning of the apothecia's attempt (B, C) at an invagination and disc formation at the more basal end of the stipe shown by the differential tissue types in circular rings from the center of the stipe cross section and small opening at the tip. Apothecia with no invagination showed no tissue differentiation in the stipe.

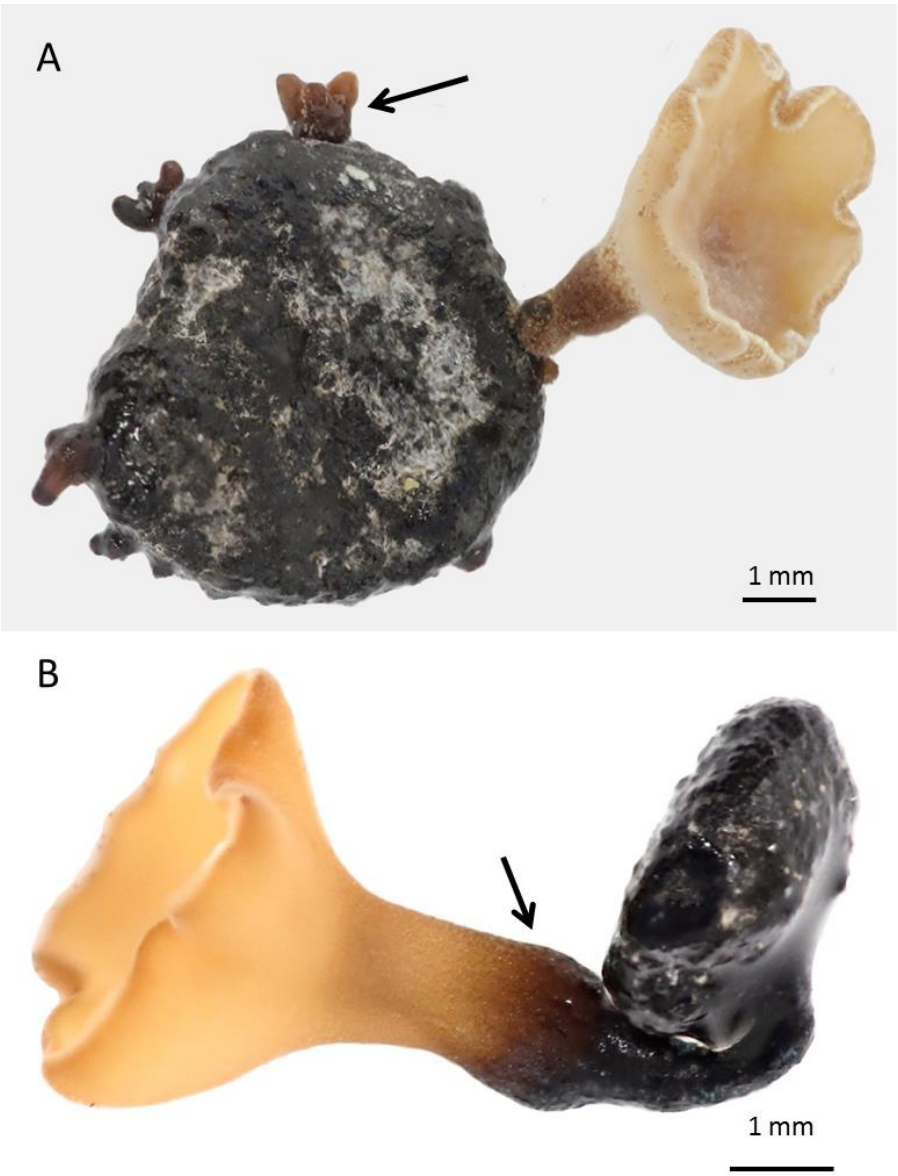


Figure 2-14. Crennulate apothecia of *mat1-2-4*. A) Arrested stipes and apothecia of the *mat1-2-4* mutant. B) Close-up of the *mat1-2-4* mutant apothecia. *mat1-2-4* apothecia showing a large number of stipes that did not develop past stage one (A, arrowed) and the commonly found widening of the stipe in the apothecia that did proceed beyond stage one (B). The apothecia that did invaginate their disc and proceed to widening of the cup did so in a variety of crenulated folded patterns.

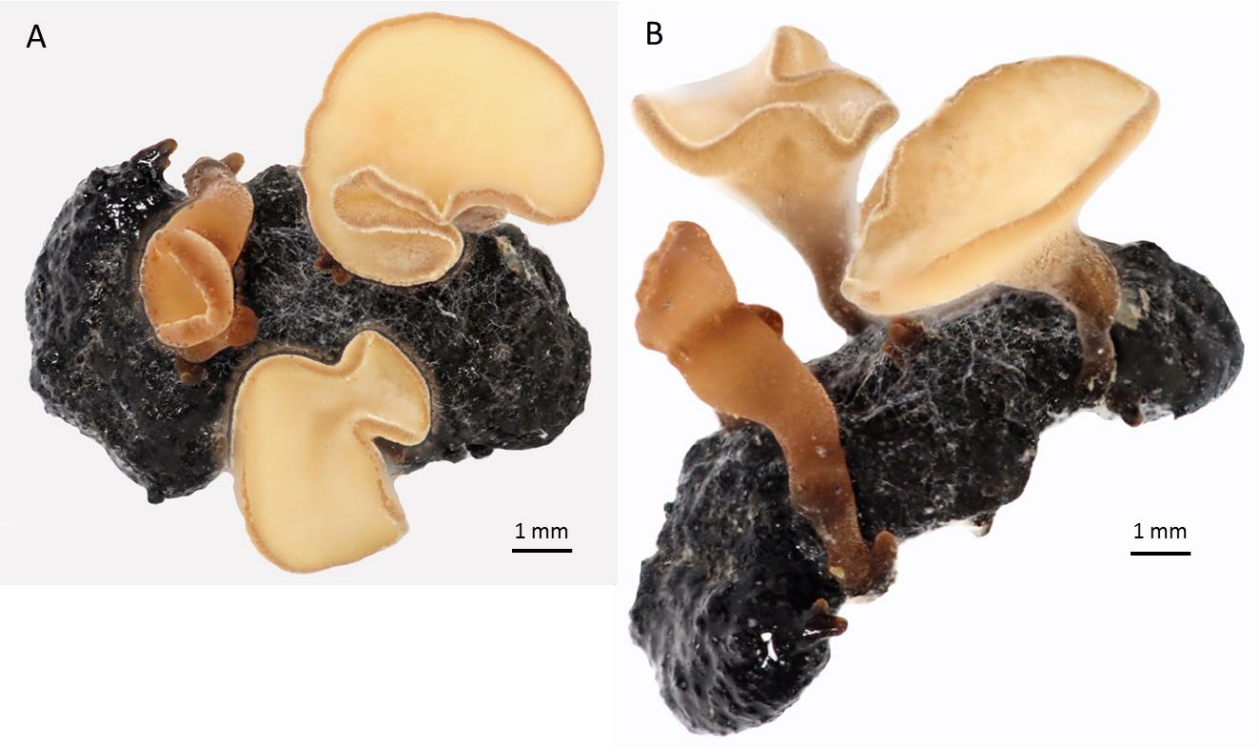


Figure 2-15. Crenulate and tubular apothecia of the *mat1-2-4 mutant*. A) Multiple apothecia of the *mat1-2-4* mutant. B) Additional apothecia of the *mat1-2-4* mutant. The *mat1-2-4* apothecia showed a wide variety of phenotypes of the stipes that proceeded beyond stage one. Apothecia commonly had widened stipes and ultra-folded crenulated discs (A, B).

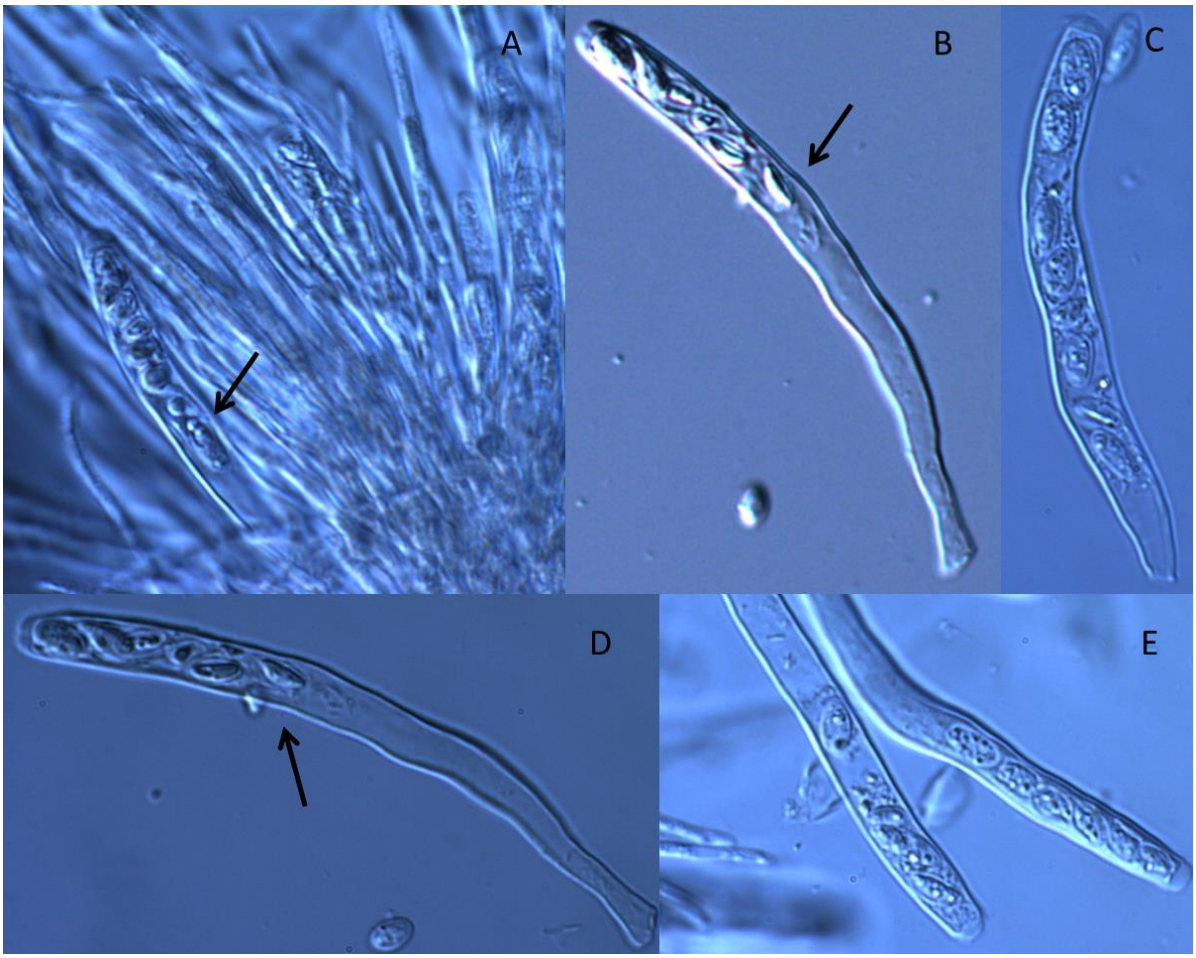


Figure 2-16. Ascus and ascospore formation in the *mat1-2-4* mutant. A) *mat1-2-4* ascus. B) *mat1-2-4* ascus. C) WT ascus. D) *mat1-2-4* ascus. E) *mat1-2-4* ascus. The ascus from *mat1-2-4* (A,B, D,E) showed an abnormal number of ascospores formed compared to wild type (C). When six ascospores were found two of the ascospores were commonly found to have a shriveled appearance (A,B,D, arrowed).

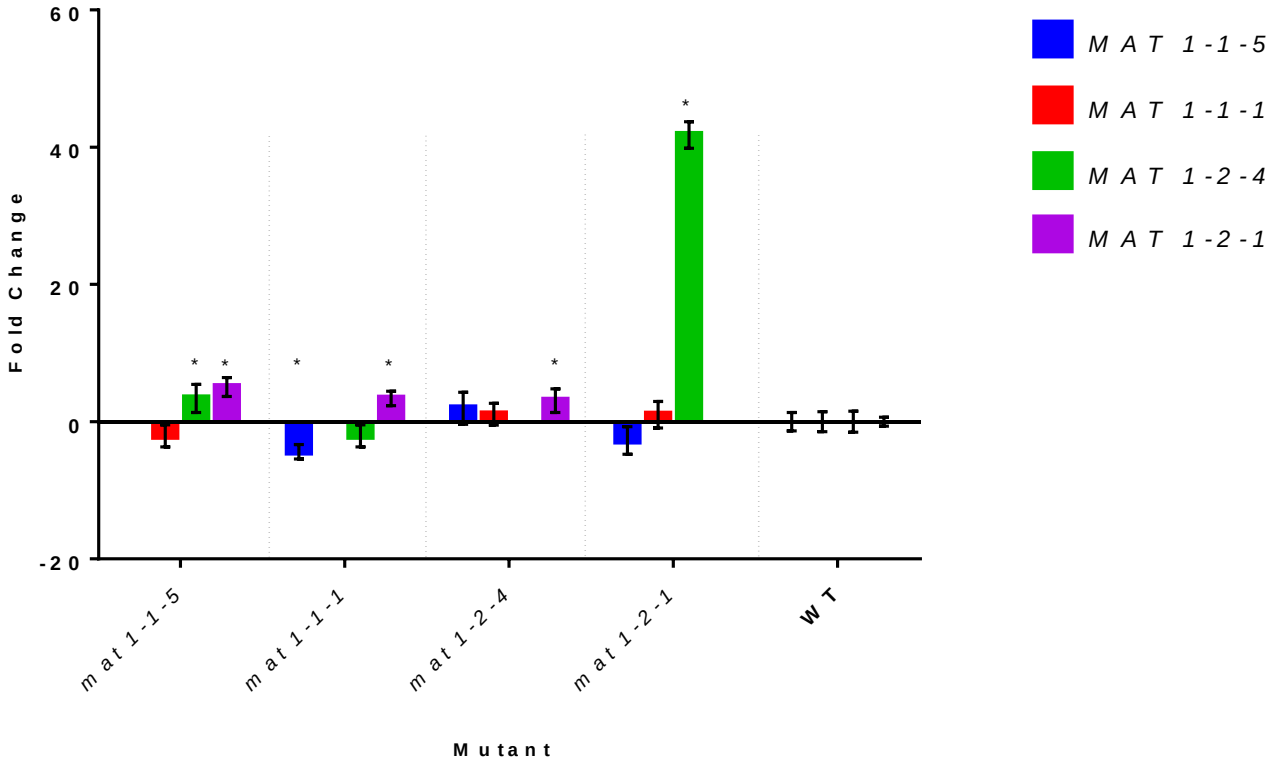


Figure 2-17. *MAT* gene expression in mycelial tissue of the *MAT* locus mutants. Gene expression in mycelial tissue of the four *MAT* locus gene mutants were compared against wild type mycelial gene expression using the $\Delta\Delta C_T$ method. Three biological replications with three technical replications were performed and statistically significant differences ($p<0.05$) are marked with a star. Each of the *MAT* locus genes were compared against wild type expression levels and the fold change in gene expression is displayed showing standard deviations for the mean.

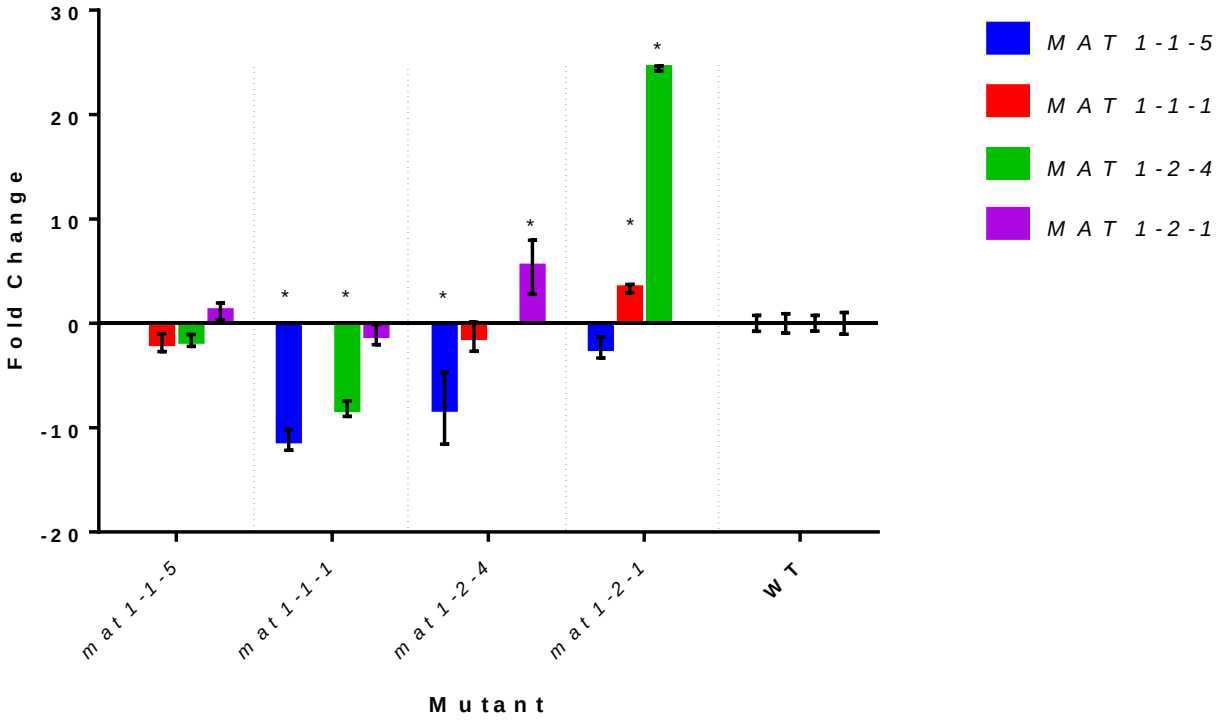


Figure 2-18. *MAT* gene expression in stage three sclerotial tissue of the *MAT* locus mutants. Gene expression in stage three sclerotial tissue for the four *MAT* locus gene mutants was compared against wild type expression using the $\Delta\Delta C_T$ method. Three biological replications with three technical replications were performed and statistically significant differences ($p<0.05$) are marked with a star. Each of the *MAT* locus genes were compared against wild type expression levels and the fold change in gene expression is displayed showing standard deviations for the mean.

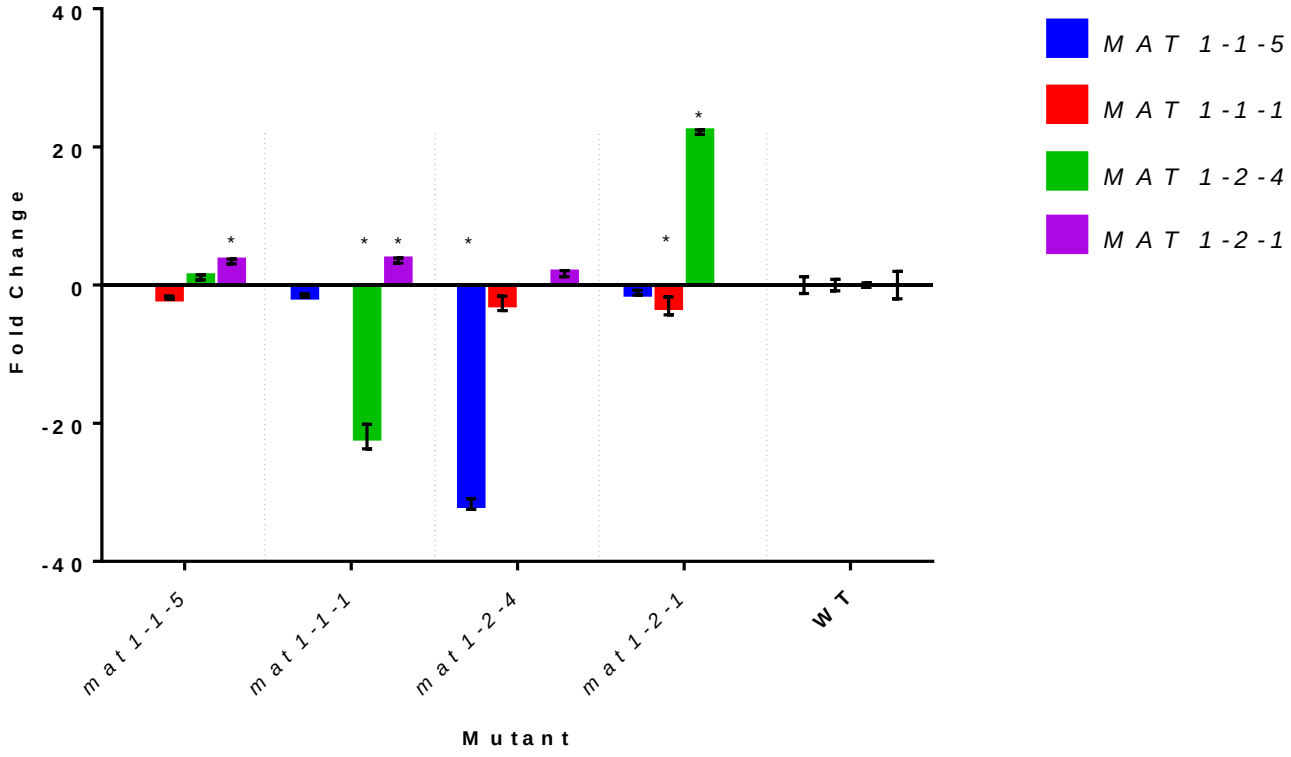


Figure 2-19. *MAT* gene expression in stage five sclerotial tissue of the *MAT* locus mutants. Gene expression in stage five sclerotial tissue for the four *MAT* locus gene mutants was compared against wild type expression using the $\Delta\Delta C_T$ method. Three biological replications with three technical replications were performed and statistically significant differences ($p<0.05$) are marked with a star. Each of the *MAT* locus genes were compared against wild type expression levels and the fold change in gene expression is displayed showing standard deviations for the mean.

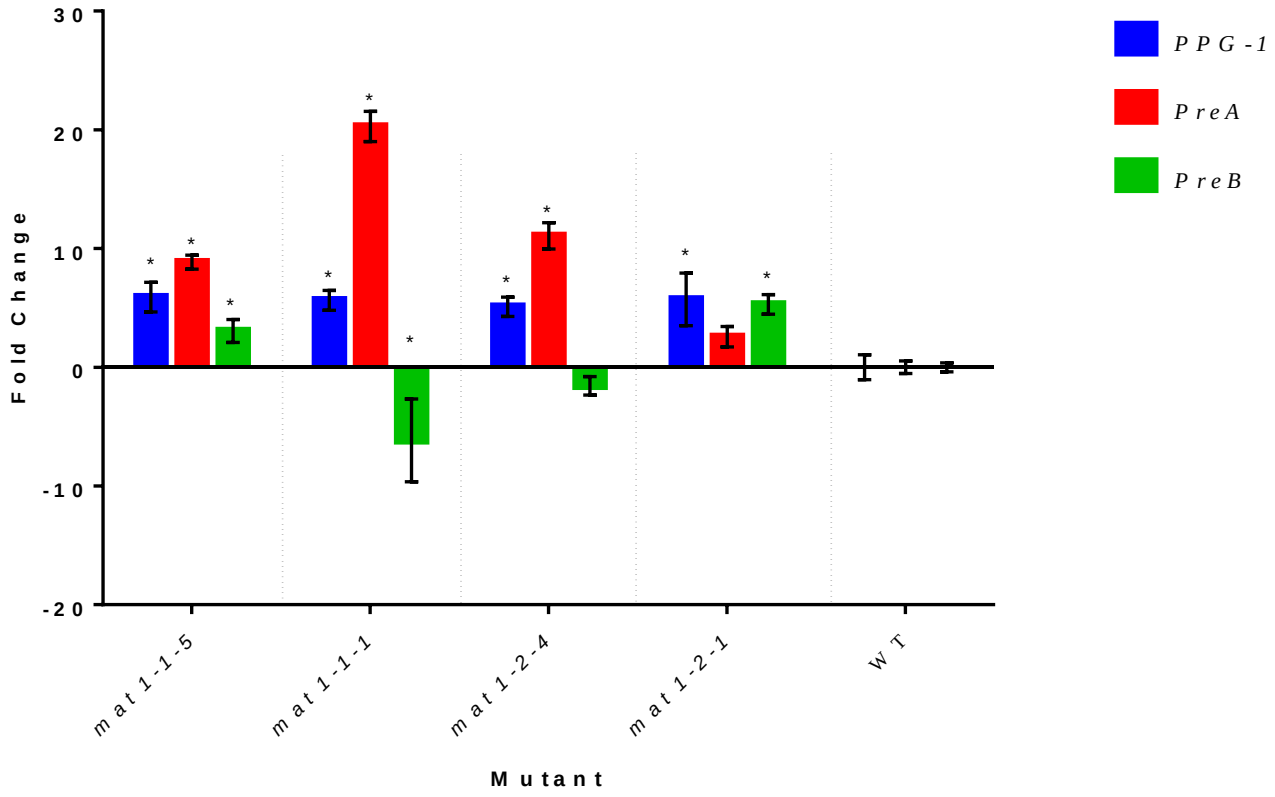


Figure 2-20. Putative pheromone and pheromone receptors gene expression in mycelial tissue of the *MAT* locus mutants. Gene expression in mycelial tissue for the four *MAT* locus gene mutants was compared against wild type expression using the $\Delta\Delta C_T$ method. Three biological replications with three technical replications were performed and statistically significant differences ($p<0.05$) are marked with a star. Each of the putative pheromone and pheromone receptor genes were compared against wild type expression levels and the fold change in gene expression is displayed with standard deviations of the mean.

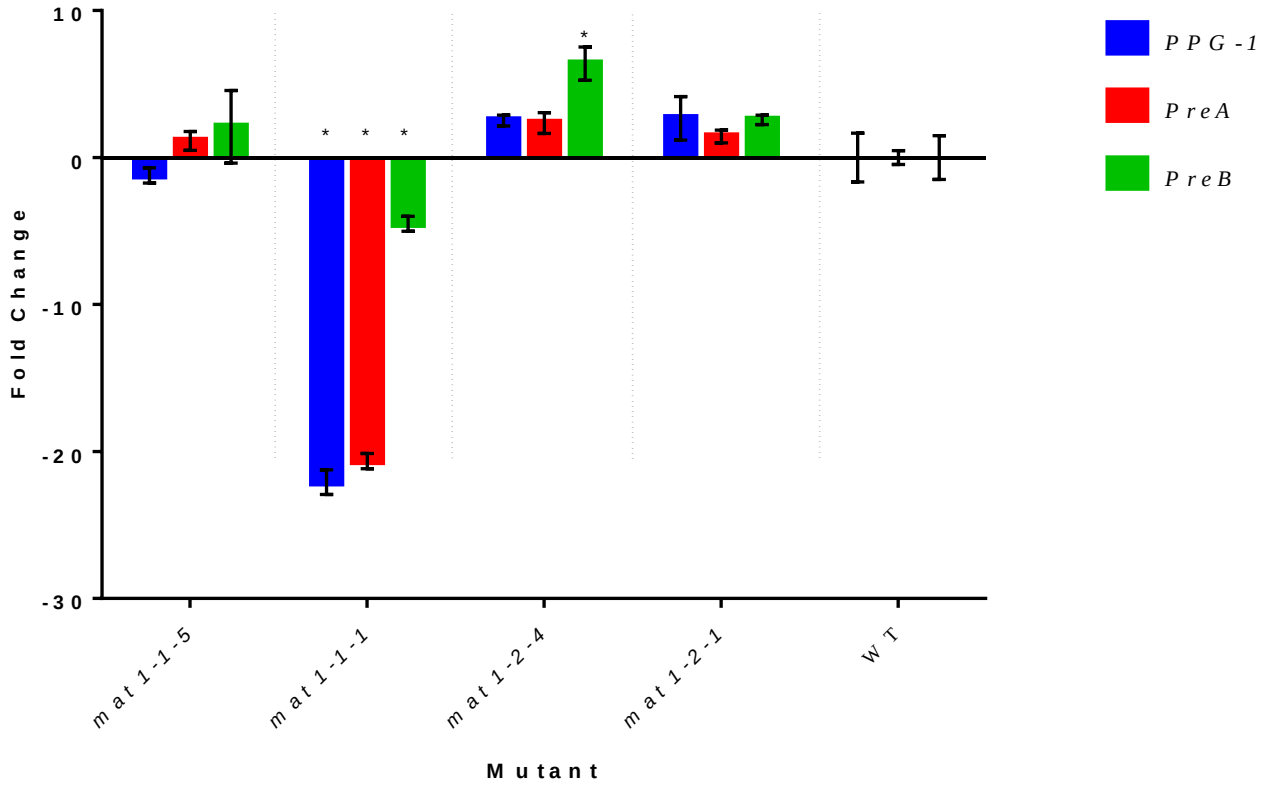


Figure 2-21. Putative pheromone and pheromone receptors gene expression in stage three sclerotial tissue of the *MAT* locus mutants. Gene expression in mycelial tissue for the four *MAT* locus gene mutants was compared against wild type expression using the $\Delta\Delta C_T$ method. Three biological replications with three technical replications were performed and statistically significant differences ($p<0.05$) are marked with a star. Each of the putative pheromone and pheromone receptor genes were compared against wild type expression levels and the fold change in gene expression is displayed with standard deviations of the mean.

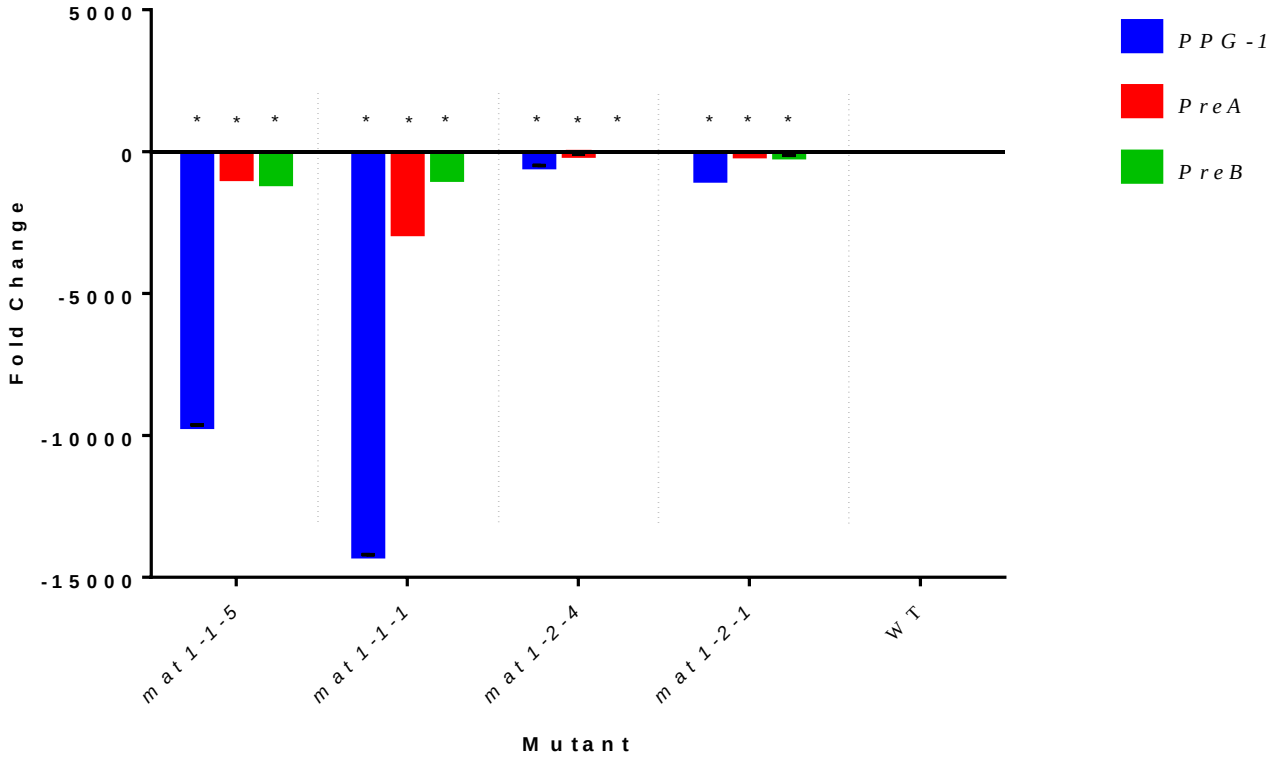


Figure 2-22. Putative pheromone and pheromone receptor gene expression in stage five sclerotial tissue of the *MAT* locus mutants. Gene expression in mycelial tissue for the four *MAT* locus gene mutants was compared against wild type expression using the $\Delta\Delta C_T$ method. Three biological replications with three technical replications were performed and statistically significant differences ($p<0.05$) are marked with a star. Each of the putative pheromone and pheromone receptor genes were compared against wild type expression levels and the fold change in gene expression is displayed with standard deviations of the mean.

CHAPTER 3
CONCLUSIONS

Mutation of the *MAT* genes of *S. sclerotiorum* manifest as distinct phenotypic alterations in the sexual life cycle and various aspects of the secondary sexual characteristics. I have determined that the *MAT1-1-1*, *MAT1-1-5* and *MAT1-2-1* genes are necessary for the initiation of apothecia while the *MAT1-2-4* gene is necessary for the proper timing and development of apothecial discs. The two core mating type gene mutants *mat1-1-1* and *mat1-2-1* appear to slow mycelial growth and decrease spermatia production. The *mat1-1-5* and *mat1-2-4* both show an increase in mycelial growth rate and decrease in spermatia production.

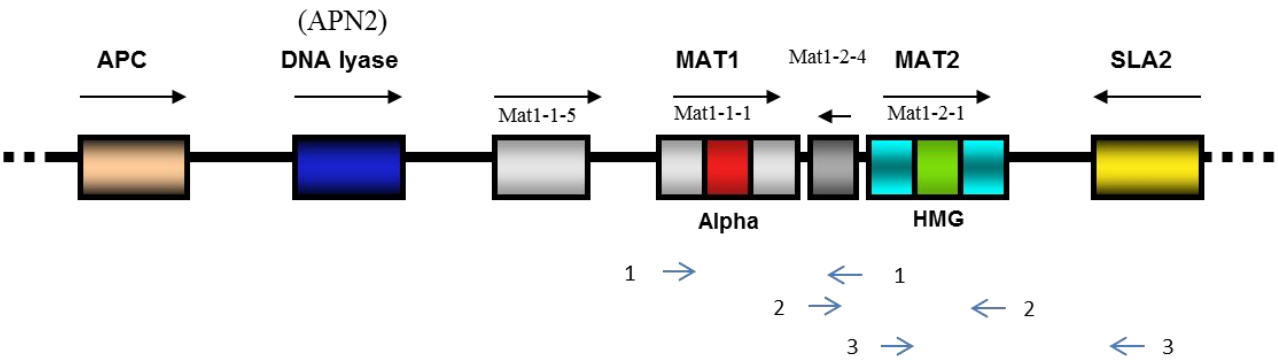
The *mat1-2-4* apothecial phenotype is consistent with phenotypic observations of the orthologous gene in *B. cinerea* (van Kan, 2011) where a disruption in disc formation and apical stipe depression was observed. The seven to twenty-eight week delay of apothecia initiation along with the various types of apothecia produced where disc elongation was attempted at premature to delayed states shows a function in the *MAT1-2-4* gene in timing of stipe development and initiation and timing of disc differentiation. The *MAT1-2-4* gene also plays a role in ascospore formation where a full eight ascospores were never observed and the possible degeneration of ascospores was observed. This could possibly be due to a genetic or epigenetic determinant, cytoskeletal instability in meiotic and mitotic divisions or nucleic acid recognition as in other Ascomycetes.

All *MAT* locus mutations showed a change in gene expression and instances where the mutant altered or reversed wild type trends in gene expression of *mat* genes. There appears to be a link between *MAT1-2-1* gene regulation and *MAT1-2-4* regulation in tissues of all types and I hypothesize that downstream regulation or their colinearity is responsible for

this. Putative pheromone and pheromone receptor transcripts were altered as seen in other *MAT* gene mutants, when measuring mycelial tissue an increase in gene expression is observed. This is reversed once the *MAT* gene mutants started stage 5 sclerotial development where very significant decreases to essential elimination of any transcripts. Future studies into the roles of the putative pheromone and pheromone receptors and the characterization of other genes regulated by the *MAT* locus hopefully will provide a more complete understanding to the sexual regulation of *S. sclerotiorum*.

APPENDIX A
THE 250 BASEPAIR INVERSION IN THE *MAT* LOCUS OF *SCLEROTINIA SCLEROTIORUM*

During investigation of the *MAT* locus of *S. sclerotiorum* probes were made for the coding sequence of the *MAT1-1-1* and *MAT1-2-1* genes. These coding sequences were located in the middle to 3' end of each gene and used for confirmation of gene knockouts which had the coding sequence replaced with a hygromycin gene cassette. A second probe was made using UTR sequences which would exhibit a size change in the banding pattern for homologously recombined knockouts that were homokaryotic. PCR was finally used as a third method of confirming the knockouts where the coding sequence probe primers were used to detect the presence or absence of the genes (Fig 2-3, C; Fig 2-5, C). When a transformant showed signs of being a homologously recombined homokaryote for the UTR probe and PCR analysis, the transformant would also show an absence of the band predicted in the coding sequence. However in some primer combinations, secondary band was observed in both the wild type and successful transformants. This band was a result of a stretch of sequence that was found in both the *MAT1-1-1* sequence and *MAT1-2-1* sequence which was later defined to be part of an inversion point (Chitrampalam *et al*., 2013). This was confirmed by me at the same time by running PCR's on tetrads isolated from a two isolates of *S. sclerotiorum* and sequencing of the inversion sites in both types to determine exactly what was being inverted (Fig A-1 through A-3). I determined that the inversion was happening 50% of the time in each ascus and that the inversion would replace the critical HMG encoding domain of *MAT1-2-1* along with the rest of the lineage specific DNA sequence and split the alpha domain sequence in *MAT1-1-1* between helices three and four of the DNA binding domain (Fig A-4, Fig A-5). *MAT1-1-1* reads directly into *MAT1-2-1* retaining critical helices 1-3 which make up the ancestral form of the alpha box sequence composed of 368 base pairs or 122 amino acids from the start site (Martin *et al*., 2010).



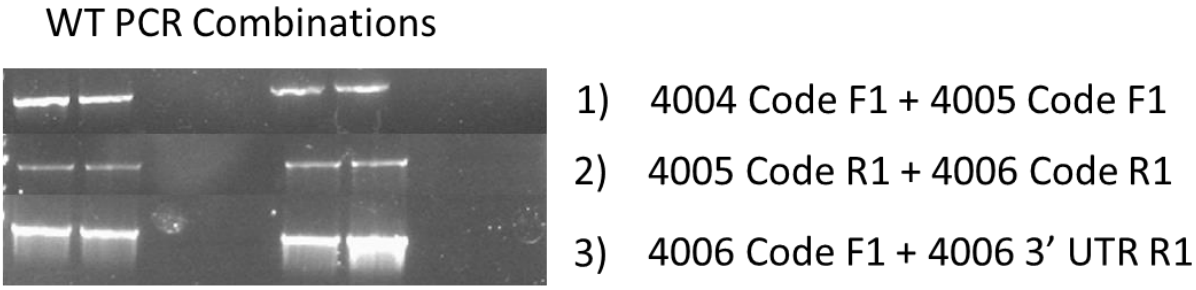


Figure A-1. PCR analysis and primer pairs used to determine "wild type" arrangement of the *MAT* locus in *S. sclerotiorum* tetrad number one.

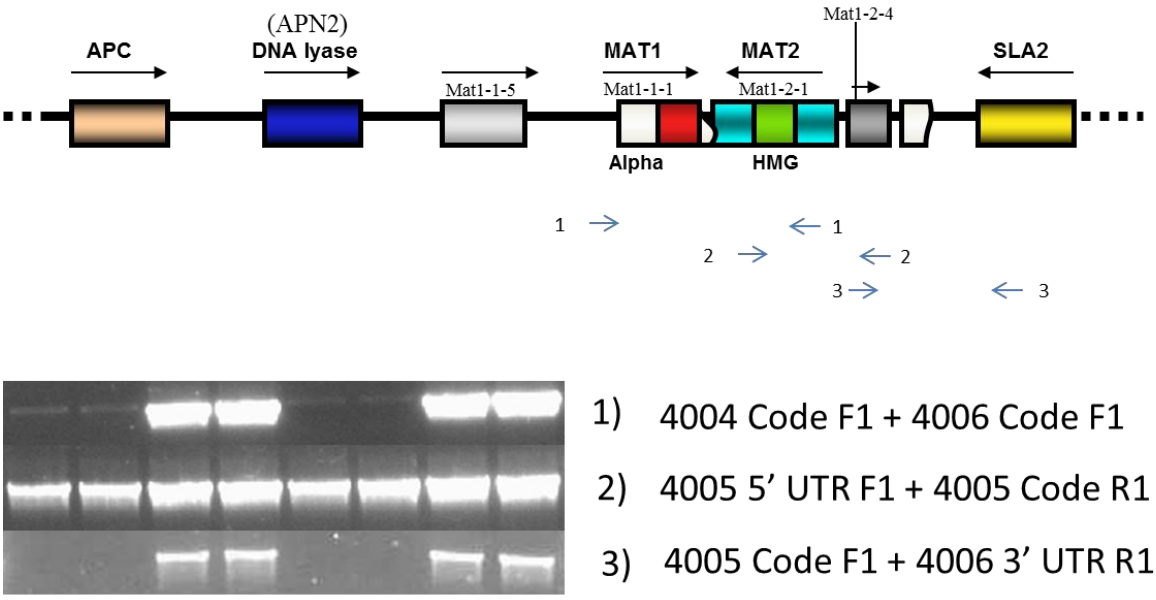


Figure A-2. PCR analysis and primer pairs used to determine "inverted wild type" arrangement of the *MAT* locus in *S. sclerotiorum* tetrad number one.

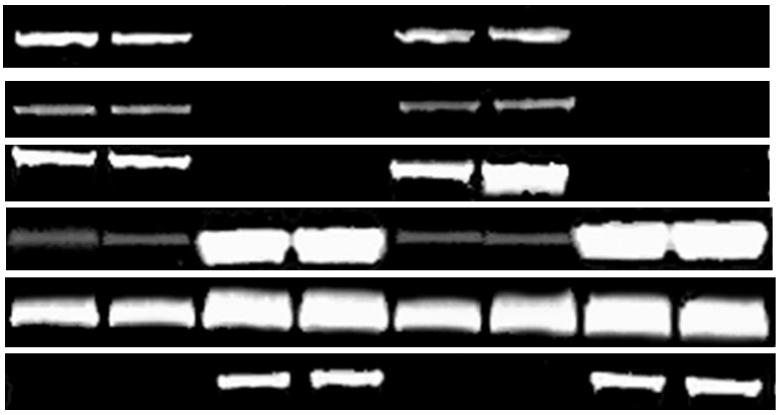


Figure A-3. PCR showing the *MAT* locus inversion in a second tetrad of *S. sclerotiorum*.

```
CLUSTAL 2.1 multiple sequence alignment


4006            MSLSTLRLGKWVPHRGTILETPLADVPYPAVDITPVEAYGIPGLSKEIYDAMRTFFNELD 60
consensus       ------------------------------------------------------------


4006            LYNALDINPWCELKDSQYLGIGTPERQILLHFYEQHTDRKGVFVRDAYVSRIFLAPLDEF 120
consensus       ------------------------------------------------------------


4006            EDKQMLAVAGFTNLCLEPLNVEHRRMMNGSWDENCTILPTNVGVTLCSGSGKKHLSPSLP 180
consensus       ------------------------------------------------------------


4006            AALKPKIPRPANEWILYRADNHIPIKKAYPGITNNEISSIIAGMWA-AETPERRLKYKIR 239
consensus       ------IPRPPNAFILYRQAHHAEVKAANPGLHNNEISVIIGKMWK-AESPEVKAYYKNL 53
                      ****.* :****  :*  :* * **: ***** **. **  **:** :  **

4006            ADLLKEAHKKA-------YPTYKYAPRKPSEKKRRASKKTLTKPTTNQLSHNINTTTSSI 292
consensus       AEEEKAEHARE-------YPDYRYTPRKPSEKKRRAT---------------------- 83
                *:  *  * :        ** *:*:***********:

4006            SFPIANNNSIDNSDLNTFGLNIHINHSNITEQQLQSENSRLTYNDQSIGMNFTSTESWQM 352
consensus       ------------------------------------------------------------


4006            VQQQDLYDFFSPRQITLGATESDNVQICQGSSSDMCWKRTDI 394
consensus       -------------------------------------------
```

Figure A-4. Protein sequence alignment of inverted *MAT1-2-1* against consensus protein sequence of HMG domain. This ClustalW alignment shows that the inverted portion of the gene is replaced and maintained with the HMG domain intact.

```
CLUSTAL O(1.1.1) multiple sequence alignment


1       ---------------------------------------------AKR----------PL
2       ---------------------------------------------AKR----------PL
3       ---------------------------------------------AKR----------PL
ss      MILNVLGNIPQKYKSAILSVLWGRDPFHAKWSILARAYTLMRDTNVRRTVSEYLALVCPY
                                                      .:*          *

1       NGFIAFRTYYL---KLF-PDT---------------------------------------
2       NGFIAFRTYYL---KLF-PDT---------------------------------------
3       NGFIAFRTYYL---KLF-PDT---------------------------------------
ss      IGILAVNDYLTDLNWIFETNEEGIVCLRQTSPSDIRSFPAHIARTTLTDLDVITFCGSQG
         *::*.. *      :*  :

1       ------------------------------------------------------------
2       ------------------------------------------------------------
3       ------------------------------------------------------------
ss      YLPAATAAGIVQGWNRMHPNANMAIQGTLPVTQHIASTQNAYGAWEMPKGVIVAPPPKPA


1       --------------------QQKDASGFLTQLW--------------------------
2       --------------------QQKDASGFLTQLW--------------------------
3       --------------------QQKDVSGFLTQLW--------------------------
ss      FDHPLTYTLSTPLPTGTLPPPPPWSTGMMAGYWSQDNGSIDLNGLENQFDQFNPTSLGDA
                             :*:::  *

1       -------------------
2       -------------------
3       -------------------
ss      NSLYVPGDISPPSDLTDS*
```

Non-Inverted Sequence:
MILNVLGNIPQKYKSAILSVLWGRDPFHAKWSILARAYTLMRDTNVRRTVSEYLALVCPYIGILAVNDYLTDLN
WIFETNEEGIVCLRQTSPSDIRSFPAHIARTTLTDLDVITFCGSQGYLPAATAA

Sequence Translocated During Inversion:
GIVQGWNRMHPNANMAIQGTLPVTQHIASTQNAYGAWEMPKGVIVAPPPKPAFDHPLTYTLSTPLPTGTL
PPPPPWSTGMMAGYWSQDNGSIDLNGLENQFDQFNPTSLGDANSLYVPGDISPPSDLTDS*

Figure A-5. MAT1-1-1 protein sequence alignment of *S. sclerotiorum* against the ancestral form of the alpha box domain. This alignment shows that ~40% of it is left behind in the inversion which contains the critical three helices of the ancestral alpha box DNA binding domain. The portion of the inverted sequence not inverted would contain helices 1-3, the primary DNA binding domains, and the remaining *MAT1-1-1* sequence of the inverted sequence that is translocated would be helix four, just outside of the crucial DNA binding domain and will contain any lineage specific sequences of the alpha box.

APPENDIX B
SCLEROTINIA TRIFOLIORUM MAT LOCUS INVESTIGATION

The mating type locus of *Sclerotinia trifoliorum* has been demonstrated to perform unidirectional mating type switching (Glass and Kudlau, 1992). A mechanism of altering the mating type locus of *Sclerotinia sclerotiorum* has recently been described and hypothesized to be present in *S. trifoliorum* (Chitramalam et al., 2013). Yet the arrangement of the *MAT* locus of *S. trifoliorum* has yet to be described. Contig analysis of *S. trifoliorum* has confirmed the presence of the four *MAT* genes found in *S. sclerotiorum* but in a different orientation. The *MAT1-2-4* reads into *MAT1-1-1* which ends with the *SLA2* gene running in the opposite direction (Fig B-1). This alone indicates a novel mechanism of *MAT* locus rearrangement is occurring since the position and orientation of the *MAT1-2-4* and *MAT1-1-1* genes in the same direction next to *SLA2* are not possible with the method described in Chitramalam *et al*., 2013. The 250 bp repeat located at the 3' end of *MAT1-1-1* in *S. sclerotiorum* would not transfer the entire portion of the *MAT1-1-1* gene and would not place it in the same direction as *MAT1-2-4*.

The *MAT1-2-1*, *MAT1-2-1* and *APN2* genes were not able to locate proximal to the identified *MAT* locus genes but sequences identifying them are located on other contigs. Amplification of the *APN2* 3' UTR region and *MAT1-1-5* coding sequence was achieved but sequencing reactions were unable to link the two genes together in sequencing reactions. This sequence may represent repetitive sequence found next to *APN2* and *MAT1-1-5* predicting an absence of the *APN2* gene or a more distal relationship than expected when compared to the *S. sclerotiorum MAT* locus. *MAT1-2-1* and the contig it was located in never produced an amplification product with any of the *MAT* locus gene primers indicating a distal placement in the genome. A short sequence (113 bp) that matched with 98% identity to the *S. sclerotiorum MAT1-2-1* sequence starts on the 5' end of *MAT1-2-4* is the only indication to date that *MAT1-2-1* exists until new sequencing data is available.

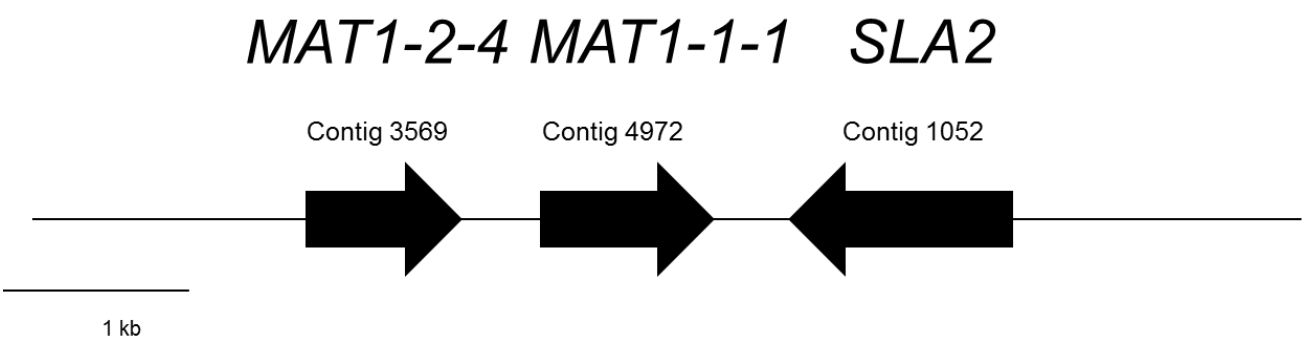


Figure B-1. The partial *MAT* locus arrangement of *S. trifoliorum*. PCR amplification of the coding sequences of contigs containing *MAT* locus gene sequences that matched *S. sclerotiorum's MAT* locus was performed and the reactions sequenced providing the orientation of two of the four members of the *S. sclerotiorum MAT* locus in *S. trifoliorum*.

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BIOGRAPHICAL SKETCH

Benjamin Doughan was born in Iowa, United States of America and grew up on a family farm with his two sisters. His parents are Dwight and Conni Doughan who farm there still. He is an avid hockey and physical fitness enthusiast. Benjamin attended the University of Northern Iowa where he majored in biotechnology and chemistry. His undergraduate research project subject was *Fusarium graminearum* where he worked on restriction fragment length polymorphism analysis to discern different isolates of the fungus. After graduation, he took a job in the private sector for a company inspecting pharmaceutical companies and identifying microorganisms. After that position, he worked at Integrated DNA Technology working on sequencing and oligonucleotide platforms.

With a plant pathology career in mind, Benjamin decided to go back and get a Master of Science in botany at the University of Northern Iowa on a full academic scholarship. His research project was on the leaf development of two species of grapes, *Ampelopsis arborea* and *Ampelopsis cordata*. This, along with some vineyard management, set him on his path to the University of Florida where he was accepted and joined Dr. Jeffrey Rollin's lab to work on *Sclerotinia sclerotiorum* and was funded by the alumni fellowship for the first four years.

While working in Dr. Rollins lab he worked on a variety of subjects such as light regulation, sclerotial mutants, potential polycomb proteins, *Sclerotinia trifoliorum* mating type locus (MAT) and finally resolving to work on the *MAT* locus and characterize the genes within *S. sclerotiorum*. Benjamin Doughan was awarded his PhD in December of 2013 and hopes to pursue a career as a research scientist in the future where he can combine his experience in botany along with plant pathology.