Studying the mating system in *Botrytis cinerea*

Maali Soliman

Master Thesis submitted to

Wageningen University

(Phytopathology Lab)

Supervised by
Dr. Jan van Kan

March 2012

Acknowledgment

I would like to express my appreciation and gratitude to Dr. Jan van Kan, Phytopathology lab, Wageningen University, for giving me the opportunity to join his group to do my master thesis. I'm also grateful to him for his supervision, guidance and valuable advice during that time. I'm thankful to Lisha Zhang, for her valuable help throughout this work. My sincere thanks to all members of the Phytopathology lab, Wageningen University, it was truly a pleasure to work in this lab. Finally, I am very much thankful to Joint Japan/World Bank Graduate Scholarship Program (JJ/WBGSP) for granting my fellowship to do my master program in Wageningen University.

Abstract

As a heterothallic ascomycete, *Botrytis cinerea* (teleomorph: *Botryotiniafuckeliana*) needs two different mating types to produce the sexual fruiting bodies. However, dual mating and homothallism can also be detected in *B. cinerea* but are considered unusual mating behaviors of that fungus. Genetic analysis of MAT idiomorphs of 18 *B. cinerea* strains showed no remarkable polymorphisms between strains that were exhibiting different sexual behavior. Progenies of RS26, a dual mating strain, crossed with SAS56 were all showing dual mating behavior except strain C30-13. Remarkably, the progeny of a crossing of RS26 with SAS405, both MAT1-2 strains, resulted in some strains possessing a MAT1-1 idiomorph. Strain Bc16, dual mating strain, was also able to self-fertilize showing homothallic behavior. Among seven random single ascospore strains resulting from self-fertilised Bc16, four were possessing a MAT1-1 idomorph and three were possessing a MAT1-2 idiomorph. Target mutagenesis of MAT1-1-1, MAT1-2-1 and MAT1-2-4 genes in three *B. cinerea* strains *e.g.* SAS56, SAS405 and RS11, resulted in about 100% ectopic integration of the hygromycin resistance gene cassettes. No homologous recombination was detected in any of the transformants.

Contents

Introduction

To save their populations from extinction is the primary aim of reproduction of living organisms. During this process the whole or part of the genetic information is passed from generation to generation according to the reproduction style. Organisms can adopt different ways of reproduction, either sexual or asexual or both. Although being considered more sophisticated, as well as time and energy consuming, sexual reproduction is the most common between Eukaryotes (Aanen& Hoekstra, 2007).

Sexual reproduction is a process by which genetically different individuals emerge from the ancestors. During gamete formation, recombination events take place due to meiotic divisions. The reshuffling of genetic information during meiosis result in a high level of genetic diversity between the offspring. This also allows selection of mutations towards beneficial traits and excluding deleterious ones. Overall, this continuous reshuffling and assembling of genes and genetic material allows the organism to evolve more rapidly due to more potent natural selection compared to organisms following an asexual life style (Aanen & Hoekstra, 2007; Otto, 2008; Kück & Pöggeler, 2009)

Sex in fungi

Reproduction was extensively studied in animals and plants but not in fungi. One of the reasons could be the complexity of their life cycles, especially in the higher fungi, and also for the fact that some fungi can switch from an asexual to sexual reproductive style during their life cycles (Nauta & Hoekstra, 1992). Most fungal species can reproduce by different means. They can simply use somatic cell division as a means of multiplying which results in a progeny genetically identical to the parent, with exception of mutations that could occasionally occur. This is called asexual reproduction, and it is considered an easy and quick way of reproducing new individuals in high numbers. Fungi known to reproduce only via this method are classified in Deuteromycota and designated as imperfect fungi (Taylor *et al.* 1999).

Many fungi, however, can take another path by sexual reproduction, which generally requires fusing of two different reproductive structures and forming sexual spores that can be of various types. This way of reproducing is quite common among higher fungi *i.e.* Ascomycota and Basidiomycota. In some fungal species, like in most Basidiomycota, the sexual phase is "obligatory" for the fungus to fulfill its life cycle (Aanen & Hoekstra 2007).

However, even among the fungal species that can sexually reproduce, they can have different mating strategies. Some fungi can be self-fertile, which means that one strain can sexually reproduce; these are designated "homothallic fungi". In other fungal species, two strains with different mating types are required for the sexual process; these are termed "heterothallic fungi". Additionally, in some conditions, some homothallic fungi can switch to the heterothallic behavior and vice versa, which implies that sex in fungi is sometimes very sophisticated (Aanen & Hoekstra, 2007).

Additionally, another mating system termed "pseudohomothallism" can be adopted by some Ascomycetes, e.g. *Neurospora tetrasperma* and *Cryphonectria parasitica* (Kronstad & Staben, 1997; McGuire *et al*., 2004). In this situation, strains are self-fertile because of heterokaryosis. After meiosis, pairs of nuclei stay together forming dikaryotic spores each with both different mating types (Faretra *et al.,* 1988)

The mating process in fungi was found to be genetically regulated. Although not having specific sex chromosomes, as in higher eukaryotes, fungi do have sex genes which control sexual identity and behavior. These genes are located in the genome in specific regions termed mating type loci "MAT loci". Fungi can have a single MAT locus which can have two or more alleles (designated as a "bipolar fungi), while other fungi have two unlinked mating loci with different numbers of alleles, (designated as "tetrapolar fungi") (Hsueh & Heitman, 2008).

Mating systems and MAT loci in ascomycetous fungi

In ascomycetous fungi, a single region in the genome, the mating locus (MAT), is controlling the mating process and behavior (Faretra *et al*., 1988). MAT loci contain genes encoding transcription factors which are responsible for regulating the mating behavior and sexual development (Coppin *et al*., 1997). In heterothallic ascomycetes the MAT locus has two alleles, MAT1-1 and MAT1-2.These mating alleles were termed "idiomorphs" since sequences in these alleles are lacking homology (Glass *et al*., 1988; Kothe, 1996).

MAT loci have been largely studied in different ascomycetous fungi, e.g. *Saccharomyces*, *Neurospora*, *Cryphonectria* (Coppin *et al*., 1997; Kronstad & Staben, 1997; McGuire *et al*., 2004). Previous studies concerning different heterothallic ascomycetes showed that each individual has only one of the two mating alleles, either MAT1-1 or MAT1-2 (Shiu & Glass, 2000). Genes in the MAT locus can differ between fungi but generally the MAT1-1 locus possesses an alpha-domain gene, while in MAT 1-2 locus contains a gene encoding a DNA-binding protein belongs to the high mobility group (HMG) proteins (Coppin *et al*., 1997). However, in homothallic ascomycetous fungi, for instance *Sclerotinia sclerotiorum*, both alpha and high mobility group domain genes were found together in a single MAT locus (van Kan *et al*., 2010; Amselem *et al*., 2011). A common feature at the MAT locus in the vast majority of Ascomycota, is that it is flanked on both sides by highly conserved genes, *i.e.* APN2 and SLA2 (Idnurm, 2011).

The homothallic fungi have both mating type genes in one locus. Therefore, they are able to self and produce the sexual fruiting bodies. For a heterothallic fungus, to sexually reproduce it has to find a partner with an opposite mating type allele, which makes sexual reproduction more complicated compared to homothallic fungi (Nelson, 1996; Kronstad & Staben, 1997). Some ascomycetous fungi can have both homothallic and heterothallic species belonging to the same genus (Nauta & Hoekstra, 1992). The genus *Cochliobolus* has species exhibiting heterothallic mating behavior and they possess either MAT1-1 or MAT1-2, while other species are homothallic and they have both MAT1-1 and MAT1-2 in their genome. It has been shown that the mating style can be changed by replacing the mating locus of a heterothallic species by the mating locus of a homothallic individual (Lu *et al.,* 2011).

Botrytis cinerea Pers. Fr.(teleomorph: *Botryotinia fuckelinia* [de Bary] Whetz.)

Botrytis cinerea is a necrotrophic plant pathogenic ascomycetous fungus with a wide host range. It can cause losses in many crops specially those growing in temperate regions (Williamson *et al*., 2007). The fungus is known to have various methods by which it can attack and colonize plant tissues (van Kan, 2006). *Botryotinia fuckeliana* is the teleomorph of *Botrytis cinerea* and it was first obtained *in vitro* by Groves and Drayton (1939). The name *Botrytis cinerea* is commonly used to describe both the anamorph and the teleomorph (Beever & Weeds, 2007).

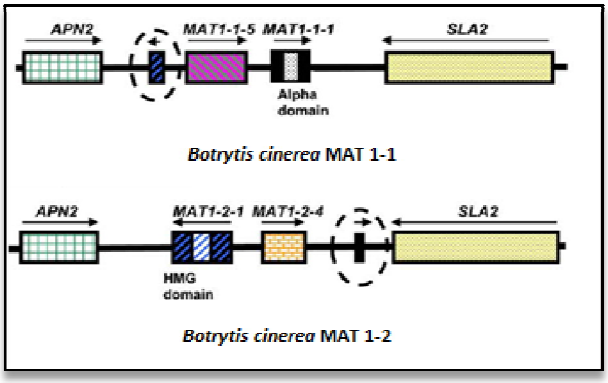

Fig 1. *Botrytis cinerea* MAT loci. alpha domain coding gene(MAT 1-1-1) and MAT 1-1-5 in MAT 1-1 locus. HMG coding gene (MAT 1-2-1) and MAT 1-2-4 gene in MAT 1-2 locus. APN2 and SLA2 genes are flanking both MAT loci. (Amselem *et al*., 2011)

As a heterothallic fungus, *B. cinerea* is normally considered to be self-sterile. The sexual reproduction in *B. cinerea* requires two different individuals with different MAT alleles. This necessity of out-crossing could be a reason why the sexual stage of the fungus is not frequently formed in nature, even though strains with different MAT loci were found to be present together in the same fields in different parts of world (Beever & Parkes, 1993; Faretra & Pollastro, 1993; Faretra *et al*., 1988).

The MAT locus in *B. cinerea* can be distinguished from other heterothallic Ascomycetes by the presence of two novel ORFs that do not have sequence homology to any previously described fungal genes. One was found in the MAT1-1 idiomorph and was designated MAT1-1-5 while the other ORF was found in the MAT1-2 idiomorph and was designated MAT1-2-4 (Fig 1) (Amselem *et al.,* 2011). The MAT1-1-5 gene was found to play a role in sex and apothecia formation since the progeny of a MAT1-1-5 mutant, crossed with a wild type MAT1-2 strain, were able to form stipes without forming mature discs (van Kan *et al.,* 2010).

Sexual fruiting bodies: "Apothecia"

Mating of *B. cinerea* strains result in forming sexual fruiting bodies called apothecia (Fig 2). The apothecium is a cup-shaped open structure in which the asci are formed (Nelson, 1996). Gregory (1949) and Polach and Abawi (1975) were able to isolate apothecia of *B. cinerea* from bean and grapevine plants. Although rarely found in nature (Faretra *et al*., 1988; Beever & Weeds, 2007), apothecia can be formed in laboratory conditions. A simple method is used by which sclerotia (female parent) of one strain is fertilized by the microconidia (male parent) of another strain with a

Fig 2.*Botrytis cinerea* sexual fruiting bodies (apothecia).

different MAT allele. Sclerotia should be cold treated before being spermatized (Faretra *et al*., 1988).

The mating process in *B. cinerea* involves cell fusion (from conidium and sclerotium) followed by nuclear fusion forming the diploid nucleus. After diploidy, meiosis takes place and result in producing 8 ascospores contained in an ascus. One ascus is emerged from a single diploid cell, and thousands of asci are formed in one apothecium (Nelson, 1996).

Aberrant mating behavior of *Botrytis cinerea*

Botrytis cinerea is not strictly a heterothallic fungus. Some strains were found to be able to mate with both mating types (Faretra *et al*., 1988; van der Vlugt-Bergmans *et al*., 1993; Angelini *et al*., 2010). This mating behavior was termed "dual mating" (van Kan *et al.*, 2010) and it is considered as an aberrant mating behavior since it is not the common mating style. From the pezizomycete fungi this was observed only in *Botrytis cinerea* (Amselem *et al*., 2011).

Faretra *et al*. (1988) reported 16% of field strains showing dual mating behavior which they designated MAT1-1/2 strains. They proposed that these strains are heterokaryotic for mating type genes. Some investigations revealed that strains showing dual mating behavior possess only one of the MAT alleles, MAT 1-1 or MAT 1-2 with no evidence of having the two alleles together (Angelini *et al*., 2010; Amselem *et al*., 2011)

On the other hand, another sexual behavior was also reported for *Botrytis cinerea*. Some strains were found to be self-fertile, hence capable of producing apothecia by itself, exhibiting a homothallic sexual style. Self-fertility was found in different *Botryotinia* species, *i.e*. *Botryotinia globosa*, *B. porri* and *B. ricini* (Faretra *et al*., 1988).

The overall aim of our investigation was to shed some light on the mating system of *B. cinerea,* and to increase our insights in dual mating behavior by:

- Amplification and sequencing of MAT idiomorphs of different *B. cinerea* strains exhibiting normal and aberrant mating behavior.
- Studying the segregation of dual mating by performing crosses with two reference strains.
- Performing knock-outs in different genes of MAT loci of a dual mater and normal strains and study the phenotype and mating behavior of the progeny of the obtained mutants.

Materials and Methods

Botrytis cinerea growth conditions for DNA isolation

Strains used are listed in Table (1) and they were provided by Dr. van Kan JAL, Phytopathology Lab, Wageningen University. They were grown on malt extract agar (MEA) medium for 5-7 days at 20°C, then mycelia were collected and freeze dried. PUREGENE DNA purification kit was used for DNA extraction following the manufacturer instructions.

Table 1: *B. cinerea* strains used for DNA isolation.

Strain name	Description
SAS56	MAT 1-1 reference strain
SAS405	MAT 1-2 reference strain
RS2	Progeny of SAS56 x SAS405 cross
RS3	Progeny of SAS56 x SAS405 cross
RS6	Progeny of SAS56 x SAS405 cross
RS11	Progeny of SAS56 x SAS405 cross
RS13	Progeny of SAS56 x SAS405 cross
RS20	Progeny of SAS56 x SAS405 cross
RS22	Progeny of SAS56 x SAS405 cross
RS26	Progeny of SAS56 x SAS405 cross
Bc16	Dual mater
RS16-12	Progeny of Bc16 self-fertilization
RS16-23	Progeny of Bc16 self-fertilization
RS16-59	Progeny of Bc16 self-fertilization
RS16-63	Progeny of Bc16 self-fertilization
RS61-71	Progeny of Bc16 self-fertilization
RS16-75	Progeny of Bc16 self-fertilization

Botrytis cinerea growth conditions for mating experiments

Crossing experiment was carried out in two rounds. Used strains are listed in Table (2). They were obtained from Dr. van Kan JAL, Phytopathology Lab, Wageningen University. Strains were grown on MEA medium at 20°C for 4 weeks until sclerotia were formed, and then they were transferred to 0°C for 4 weeks to allow full maturation and cold conditioning of the sclerotia.

Table 2: *B. cinerea* strains used for mating experiments.

Strain name	Description
SAS56	MAT 1-1 reference strain
SAS405	MAT 1-2 reference strain
RS11	Progeny SAS56 x SAS405 cross - Dual mater
RS26	Progeny SAS56 x SAS405 cross - Dual mater
Bc12	Dual mater
Bc16	Dual mater
C29-1	Progeny of SAS405 x RS26 cross
C29-2	Progeny of SAS405 x RS26 cross
C29-3	Progeny of SAS405 x RS26 cross
C29-4	Progeny of SAS405 x RS26 cross
C29-5	Progeny of SAS405 x RS26 cross
C29-6	Progeny of SAS405 x RS26 cross
C29-7	Progeny of SAS405 x RS26 cross
C29-8	Progeny of SAS405 x RS26 cross
C29-9	Progeny of SAS405 x RS26 cross
C29-10	Progeny of SAS405 x RS26 cross
C29-11	Progeny of SAS405 x RS26 cross
C29-12	Progeny of SAS405 x RS26 cross
C29-13	Progeny of SAS405 x RS26 cross
C29-14	Progeny of SAS405 x RS26 cross
C29-15	Progeny of SAS405 x RS26 cross
C30-1	Progeny of SAS56 x RS26 cross
C30-2	Progeny of SAS56 x RS26 cross
C30-4	Progeny of SAS56 x RS26 cross
C30-5	Progeny of SAS56 x RS26 cross
C30-7	Progeny of SAS56 x RS26 cross
C30-9	Progeny of SAS56 x RS26 cross
C30-10	Progeny of SAS56 x RS26 cross
C30-11	Progeny of SAS56 x RS26 cross
C30-12	Progeny of SAS56 x RS26 cross
C30-13	Progeny of SAS56 x RS26 cross

Sexual crosses

Crossings were performed according to Faretra *et al*. (1988). Sclerotia of each strain were collected and washed (brushed) gently in sterilized water to remove the hydrophobic layer from the surface. Sclerotia were used as female parents while suspensions of microconidia of each strain were used as male for spermatizing the sclerotia. Each strain was crossed reciprocally with both reference strains as male and female. For the 4 dual maters, sclerotia of each strain were spermatized with its own microconidia suspension and non-spermatized sclerotia were used as control by submerging them in sterilized water. Three replicates were set for each cross. Crossings were kept at 12°C in dark for 2 weeks, then in continuous light at the same temperature for about 3 months. Development of apothecia and stipes were scored during that time.

MAT loci amplification

Primers used to amplify the MAT idiomorphs are listed in Table (3). For each idiomorph, 5 primer pairs were used to amplify 5 overlapping DNA fragments covering the whole idiomorph region.

Table 3. PCR primers used to amplify fragments of the MAT loci of *B. cinerea* strains.

Primer Name	Primer sequence 5'-3'	
BotMAT3	GTGACCAGGAAACAGCTATGACC	ACATACTCGCATTAGTGGAAC
BotMAT5	GTGACTGTAAAACGACGGCCAGT	TCCGTATTCACAATCCATCC
BotMAT101	GTGACTGTAAAACGACGGCCAGT	CGAGCTAGCTGCAGCACTAGAG
BotMAT102	GTGACCAGGAAACAGCTATGACC	CCGTTCCCCTATCAGTCAACA
BotMAT103b	GTTTTCCCAGTCACGAC	CTGCTCCTGGAGAAGCATTC
BotMAT104	GTGACCAGGAAACAGCTATGACC	CTCCGGCCTGTCTCCAGTCTT
BotMAT105	GTGACTGTAAAACGACGGCCAGT	GATTTTGACGGTGCTTGGGAA
BotMAT106b	CAGGAAACAGCTATGAC	GGTATAAGCGCGAGCAATG
BotMAT107new	GTGACTGTAAAACGACGGCCAGT	GTTCGAACTTTCGTGGCACCG
BotMAT108	GTGACCAGGAAACAGCTATGACC	CAGCGAGTATAAACGTCCATCC
BotMAT201	GTGACTGTAAAACGACGGCCAGT	GGGCATGGAAGATGAGGCAAG
BotMAT202	GTGACCAGGAAACAGCTATGACC	GCTGGAGCTACTCCTTGTTCTAC
BotMAT203	GTGACTGTAAAACGACGGCCAGT	GAACACTGCGGAGCCAGAAATGAC
BotMAT204	GTGACCAGGAAACAGCTATGACC	GATGGTATGGCAGCTGAGATG
BotMAT205	GTGACTGTAAAACGACGGCCAGT	GAGAGTAAGTTCGGTCCATGCTTG
BotMAT206	GTGACCAGGAAACAGCTATGACC	GACTGCAACGAATTAGTATAGGCAG
BotMAT207	GTGACTGTAAAACGACGGCCAGT	ACTGACGCAGGCGGTCTCTAC
BotMAT208	GTGACCAGGAAACAGCTATGACC	GTGGTGGTGAAGGGACATCTTC

The amplification was carried out in 50 µl reaction volume using 50-100 ng genomic DNA, 10 µl 10xGotaq buffer + MgCl2 (Promega), 2.0 µl 2.5mM dNTPs mixture, 1.0 µl of 10 µM of each primer, and 0.2 µl of 5 U/µl Taq DNA polymerase (Promega). PCR conditions used for amplification were: an initial cycle of 95°C for 5 min, 30 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 4 min and final extension at 72°C for 5 min. The amplified fragments were purified from PCR mixtures using GFX PCR DNA purification Kit (GE Health Care) according to the manufacturer manual. DNA fragments were sequenced by Macrogen Inc., Amsterdam, NL.

Checking MAT idiomorphs using PCR

B. cinerea strains used in crossings were also checked for the MAT idiomorphs. Two primer pairs were used to amplify unique parts in each MAT idiomorph. Primer pair BotMAT 105 / BotMAT 108 was used to check for the presence of the MAT 1-1 idiomorph, while primer pair BotMAT 201 / BotMAT 204 was used to check for MAT 1-2. Amplification was carried out in 25 µl reaction mixture using 50-100 ng genomic DNA, 5 µl 10xGotaq buffer + MgCl2 (Promega), 1.0 µl 2.5mM dNTPs mixture, 0.5 µl of 10 µM of each primer, and 0.1 µl of 5 U/µl of Taq DNA polymerase. PCR conditions used for amplification were: an initial cycle of 95°C for 5 min, 30 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 4 min and final extension at 72°C for 5 min. using gel electrophoresis PCR products were examined on 1% agarose gel for the presence of amplified fragments.

Target mutagenesis of genes in the MAT loci of *B. cinerea*

Designing primers for gene replacement

MAT 1-1-1, MAT1-2-1 and MAT 1-2-4 genes were singly targeted for mutagenesis. The strategy used in this experiment was described by Kars *et al*. (2005). This strategy was based on homologous recombination and one step replacement of the target gene (Fig 3). Three primer pairs were designed for each gene. Two primers were chosen outside the ORF

borders (5.1, 3.1) , and another primer pair inside the ORF (5.3, 3.3).These two pairs were used to amplify 600-800 bp flanking fragments. Primers 5.3 and 3.3 had an extension sequence complementary to the hygromycin cassette. The other two primers (5.2, 3.2) were designed inside the flanking fragments adjacent to 5.1 and 3.1 respectively (Fig. 3) (Table4).

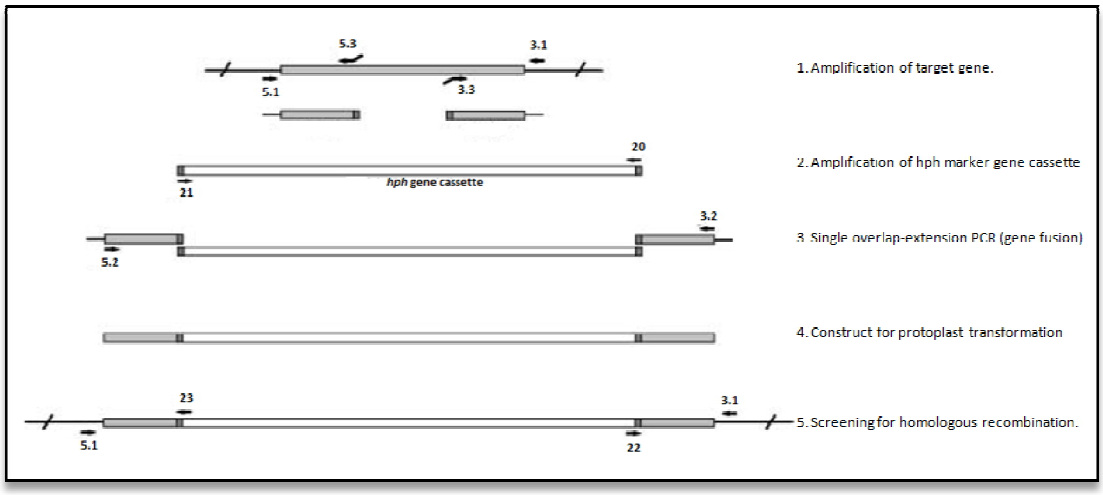

Fig 3. Gene replacement strategy and screening method (Kars *et al.,* 2005).

Amplification of constructs

SAS56 strain was used for targeting MAT 1-1-1 gene while SAS405 and RS11 were used for targeting MAT 1-2-1 and MAT 1-2-4 genes. Strains were grown on MEA medium and genomic DNA was extracted as describe above. DNA extractions were used to amplify the two flanking regions of each construct. Amplification was carried out in 50 µl using 50-100 ng genomic DNA, 10 µl 10xGotaq buffer + MgCl2 (Promega), 2.0 µl 2.5mM dNTPs mixture, 1.0 µl of 10 µM of each primer, and 0.2 µl of 5 U/µl Taq DNA polymerase (Promega). PCR conditions used for amplification were: an initial cycle of 95°C for 5 min, 30 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 4 min and final extension at 72°C for 5 min.

Using primers 20 and 21, the hygromycin resistance gene cassette (*hph*) was amplified from a plasmid DNA. Amplification reaction was carried out in 50 µl reaction mixture containing20 ng DNA, 10 µl 10xGotaq buffer + MgCl2 (Promega), 2.0 µl 2.5mM dNTPs mixture, 1.0 µl of 10 µM of each primer, and 0.2 µl of 5 U/µlTaq DNA polymerase (Promega). PCR conditions used for amplification were as follows: an initial cycle of 95°C for 5 min, 30 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 4 min and final extension at 72°C for 5 min.

Table 4: PCR primers used for amplification of target genes fragments, replacement constructs, and for screening of transformants.

Target gene	Primer name	Primer sequence 5'-3'
MAT 1-1-1	MAT 1-1-1 5.1	GAGGAAGTTCCTTAGCTCGT
	MAT 1-1-1 5.2	CTCACCTTCGCATGTCTTGA
	MAT 1-1-1 5.3	CTCGGCGCGCCGAAGCTTGACACTGAGGATAGCAGACT
	MAT 1-1-1 3.1	ACCTAACATCGCCAACTATC
	MAT 1-1-1 3.2	CACAGAGAGGGAGAGAATTTG
	MAT 1-1-1 3.3	GGGTACCGAGCTCGAATTCCTATTGCTTACACTCCTCCC
MAT 1-2-1	MAT 1-2-1 5.1	CTCTCTCCTCTCTCCGACTA
	MAT 1-2-1 5.2	CGGCACTGTCCTCGAAACGC
	MAT 1-2-1 5.3	CTCGGCGCGCCGAAGCTTGATCTTTGGCTTGAGACCAG
	MAT 1-2-1 3.1	GTGAATGGTGATGGCTAGCAT
	MAT 1-2-1 3.2	ATGGTGTGTCATCGAAGGCACA
	MAT 1-2-1 3.3	GGGTACCGAGCTCGAATTCCTAGAGCAACAGCTTCAGCC
MAT 1-2-4	MAT 1-2-4 5.1	GCGTTTCGAGGACAGTGCCG
	MAT 1-2-4 5.2	TAGTCGGAGAGAGGAGAGAG
	MAT 1-2-4 5.3	CTCGGCGCGCCGAAGCTTACAGCTGAGAACATGCGCTG
	MAT 1-2-4 3.1	CTTGAGTGCCACAAAAGGTG
	MAT 1-2-4 3.2	AGAGAGTTCTGGCGATGTGT
	MAT 1-2-4 3.3	GGGTACCGAGCTCGAATTCGAACCATGACTCAAGCAGAG
hph selection marker cassette	Cassette-5 (20)	GAATTCGAGCTCGGTACCCGGGGA
	Cassette-3 (21)	CAAGCTTCGGCGCGCCGAG
	Screen-3 (22)	GGGTACCGAGCTCGAATTC
	Screen-5 (23)	GTAACCATGCATGGTTGCCT

To join the two amplified fragments of each gene with the hygromycin cassette, a one step overlap-extension PCR was carried out using primer pair 5.2/3.2 of each gene. The reaction was done in 50 µl volume using 20 ng of DNA template of each flaking fragment and *hph* cassette, 5µl reaction buffer+$MgCl_2$, (Roche) , 1µl of 10µM of each primer, 4µl of 2.5 mM dNTPs mixture, 0.3 µl of 3.5U/µl of Expand High Fidelity polymerase (Roche). The PCR conditions were as follows: 94°C for 2min then 9 cycles of 94°C for 20 sec., 58°C of 30 sec., then 68°C for 3 min. followed by 19 cycles of 94°C for 20 sec., 58°C for 30 sec., and 68°C for 3 min this was followed by final extension at 72°C for 7min. PCR products were precipitated in 100% ethanol with NaAc , and kept in -20°C until they were used for transformation.

Protoplast formation

Strains SAS 56, SAS 405 and RS11 were used for transformation. Each strain was grown on MEA medium at 20°C in dark for 3 days and then exposed to near UV light at the same temperature for 3 days to enhance conidia formation. Spores were then collected in sterilized distilled water and filtrated through sterile fiberglass wool to get rid of mycelium debris. Spore suspension was centrifuged to separate and concentrate macroconidia. The pellet was re-suspended in sterilized MQ water and 0.5 liter of 1% malt extract (Difco) was inoculated with macroconidia suspension (10^8 –10^9 conidia). Inoculated medium was incubated at room temperature shaking for 2h, then was transferred to 20°C and 140 rpm for 16-20h. Cultures were then filtered using 22.4µm filter system, to separate young mycelia, and mycelia were washed twice with KC buffer (0.6 M KCl, 50mM $CaCl_2$). Mycelia were then collected in KC and treated with Glucanex (5 mg/ml) and incubated at 25°C and 140 rpm. After 2 hours, cultures were checked microscopically for protoplast formation. Protoplasts were then filtrated through 22.4 µm filter system, filtrates were collected

centrifuged at 1200x g for 10 min. Protoplasts were re-suspended in KC to a final concentration 10^7protoplasts / 100 µl and then incubated on ice for 5 min.

Transformation

DNA constructs were precipitated in ethanol with NaAc and re-suspended in 95 µl KC then incubated on ice for 5 min. Five µl of 5 mM spermidin were added and re-incubated on ice for 5 min then, 100 µl of protoplast suspension was added to the DNA constructs and incubated for 5 min. A hundred µl of filter sterilized PEG solution (25% PEG 3350 in 10 mMTris 7.4, 50 mM $CaCl_2$) was added to the transformation mixture and incubated for 20 min. An additional amount of 500 µl PEG were added to the mixture and further incubated for 10 min. The mixture was then brought up to 1000 µl by adding 200 µl KC. Half of the transformation mixture was mixed with 100 ml of SH agar medium (0.6 M sucrose, 5 mM HEPES (pH 6.5), 1 mM $NH_4H_2PO_4$, 1.2% agar) at 40 °C and plated (10 ml/plate). Plates were incubated overnight at 20 °C.

After incubation, 10 ml SH agar medium mixed with hygromycin (100 µg/ml) were added to each plate except for the control plates. Plates were checked every day for the formation of surface growing colonies which were then picked up and transferred to MEA plates amended with 100 µg/ml hygromycin for further selection of transformants. After growth for few days, sub-cultures were made of these transformants on non-selecting MEA plates which were then incubated at 20 °C in dark for few days then in near UV light to allow conidiation. Meanwhile, parts of the mycelia were harvested from each transformant for DNA extraction.

Screening for targeted mutagenesis.

DNA was extracted from transformants using the DNA extraction method described earlier. Two primer pairs, 5.1/23 and 3.1/22, of each gene were used to check for targeted insertions. Two more amplifications were added using 5.3/5.1 primer pair of each gene and another primer pair to amplify part of hph gene to check for its insertion. Amplifications were carried out in 25 µl reaction mixtures each containing: 50-100 ng genomic DNA, 5 µl 10xGotaq buffer + MgCl2 (Promega), 1.0 µl 2.5mM dNTPs mixture, 0.5 µl of 10 µM of each primer, and 0.1 µl of 5 U/µl of Taq DNA polymerase. PCR conditions used for amplification were: an initial cycle of 95°C for 5 min, 30 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 4 min and final extension at 72°C for 5 min.

Results

Sequence analysis of the MAT locus in *B. cinerea* strains

The MAT loci of 18 *B. cinerea* strains (Table 1) were amplified in overlapping fragments of about 1 kbp, and the PCR products were sequenced. Sequences were assembled and aligned using Vector NTI. The MAT sequences of B05.10 (MAT1-1) and T4 (MAT1-2) strains were added to the alignment as references.

No polymorphisms were detected for the sequence of the MAT1-1 locus in the progeny from SAS56 x SAS405 crossing (strains RS2, RS6, RS13, RS22), or in the progeny of self-fertilized Bc16 (strains RS16-56, RS16-59, RS16-63, RS16-71),. The MAT loci of all these strains were identical to the MAT locus of B05.10.

By contrast, aligning the sequences of the MAT1-2 locus in progeny from a cross SAS56 x SAS405 (strains RS3, RS11, RS20, RS26), didn't show any differences between them, but were remarkably different from the sequence present in the T4 strain.

RS16-23 and RS16-75 are progeny of self-fertilization of Bc16 and they possess a MAT1-2 locus. The MAT locus sequence in these strains and in Bc12 (behaving as a dual mater) were identical to the sequence of the MAT locus of strain T4, while SAS405 contained several polymorphisms. Two single nucleotide polymorphisms were found in the MAT1-2-1 gene but these didn't cause change in the amino acid sequence. A single nucleotide polymorphism was found within the MAT1-2-4 gene and caused an amino acid substitution from phenylalanine to valine. Remarkably, a sequence duplication of 12 nucleotides inside the coding region of MAT1-2-1 gene was noticed in T4, Bc12, Rs16-23 and Rs16-75 which was not found in SAS405 (Fig 4).

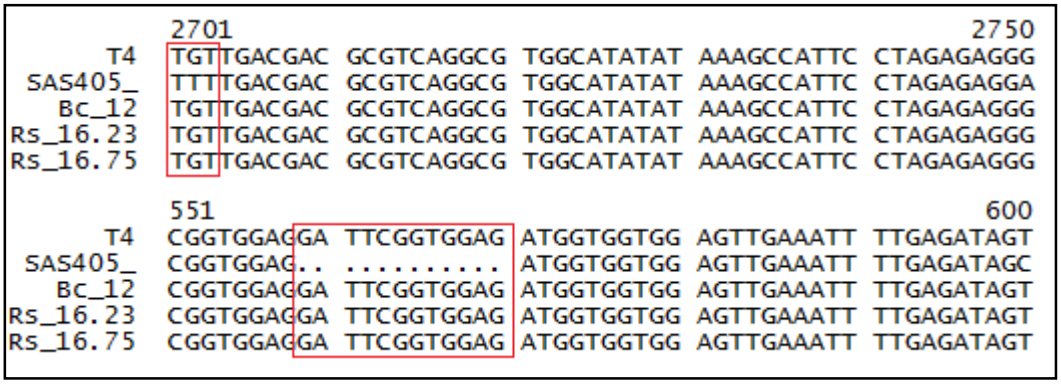

Fig 2. Multiple sequence alignment of *B. cinerea* strains consisting of MAT1-2 locus, showing amino acid substitution and sequence duplication.

Mating behavior and MAT locus analysis of different *B. cinerea* strains.

To test the mating behavior, strains mentioned earlier (Table 2) were crossed reciprocally with SAS56 and SAS405 using sclerotia and microconidia.

Cross of dual mater strain RS26 with MAT 1-1 reference strain

Strain RS26 was able to mate with both SAS56 and SAS405, thus confirming the dual mating behavior found in a previous investigation. Single ascospore progeny strains generated from a previous cross of RS26 with SAS56 were all, except for strain C30-13, found to be compatible with both SAS56 and SAS405, thus exhibiting a dual mating behavior (Table 3). The idiomorphs in all strains were verified by amplifying the alpha domain and HMG genes using two primer pairs. RS26 was found to contain the MAT1-2 locus. Among 10 single ascospore progeny of the cross RS26 x SAS 56, 2 strains were found to contain the MAT 1-1 idiomorph and 8 were found to contain the MAT1-2 idiomorph.

Table 3. Mating behavior and MAT locus analysis of random ascospore strains, progeny of SAS56 x RS26 cross.

Strains	Mating behaviour			MAT 1-1 primer BotMAT 105/ 108	MAT 1-2 primer BotMAT 201/ 204
	SAS 56 cross	SAS 405 cross	Self fertility		
SAS 56	-	+	-	+	-
RS26	+	+	-	-	+
C30-1	+	+	-	-	+
C30-2	+	+	-	-	+
C30-4	+	+	-	-	+
C30-5	+	+	+	+	-
C30-7	+	+	-	-	+
C30-9	+	+	-	-	+
C30-10	+	+	-	-	+
C30-11	+	+	+	+	-
C30-12	+	+	-	-	+
C30-13	+	-	-	-	+

Cross of dual mater strain RS26 with MAT 1-2 reference strain

Fifteen single ascospore progeny resulting from a previous cross of RS26 with SAS405 (both containing a MAT1-2 idiomorph), were tested for mating behavior. Five strains were able to mate with both mating references, and thus showed a dual mating behavior. Two strains were able to mate only with SAS 405. The idiomorph in all strains was verified by amplifying the alpha domain and HMG genes using two primer pairs. This screening showed unexpected results, since7 strains were found to contain the MAT 1-1 idiomorph (Table 4). Remarkably, strain C29-10 was found to possess both MAT idiomorphs.

Table 4. Mating behavior and MAT locus analysis of random ascospore strains, progeny of RS26 x SAS405 cross.

Strains	Mating behaviour			MAT 1-1 primer BotMAT 105/108	MAT 1-2 primer BotMAT 201/204
	SAS 56 cross	SAS 405 cross	Self fertility		
SAS 405	+	-	+	-	+
RS26	+	+	-	-	+
C29-1	-	+	ND	+	-
C29-2	+	+	ND	+	-
C29-3	+	-	ND	-	+
C29-4	+	+	+	-	+
C29-5	+	+	ND	-	+
C29-6	+	+	ND	-	+
C29-7	+	+	ND	+	-
C29-8	-	+	ND	+	-
C29-9	+	-	-	-	+
C29-10	+	+	+	+	+
C29-11	+	+	+	+	-
C29-12	+	-	-	-	+
C29-13	+	-	-	-	+
C29-14	+	+	+	+	-
C29-15	+	+	-	-	+

ND: not done.

Cross of MAT1-1 reference SAS 56 with MAT1-2 reference SAS 405

Previous investigation (van der Vlugt-Bergmans, unpublished) on the mating behavior of single ascospore progenies from cross SAS56 X SAS405 showed differences between strains. RS2, RS6 and RS13 were able to cross only with SAS405 strain while RS3 was able to cross only with SAS56. By contrast, strains RS11 and RS26 were found to exhibit dual mating behavior, this result was confirmed in our investigation. In addition, strain RS11 showed homothallism since it was able to self-fertilize and form apothecia (Table 5). The entire MAT idiomorphs of SAS56, SAS405 and their progeny were amplified in five overlapping fragments and were sequenced. Dual maters, RS11 and RS26, were found to possess a MAT1-2 idiomorph. Unexpectedly, the reference strain SAS405 was in one case able to form apothecia when the sclerotia were treated with water.

Table 5. Mating behavior and MAT locus analysis of SAS56, SAS405 and random ascospore strains resulted from their cross.

	Mating behaviour			MAT 1-1 (BotMAT primer pairs)					MAT 1-2 (BotMAT primer pairs)				
Strain	**SAS 56 cross**	**SAS 405 cross**	**Self fertility**	**5/ 102**	**101/ 104**	**103b/ 106b**	**105/ 108**	**107_{new} /3**	**5/ 202**	**201/ 204**	**203/ 206**	**205/ 208**	**207/ 3**
SAS 56	-	+	-	+	+	+	+	+					
SAS 405	+	-	+						+	+	+	+	+
RS 2	-	+	ND	+	+	+	+	+					
RS 3	+	-	ND						+	+	+	+	+
RS 6	-	+	ND	+	+	+	+	+					
RS11	+	+	+						+	+	+	+	+
RS26	+	+	-						+	+	+	+	+
RS 13	-	+	ND	+	+	+	+	+					
RS 20	+	-	ND						+	+	+	+	+
RS 22	ND	ND	ND	+	+	+	+	+					

ND=Not done.

Self-fertile strain Bc16 and its progeny

Our investigation confirmed Bc16 to be a dual mater. In a self cross by treating sclerotia with microconidial suspension of Bc16, the strain was able to self fertilize showing a homothallic trait (Table 6). A progeny of 7 strains resulted from Bc16 self-fertilization showed different mating behaviors (van der Vlugt-Bergmans, unpublished). Three strains were compatible with SAS56, 3 strains were compatible with SAS405, and RS16-56 was compatible with both references. MAT idiomorphs were amplified from all strains. Although Bc16 itself has a MAT1-1 idiomorphs, three of its progeny were found to contain a MAT 1-2 idiomorph.

Targeted mutagenesis of genes in the MAT loci of *B. cinerea* strains

The MAT1-1-1, MAT1-2-1 and MAT1-2-4 genes were targeted for mutation in three *B. cinerea* strains, in order to study their function during the sexual process. The used method was disrupting genes by introducing a hygromycin selectable marker gene inserted by homologous recombination.

Transformation of *B. cinerea* strains SAS56, SAS405 and RS11with the knockout constructs resulted in a total 67 transformants which were able to grow on hygromycin, and 55 of these transformants were shown by PCR to contain the hygromycin resistance gene. These transformants were checked for integration of the hygromycin cassette in the target gene. Results revealed that homologous recombination did not occur and the hygromycin cassette was inserted elsewhere in the genome. To confirm these results, all the tested transformants were checked for the presence of the wild type 5' flank region, and it was present in all of them (Appendix 1).

Table 6. Mating behavior and MAT locus analysis of Bc16 and random ascospore strains resulted from its self-fertilization.

	Mating behaviour			MAT 1-1 (BotMAT primer pairs)					MAT 1-2 (BotMAT primer pairs)				
Strain	**SAS 56 cross**	**SAS 405 cross**	**Self fertility**	**5/ 102**	**101/ 104**	**103b/ 106b**	**105/ 108**	**107$_{new}$ /3**	**5/ 202**	**201/ 204**	**203/ 206**	**205/ 208**	**207/ 3**
Bc16	+	+	+	+	+	+	+	+					
RS16-12	+	-	ND						+	+	+	+	+
RS16-23	+	-	ND						+	+	+	+	+
RS16-56	+	+	ND	+	+	+	+	+					
RS16- 59	-	+	ND	+	+	+	+	+					
RS16-63	-	+	ND	+	+	+	+	+					
RS16-71	-	+	ND	+	+	+	+	+					
RS16-75	+	-	ND						+	+	+	+	+

ND=Not done

Discussion

The teleomorph of the plant pathogen *B. cinerea* was not in the spotlight of research concerning this fungus. The rarity of the occurrence of the sexual stage of *B. cinerea* in nature led scientists to conclude that it does not play a role in the disease cycle (Grindle, 1979). Another reason for not being an attractive subject of research is the time-consuming procedure required to obtain the sexual stage *in vitro* (Faretra *et al.,* 1988). However, the sexual stage as part of life cycle of a fungus offers a substantial source of genetic diversity (Aanen & Hoekstra 2007).

Mating behavior and MAT loci analysis of different *B. cinerea* strains

Transition from one type of mating behavior to another is known to occur occasionally in some ascomycetous fungi (Lin & Heitman, 2007). Dual mating in *B. cinerea* was previously mentioned in some investigations and was found in field isolates, as well as *in vitro* ascospore progenies (Faretra *et al*., 1988; Lorenz & Eichhorn 1983). Faretra and Pollastro (1996) performed an analysis on complete tetrads from several crosses and demonstrated, according to the mating behavior, that in each ascus the two mating alleles are usually present in a 1:1 ratio (2 pairs of spores with each allele). However, they also identified tetrads, in which some progeny strains were showing dual mating behavior. This behavior was always occurring in ascospores located in a position where the MAT 1-2 behavior was expected, thus they proposed that dual mating is due to gene conversion and that it is unidirectional (Faretra & Pollastro, 1996).

Data from our investigation revealed that most of the strains showing homothallism and dual mating behavior possess the MAT 1-2 idiomorph. However, there were also strains possessing the MAT 1-1 idiomorph, which suggests that this behavior is not linked to the MAT idiomorphs and it is not a unidirectional gene conversion. This could suggest the existence of another locus or gene that may contribute in or control this behavior and it is present somewhere in the genome outside the MAT locus (Amselem, 2011; van Kan *et al.,* 2010). This was also hypothesized to explain homothallism in heterothallic species of the fungus *Neurospora* (Glass *et al.,* 1990).

In our investigation, segregation of the two different MAT idiomorphs was found in a progeny obtained from a self-fertilized parent (strain Bc16) or in progeny from parents possessing the same idiomorphs (RS 26 x SAS 405). The possibility that a change occurred in one of the parental cells to be able to cross and give progenies with different mating types, could explain this unexpected results. In the ascomycete *Saccharomyces cerevisiae*, cells are able to switch from one mating type to the other and then cross with a neighbor cell which has a different mating type, thus the fungus changes from self sterility to self-fertility (Klas, 1992). This gene switching in *S. cereviseae* was explained by the presence of a silent part of the opposite MAT cassettes in the genome, which can be transferred from a silent to an active position by recruiting a specific endonuclease enzyme essential for that process (Nelson, 1996). Switching of the mating type could also be suggested in *B. cinerea* but a different mechanism could be involved. Mating type switching was also described in other

ascomycetous fungi such as *Cryphonectria parasitica* and *Sclerotinia trifoliorum* (Lin &Heitman, 2007; Nelson, 1996).

Results also revealed the presence of dual mating in progeny originating from normally behaving, heterothallic parents (SAS56 x SAS405). Two out of 8 tested strains of this progeny showed dual mating behavior. When crossing the dual mating strain RS26 with reference strains SAS56 or SAS405, the majority of the tested progeny showed dual mating behavior which suggests that the dual mating trait is inheritable and can be transferred from generation to generation.

Self-fertility is a common feature of the mating system of the heterothallic fungus C*ryphonectria parasitica*. Strains of *C. parasitica* were found to be heterokaryotic. Therefore, each spore contains two types of nuclei, each with a different MAT idiomoph. Heterokaryosis was also found in other heterothallic ascomycetous fungi *e.g. Neurospora tetrasperma*. In our investigation we were able to check the presence of MAT idiomorphs in the tested strains. We found that each strain that shows dual mating behavior has only one of the two MAT idiomorphs, with one exception. In strain C29-10 we could detect both mating idiomorphs. Further investigation could be proposed to confirm the presence of the two mating idiomorphs in that strain, by performing multiple rounds of single spore purification technique and then detecting the presence of MAT idiomorphs.

For further investigations, replacing the MAT idiomorph of a normally behaving, heterothallic *B. cinerea* strain by the same type of mating idiomorph from a dual mating strain and studying the mating behavior of this transformant, could give an answer as to what extent the MAT loci control the mating process and the dual mating behavior.

Sequence analysis of the MAT loci of *B. cinerea* strains.

MAT idiomorphs of some *B. cinerea* strains were amplified and sequenced. Aligning the MAT1-2 sequences of SAS 56 x SAS 405 progeny revealed no polymorphisms between normally behaving heterothallic strains and dual mating strains. This led us to conclude that there is no correlation between the sequence of the MAT locus and the mating behavior.

On the other hand polymorphisms were not detected when aligning RS16-23, RS16-75 (progeny from self-fertilized strain Bc16, carrying a MAT1-2 idiomorph) in addition to Bc12 (a dual mater), and to strain T4. Some polymorphisms were detected, however, when these strains were aligned to SAS 405. One of the polymorphisms was in the MAT1-2-4 gene causing amino acid substitution in addition to a duplication in a sequence within the MAT1-2-1 gene. T4 is a fully sequenced *B. cinerea* strain that was added as a reference to compare the aligned MAT 1-2 strains to it.

Generally these results support the tendency that dual mating and homothallism in *B. cinerea* are controlled by another region or gene present somewhere else in the genome, outside the MAT loci. Sequencing the whole genome of dual mating strains and aligning with normal behaving strains would help to prove this assumption.

Targeted mutagenesis of genes in the MAT locus

Different methods were used to generate gene knock-outs in many filamentous fungi. Targeted mutations were made in *Neurospora* and led to loss of function of the mating-type genes (Colot *et al*., 2006). The MAT 1-2-1 gene was mutated in *Fusarium verticilloides* and affected the transcription of different genes involved in different biological processes (Keszthelyi, 2007).

During our study we tried to target genes in both MAT loci for mutation by introducing a DNA insert with a hygromycin resistance cassette, to be integrated by homologous recombination. Although we obtained a high number of transformants (colonies resistant to hygromycin), PCR analyses showed that all transformants contained an ectopic insertion, therefore, the targeted genes were not mutated. This result shows that homologous recombination did not occur.

Gene disruption using homologous recombination based strategies is considered a powerful tool to study gene function in filamentous fungi. However, the efficiency of the homologous recombination varies widely between fungi species (Ninomiya *et al*., 2004). In addition, this process depends largely on the length of the homologous regions. In a previous investigation studying the effect of the length of the homologous regions in the efficiency of targeted mutagenesis in *B. cinerea*, results revealed that using flanking regions ranged from 100 bp to 2000 bp gave replacement efficiency ranged from 6 – 60% respectively (Noda *et al*., 2007). During our study the length of the homologous regions in the three different genes were in the range 500-700 bp which could be relatively short for effective recombination to occur. We suggest that by using larger regions (>1 kb) the efficiency of homologous recombination could be improved.

Although homologous recombination is considered the primary DNA repair mechanism in most of the living organisms, the non-homologous end joining is another way of DNA repair. The non-homologous end joining mechanism causes the ectopic integration of DNA constructs during target mutagenesis process. Disrupting genes encoding Ku70 or Ku80 proteins, responsible for the non-homologous end joining in *B. cinerea*, was found to increase the efficiency of homologous recombination and thus the mutagenesis rate (Choquer *et al*., 2008)

Furthermore, homologous recombination may also vary between different regions within the genome. Previous studies showed that targeted mutagenesis using homologous recombination was successfully done in *B. cinerea* for different genes outside the MAT locus. Using gene silencing as an alternative approach to study functions of MAT loci genes could also be proposed.

Conclusion

In our study we tried to get a closer image of the mating behavior of *B. cinerea* and concentrate on the homothallism and dual mating as exceptional mating styles of this heterothallic fungus.

We performed crossings with some single ascospore progenies of a dual mating and homothallic strains and we were able to conclude that dual mating is not an acquired trait but it can be passed through generations, thus it is inherited.

If the MAT idiomorphs are solely responsible for the mating behavior of the fungus we would have been able to find remarkable differences between MAT idiomorphs of strains that were behaving differently. In our investigation alignment of the sequenced MAT idiomorphs of different *B. cinerea* strains did not show polymorphisms between them. Therefore our conclusion was that the transition in the mating behavior is not controlled by the MAT idiomorphs and it is possible that another locus or gene(s) somewhere else in the genome is responsible for that behavior.

How genes in the MAT idiomorphs are involved in the mating process, needs to be dissected. We tried in vain to make mutations targeting the genes in the MAT idiomorphs in both normal behaving and dual mating strains. Disrupting those genes and studying the phenotype of the transformed strains could help to know their roles in the mating process. Further investigations are needed to understand the sexual behavior of *B. cinerea* and to know what could be the genetic explanation for the unusual turn in this behavior.

References

Aanen, D. K., Hoekstra, R. F. 2007. Why sex is good: on fungi and beyond. In: Heitman, J., Kronstad, J. W., Taylor, J. W., Casselton, L. A. (Eds.) Sex in fungi: Molecular determination and evolutionary implications. ASM Press, Washington, pp. 527-537.

Amselem, J., Cuomo, C.A., van Kan, J.A.L., Viaud, M., Benito, E.P., *et al*. 2011. Genomic analysis of the necrotrophic fungal pathogens *Sclerotiniasclerotiorum* and *Botrytis cinerea*. PLoS Genet. 7(8):e1002230.doi:10.1371/journal.pgen.1002230.

Angelini, R. M. D. M., Rotolo, C., Pollastro, S., Faretra, F. 2010. Molecular characterization of the mating type (MAT1) locus in *Botryotiniafuckeliana* (*Botrytis cinerea*). XV International Botrytis Symposium.

Beever, R. E., Parkes, S. L. 1993. Mating behavior and genetics of fungicide resistance of *Botrytiscinerea* in New Zealand. N. Z. J. Crop Hort. Sci. 21: 303-310.

Beever, R. E., Weeds, P. L. 2007. Taxonomy and genetic variation of *Botrytis* and *Botryotinia*. In: Elad, Y., Williamson, B., Tudzynski, P., Delen, N. (Eds.) Botrytis: Biology, pathology and control. Dordrecht, The Netherlands: Kluwer Academic Press. pp. 29-52.

Choquer, M., Robin, G., Le Pécheur, P., Giraud, C., Levis, C., Viaud, M. 2008. *Ku70* and *Ku80* deficiencies in the fungus *Botrytis cinerea* facilitate targeting of genes that are hard to knock out in a wild-type context. FEMS Microbiol. Lett. 289:225-232.

Colot, H. V., Park, G., Tumer, G. E., Ringelberg, C., Crew, C. M., Litvinkova, L., Weiss, R. L., Borkovich, K. A., Dunlap, J. C. 2006. A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. 103: 10352-10357.

Coppin, E., Debuchy, R., Arnaise, S., Picard, M. 1997. Mating types and sexual development in filamentous ascomycetes. Mcrobiol. Mol. Biol. Rev. 61:411-428.

Faretra F, Antonacci E, Pollastro S. 1988. Improvement of the technique used for obtaining apothecia of *Botrytinia fuckeliana* (*Botrytis cinerea*) under controlled conditions. Annuli di microbiologia ed enzimologia. 38:29-40.

Faretra, F., Pollastro, S. 1993. Genetics of sexual compatibility and resistance to bezimidazole and dicarboximide fungicides in isolates of *Botryotinia fuckeliana* (*Botrytis cinerea*) from nine countries. Plant Pathol. 42: 48-57.

Faretra, F., Pollastro, S. 1996. Genetic studies of the phytopathogenic fungus *Botryotinia fuckeliana* (*Botrytis cinerea*) by analysis of ordered tetrads. Mycol. Res. 100:620-624.

Glass. N.L., Vollmer, S. J., Staben, C., Grotelueschen, J.,Metzenberg, R.L., Yanofsky, C. 1988. DNAs of the two mating type alleles of *Neurospora crassa* are highly dissimilar. Science 241:570-573.

Glass, N. L., Metzenberg, R. L., Raju, N. B. 1990. Homothallic sordariaceae from nature: the absence of strains containing only the a mating type sequence. Exp. Mycol. 14: 274–289.

Gregory, P. H. 1949. Studies on *Sclerotinia* and *Botrytis* II. De Bary's description and specimens of *Peziza fuckeliana*. Trans. Brit. Mycol. Soci. 30: 1-13.

Grindle, M. 1979. Phenotypic differences between natural and induced variants of *Botrytis cinerea*. J. Gen. Microbiol. 111:109-120.

Groves, J. W., Drayton, F. L. 1939. The perfect stage of *Botrytis cinerea*. Mycologia 31: 485-489.

Heitman, J., Krosnstad, J. W., Yaylor, J. W., Casselton, L. A. 2007. Sex in fungi: Molecular determination and evolutionary implications. ASM Press, Washington DC 542pp

Hsueh, Y. P., Heitman, J. 2008. Orchestration of sexual reproduction and virulence by the fungal mating-type locus. Curr. Open. Microbiol. 11:517-524.

Idnurm, A. 2011. Sex and speciation: The paradox that non-recombining DNA promotes recombination. FungalBiol. Rev. 25:121-127.

Keszthelyi, A., Jeney, A., Kerenyi, Z., Mendes, O., Waalwijk, C., Hornok, L. 2007. Tagging target genes of the MAT 1-2-1 transcription factor in *Fusarium verticilloides* (*Gibberella fujikuroi* MP-A). Antonie van Leeuwenhoek 91:373–391.

Klar, A. J. S. 1992. Molecular Genetics of Fission Yeast Cell Type: Mating Type and Mating-type Interconversion. In: Jonees, E. W., Pringle, J. R., Broach, J. R. (Eds.), The molecular and cellular biology of the yeast Saccahromyces. Cold Spring Harbor Laboratory Press, pp. 745-777.

Kronstad. J.W., Staben, C. 1997. Mating type in filamentous fungi. Ann. Rev. Genet. 31: 245-276.

Kothe, E. 1996. Tetrapolar fungal mating types: sexes by the thousands. FEMS Microbiol. Rev. 18:65-87.

Kück, U., Pöggeler, S. 2009. Cryptic sex in fungi. Fungal Biol. Rev. 23: 86-90.

Lin, X., Heitman, J. 2007. Mechanisms of homothallism in fungi and transitions between heterothallism and homothallism. In: Heitman, J., Kronstad, J. W., Taylor, J. W., Casselton, L. A. (Eds.) Sex in fungi: Molecular determination and evolutionary implications. ASM Press, Washington, pp. 35-57.

Lu, S. W., Yun, S. H., Lee, T., Turgeon, B. G. 2011. Altering sexual reproduction mode by interspecific exchange of MAT loci. Fungal Genet. Biol. 48:714-724.

McGuire, C., Marra, R. E. Milgroom, M. G. 2004. Mating-type heterokaryosis and selfing in *Cryphonectria parasitica*. Fungal Genet. Biol. 41:521-533.

Nauta, M. J. Hoekstra, R. F. 1992. Evolution of reproductive systems in filamentous ascomycetes. I. Evolution of mating types. Heredity 68:405-410.

Nelson, M. A. 1996. Mating systems in ascomycetes: a romp in the sac. Tend Genet. 12:69-74.

Ninomiya, Y., Suzuki, K., Ishii, C., Inoue, H. 2004. Highly efficient gene replacement in *Neurospora* strains deficient for nonhomologous end-joining. PNAS. 17:12248-12253.

Noda, J., Brito, N., Espino, J. J., Gonzalez, C. 2007. Methodological improvement in the expression of foreign genes and in gene replacement in the phytopathogenic fungus *Botrytis cinerea*. Mol Plant Pathol. 8:811-816.

Otto, S. 2008. Sexual reproduction and the evolution of sex. Nature Education 1(1).

Polach, F. J., Abawi, G. S. 1975. Occurrence and biology of *Botryotinia fuckeliana* on beans in New York. Phytopathol. 65: 657-660.

Shiu, P. K. T., Glass, N. L. 2000. Cell and nuclear recognition mechanisms mediated by mating type in filamentous ascomycetes. Curr. Opin. Microbiol. 3:183-188.

Taylor, J. W., Jacobson, D. J., Fisher, M.C. 1999. The evolution of asexual fungi: reproduction, speciation and classification. Ann. Rev. Phytopathol. 37: 197-246.

van der Vlugt-Bergmans, C. J. B., Brandwagt, B. F. van't Klooster, J. W. and van Kan, J. A. L. 1993. Genetic variation and segregation of DNA polymorphisms in *Botrytis cinerea*. Mycol. Res. 97:1193-1200

van Kan, J. A. L. 2006. Licensed to kill: the lifestyle of a ncerotrophic plant pathogen. Trend Plant Sci. 11: 247-253.

van Kan, J. A. L., Duarte, J., Dekkers, E., Dyer, P. S., Kohn, L. M. 2010. The *Botrytis cinerea* mating type loci. XV international Botrytis Symposium

Williamson, B., Tudynski, B., Tudzynski, P., van Kan, J. A. L. 2007. *Botrytis cinerea*: the cause of grey mould disease. Mol. Plant Pathol. 8:561-580.

Appendix 1

Targeted mutagenesis of MAT loci genes in three *B. cinerea* strains.

Strain	Transformant	3' flank region (3.1- 22 screening primer)	5' flank region (5.1-23 screening primer)	*hph* gene	5' flank region (5.1-5.3)
RS 11 MAT1-2-1 gene	1	-	-	+	+
	2	-	-	+	+
	3	-	-	+	+
	4	-	-	+	+
	5	-	-	+	+
	6	-	-	-	+
	7	-	-	+	+
	8	-	-	+	+
	9	-	-	+	+
	9''	-	-	-	+
	10	-	-	+	+
	11	-	-	+	+
	12	-	-	+	+
	13	-	-	-	+
	14	-	-	+	+
	15	-	-	+	+
	16	-	-	+	+
	17	-	-	+	+
	18	-	-	-	+
	19	-	-	-	+
	20	-	-	-	+
	21	-	-	+	+
	22	-	-	+	+
	23	-	-	-	+
RS11 MAT1-2-4gene	1	-	-	+	+
	2	-	-	-	+
	3	-	-	-	+
	4	-	-	+	+
	5	-	-	-	+
	6	-	-	+	+
	7	-	-	+	+
	9	-	-	+	+
SAS405 MAT1-2-1 gene	1	-	-	+	+
	2	-	-	+	+
	3	-	-	-	+
	4	-	-	+	+
	5	-	-	+	+
	6	-	-	+	+
	7	-	-	+	+
	8	-	-	+	+
	9	-	--	+	+
	10	-	-	+	+
	11	-	-	-	+
	12	-	-	+	+
	13	-	-	+	+
	14	-	-	+	+
	15	-	-	+	+

Strain	Transformant	3' flank region (3.1- 22 screening primer)	5' flank region (5.1-23 screening primer)	*hph* gene	5' flank region (5.1-5.3)
SAS405	16	-	-	+	+
MAT1-2-1 gene	17	-	-	+	+
	1	-	-	+	+
	2	-	-	+	+
	3	-	-	+	+
	4	-	-	+	+
SAS405	5	-	-	+	+
MAT 1-2-4 gene	6	-	-	+	+
	7	-	-	+	+
	8	-	-	+	+
	9	-	-	+	+
	10	-	-	+	+
	1	-	-	+	+
	2	-	-	+	+
	3	-	-	+	+
SAS56	4	-	-	+	+
MAT1-1-1 gene	5	-	-	+	+
	6	-	-	+	+
	7	-	-	+	+
	8	-	-	+	+