Aspects of sexual reproduction in *Mycosphaerella* species on wheat and barley

genetic studies on specificity, mapping, and fungicide resistance

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Contents

This thesis is dedicated to my husband and best friend, Jonathan, and my two beautiful daughters, Hannah and Hope.

The fear of the Lord is the beginning of knowledge.
– Proverbs Chapter 111, Verse 10, Holy Bible

CHAPTER 1

General introduction and outline of thesis

The *Mycosphaerella* genus

Mycosphaerella is the largest genus in the family Mycosphaerellaceae and of the order Dothideales, having more than 3,000 names (Aptroot 2006). In addition, at least 40 anamorph genera (with no known sexual stage) are associated with *Mycosphaerella*, including over 3,000 species of *Cercospora* (Crous and Braun 2003) and over 1,000 species of *Septoria* (Crous and Braun 2003; Kirk et al. 2001). *Mycosphaerella* species are loculoascomycetes that form specialized fruiting bodies, called pseudothecia, which contain asci with sexual ascospores. Both *Mycosphaerella* and *Septoria* species also produce asexual pycnidiospores in flask-like structures called pycnidia. Many *Mycosphaerella* and *Septoria* species are plant pathogenic and cause diseases on an extremely diverse range of hosts, including wheat, bananas, strawberries, eucalyptus, tomatoes, and oranges (Farr et al 1995).

Mycosphaerella graminicola (Fuckel) J. Schröt. in Cohn (anamorph *Septoria tritici* Roberge in Desmaz.) causes septoria tritici blotch of wheat. This disease is a problem worldwide (Eyal and Levy 1987; Halama 1996; Hardwick et al. 2001; Jorgensen et al. 1999) and is controlled primarily by the use of fungicides, including strobilurins and azoles (Gisi et al. 2005; Oerke et al. 1994). *M. graminicola* infects both hexaploid bread wheat (*Triticum aestivum* L., AABBDD, 2n=42) and tetraploid durum wheat (*T. turgidum* L. (Thell.) subsp. *durum* L., AABB, 2n=28) species. Sequencing of the internally transcribed spacer region (ITS) of the ribosomal DNA, as well as the β-tubulin, actin, and calmodulin genes, did not show any clear distinction between isolates of *M. graminicola* infecting bread wheat when compared to those infecting durum wheat (Groenewald and Crous. *unpublished*, Figure 1). Nevertheless, isolates of *M. graminicola* exhibit both host species specificity and cultivar specificity (Kema et al. 1996a,b; Kema and van Silfhout 1997). Host species specificity refers to avirulence to all cultivars within one or the other wheat species, whereas cultivar specificity refers to virulence and avirulence on particular cultivars of either bread wheat or durum wheat.

Septoria passerinii Sacc. causes septoria speckled leaf blotch (SSLB) of barley (*Hordeum vulgare*, diploid, HH, 2n=14). Like septoria tritici blotch, this disease has been reported around the globe, but it is of particular importance in the Upper Midwest region of the United States and the Prairie Provinces of Canada (Cunfer and Ueng 1999; Mathre 1997). *S. passerinii* and *M. graminicola* share many similarities. *S. passerinii* clusters closely to *M. graminicola* in phylogenetic studies based on ITS sequences (Goodwin et al. 2001; Goodwin and Zismann 2001) (Figure 2). Both pathogens have a dimorphic lifestyle, in which they

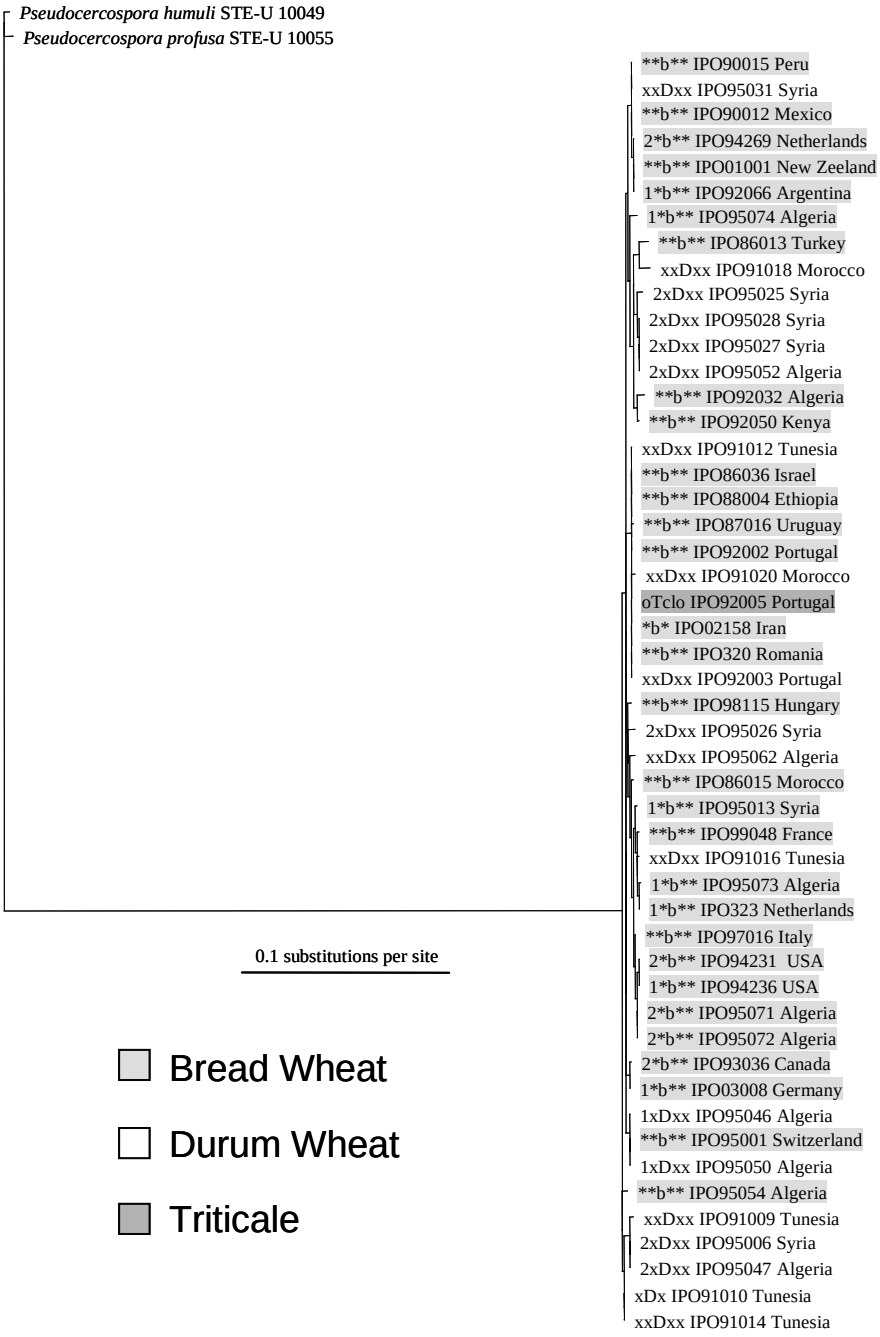

Figure 1. Neighbor-joining tree of bread wheat-derived and durum wheat-derived isolates of *Mycosphaerella graminicola* from different locations throughout the world obtained from combined ITS, actin, calmodulin, and β-tubulin sequence data (Groenewald and Crous, unpublished).

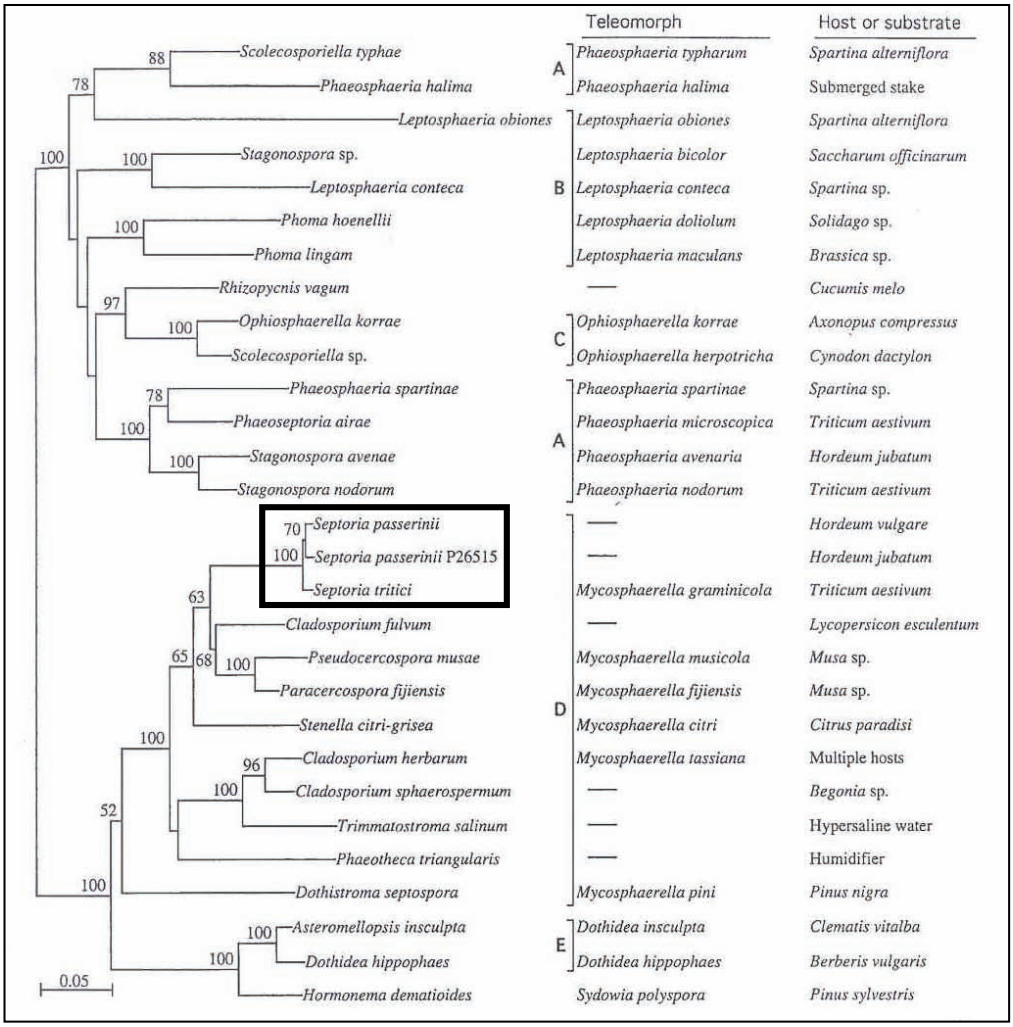

Figure 2. Unrooted neighbor-joining tree from fungal species related to *Mycosphaerella graminicola* based on ITS sequences (Goodwin and Zismann 2001).

produce yeast-like cells by blastic conidiogenesis under rich culture conditions, but grow filamentously under poor cultural and *in planta* conditions. The pathogenesis of both pathogens is similar. Small chlorotic flecks begin to appear 10-14 days after inoculation, and these slowly develop into larger chlorotic blotches that eventually turn necrotic. At 14-19 days after inoculation, pycnidia will form in the necrotic lesions, and these pycnidia contain slender, multi-celled pycnidiospores that serve as asexual primary inoculum.

One major difference between *M. graminicola* and *S. passerinii* is that *M. graminicola* has a year round and very active sexual cycle (Hunter et al. 1999; Kema et al. 1996c; Shaw and Royle 1989; Zhan et al. 1998, 2003), whereas there have been no reports of a sexual cycle for *S. passerinii*. Interestingly, the mating-type genes of the latter fungus were cloned based on homology with the *M. graminicola* mating-type genes, and subsequently,

isolates of *S. passerinii* with opposite mating types were commonly found in natural populations, even on the same leaf (Goodwin et al. 2003). These results, in addition to molecular data, suggest that this pathogen has an active, yet unknown, sexual cycle in nature (Goodwin et al. 2003).

Fungal genetics

Genetics is the scientific study of heredity, which is how particular qualities or traits, encoded by genes, are transmitted from parents to progeny. In fungi, the most common way for progeny to inherit recombinations of genes from both parents is through generative reproduction via a sexual cycle. For fungal pathogens with known sexual cycles, the frequency and impact of these cycles in nature can greatly differ. For example, field populations of the rice blast pathogen *Magnaporthe grisea* (anamorph *Pyricularia grisea*) are largely clonal (reviewed by Talbot 2003), which suggests that sexual recombination does not occur frequently in nature and that ascospores are not a major source of disease inoculum. In contrast, populations of *M. graminicola* have extremely diverse genotypes in natural populations throughout the world (Linde et al. 2002; Zhan et al. 2003). Sexual recombination in nature occurs many times per growing season (Hunter et al. 1999; Kema et al. 1996c), and ascospores are a major source of disease inoculum (Zhan et al. 1998).

An example of sexual reproduction in *Mycosphaerella* species is shown in Figure 3 (adapted from Alexopoulos et al. 1996). In *Mycosphaerella*, the organs in which the gametes are produced are spermagonia, which produce the male spermatia, and ascogonia, which produce the female trichogynes (Crous 1998). A spermatium fertilizes a trichogyne after the cytoplasm of two parent mycelia fuse (plasmogamy). Next, nuclei undergo simultaneous mitosis, and septa will develop to yield a dikaryotic cell having two haploid nuclei (a dikaryote). Such cells will undergo mitosis that leads to formation of ascogenous hyphae containing nuclei from each parent. Ascus mother cells will form, and genetically distinct nuclei will then fuse into a zygote during karyogamy. The zygote nucleus undergoes meiosis, resulting in four haploid nuclei. Each of these nuclei will then undergo mitosis to yield four twin pairs of daughter cells (ascospores), which form within asci within fructifications called pseudothecia.

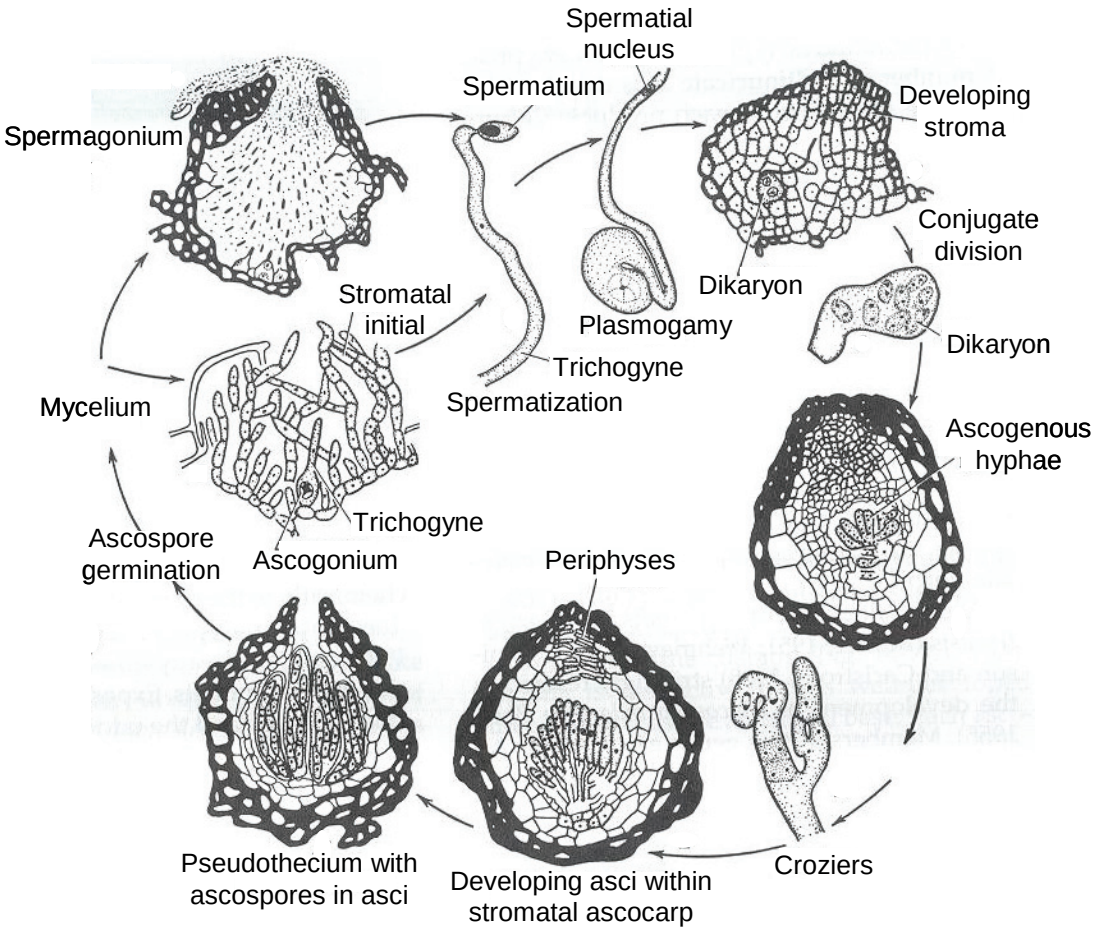

Figure 3. Assumed process of sexual reproduction in *Mycosphaerella graminicola* (adapted from Alexopoulos et al. 1996 from the example of sexual reproduction in *Mycosphaerella* species).

Recombination during meiosis can occur by both chromosomal reassortment and crossing over. Reassortment of homologous chromosomes results in the redistribution of entire chromosomes from the parents in progeny. Crossing over results in the exchange of genetic material between non-sister chromatids of homologous chromosomes, thereby forming new chromosomes containing parts from each parent. Fungal geneticists study the reassortment of genes in progeny from such sexual crosses to identify functions associated with these genes. The heritable traits of progeny can be visually scored, such as fungal colony pigmentation, but these traits can also be scored molecularly, such as by polymerase chain reaction (PCR) detection of the presence or absence of a particular locus.

All heritable traits and genetic sequences in a segregating progeny population can be scored as "markers" and incorporated together to create genetic linkage maps. When enough progeny and markers are available, these maps can give a good idea about the approximate number of chromosomes present in terms of linkage groups and can also place the genetic markers in the correct order on these linkage groups. In this way, traits of interest can be localized by using markers that segregate with these traits, and fine-mapping strategies can be used to further delimit the regions and eventually clone genes involved in regulation and control of the desired traits. There are many methods used to obtain molecular markers, including PCR-based techniques such as Random Amplification of Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and Diversity Arrays Technology (DArT).

Specificity and genetics in *M. graminicola*

Host species specificity among isolates of *M. graminicola* has been generally accepted for a long time, as it has been typically reported that isolates from bread wheat do not cause disease on durum wheat cultivars, and *vice versa* (Eyal et al. 1973; Saadaoui 1987; van Ginkel and Scharen 1988; Kema et al. 1996a). Cultivar specificity, however, has been controversial, mainly due to the presence of quantitative virulence and the lack of hypersensitive responses (Eyal et al. 1973, 1985; Johnson 1992; Parlevliet 1993; van Ginkel and Scharen 1988).

In 1996, there was a breakthrough, as genetic studies became possible for *M. graminicola* with the development of an *in planta* crossing protocol on wheat (Kema et al. 1996c). A mapping population was generated from two Dutch bread wheat-derived isolates of *M. graminicola*, IPO323 (avirulent on cv. Shafir) and IPO94269 (virulent on cv. Shafir). Half of the progeny was virulent and half avirulent on cv. Shafir, which led to the conclusion that avirulence in isolate IPO323 was due to a single locus (Kema et al. 2000). The locus conferring avirulence was mapped on a genetic linkage map created via the use of AFLP and RAPD molecular markers (Kema et al. 2002). Further analyses of resistance in the host provided final proof of the much-debated existence of a gene-for-gene interaction between isolates of *M. graminicola* and cultivars of wheat (Brading et al. 2002). Since then, 12 resistance genes have been mapped in bread wheat (Chartrain et al. 2005), some of which are currently being incorporated into breeding programs (Goodwin et al. 2004).

Disease control methods

The best way to control septoria tritici blotch is through the use of resistant wheat cultivars. Although there have been 12 resistance genes identified in bread wheat cultivars in recent years, the targeted incorporation of these genes in breeding programs is still underway. Another effective way to control *M. graminicola* is through the use of systemic fungicides such as azoles and strobilurins. Azoles have been extensively used for over 20 years, and no major resistance has developed (Gisi et al. 2000; Suty and Kuck 1996). Conversely, strobilurins were first introduced in 1996, and high levels of resistance in populations of *M. graminicola* were already reported in 2003 (Gisi et al. 2005).

Outline of the thesis

Research presented in this thesis sheds light on the underestimated capacity for survival of *M. graminicola* via mating and the passing on of essential genes to future generations. We generated a high-density genetic linkage map from progenies of *in planta* crosses between virulent and avirulent isolates on hosts resistant to one of the parents, a phenomenon that is in itself intriguing. We further went on to characterize these progenies for virulence on a set of seven differential wheat cultivars, and these results were used to map nine segregating quantitative trait loci involved in (a)virulence. Many additional crosses between avirulent and virulent isolates were also successful, showing that a resistant host species or cultivar restricts or inhibits disease development but does not prevent sex in *M. graminicola*. *In planta* detection of avirulent isolates in resistant hosts showed that these avirulent isolates can increase in biomass over time despite the fact that no visual disease symptoms develop. *In planta* successful crosses between isolates of *M. graminicola* were also observed and characterized under fungicide pressure when one isolate was sensitive and one isolate was resistant to strobilurins, which reveals yet another situation in which a stressed isolate of *M. graminicola* can still complete a sexual cycle.

Septoria passerinii was thought to be asexual for the past 130 years, but co-inoculating isolates with opposite mating types *in planta* resulted in the generation of sexual progeny, which again proves that sex in fungi is more prevalent than is currently acknowledged.

Chapter 1 gives an introduction to the biology of *Mycosphaerella graminicola* and *Septoria passerinii*, as well as an introduction to haploid genetics in *Mycosphaerella* species.

Concepts of host species and cultivar specificity in the *M. graminicola*-wheat pathosystem are discussed.

In **Chapter 2**, interactions between avirulent and virulent isolates on different wheat cultivars and species are described. Competition aspects are addressed using quantitative PCR, and the findings are related to sexual development in *M. graminicola*. We conclude that *in planta* mating is common for isolates of *M. graminicola* even when one of the isolates is avirulent, which provides this pathogen with a unique opportunity to circumvent host resistance and to transfer avirulence genes to next generations.

Chapter 3 describes the construction and comparisons of two high-density genetic linkage maps of *M. graminicola* using segregations of DArT markers in progenies generated from a cross between two Dutch bread wheat-derived isolates and a cross between a Dutch bread wheat-derived isolate and an Algerian durum wheat-derived isolate.

Chapter 4 describes the genetic basis of specificity in *M. graminicola*. Virulence of progeny from a cross between a Dutch bread wheat-derived isolate and an Algerian durum wheat-derived isolate was assessed on four bread wheat and three durum wheat cultivars. These data were used to identify nine QTLs involved in (a)virulence on cultivars of either bread wheat or durum wheat or both on the genetic linkage map described in Chapter 3.

Chapter 5 describes the fitness, pathogenicity, and sexual development of *M. graminicola* on wheat plants that were preventively treated with a stobilurin fungicide. We conclude that these fungicides do not prevent sex. This finding has implications for the development and distribution of fungicide resistance in the Western European *M. graminicola* population.

Chapter 6 describes the generation of a functional *Mycosphaerella* teleomorph in the presumed asexual barley pathogen *S. passerinii* using an *in planta* crossing method. *S. passerinii* isolates with opposite mating types were co-inoculated onto susceptible barley cultivars, and ascospores discharged from leaves were positively identified as sexual progeny. A previously unknown sexual cycle explains high genetic variation among isolates in nature.

Chapter 7 provides a summarizing discussion of the thesis. The importance of the ability of *M. graminicola* to sexually recombine even in hostile environmental conditions such as fungicide applications and resistant cultivars is emphasized. The consequences with respect to population genetics and durability of resistance are discussed.

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CHAPTER 2

Avirulence does not prevent sexual propagation of the fungal plant pathogen *Mycosphaerella graminicola*

Sarah B. Ware, Caucasella Díaz-Trujillo, and Gert H. J. Kema

ABSTRACT

The role of sexual reproduction in the survival of fungal organisms is an important field of research, owing to the fact that sexual propagules can travel over longer distances and disseminate genetic variation efficiently. Such processes can be responsible for quick distribution of fungicide resistance or pathogen virulence. We studied the possibility of *in planta* generation of sexual progeny of the fungal wheat pathogen *Mycosphaerella graminicola* involving avirulent parents. In addition to being cultivar-specific, isolates of *M. graminicola* generally show host species specificity, in which bread wheat-derived isolates are non-pathogenic on durum wheat cultivars, and *vice versa*. Hence, progeny from a cross between a bread wheat-derived isolate and a durum wheat-derived isolate would enable us to study the genetic basis of host species specificity. Nineteen crosses between isolates with different host species and cultivar specificities were performed on either bread wheat or durum wheat cultivars, as well as on the non-host barley. We show that avirulence does not prevent sex, as all crosses were successful and resulted in viable ascospores as long as one parent was virulent on a particular wheat cultivar. *In planta* biomass accumulation of avirulent isolates on resistant hosts was assessed by TaqMan® quantitative PCR using mating-type-specific probe and primer combinations, and results showed that the biomass of avirulent isolates on resistant host species and cultivars was either maintained or increased over time. We discuss the implications of these results for the dynamics of virulence and avirulence loci in natural populations in relation to durability of resistance in wheat cultivars and the potential to study the genetic basis of host species specificity in the wheat-*M. graminicola* pathosystem.

INTRODUCTION

In the process of co-evolution in pathosystems, gene-for-gene interactions between resistance genes and avirulence effectors have developed between hosts and pathogens (Janzen 1980). Such gene-for-gene interactions have been proven to exist between plants and viruses, bacteria, fungi, and oomycetes (Chisholm et al. 2006; Kang et al. 2005; Person 1959). These interactions have co-evolved between plants and both asexual and sexual fungi, such as in the tomato-*Cladosporium fulvum* and flax-*Melampsora lini* pathosystems, respectively (Chisholm et al. 2006; Flor 1956; Joosten and de Wit 1999). Fungal avirulence genes have been cloned by reverse genetics and by map-based cloning. Map-based methods have so far resulted in the cloning of avirulence genes from only three species of plant pathogenic fungi. The genes identified include four effectors from the rice blast pathogen *Magnaporthe grisea* (Sweigard et al. 1995; Valent 1997), four from the flax rust pathogen *M. lini* (Catanzariti et al. 2005, Dodds et al. 2004), and two from the oilseed rape stem canker pathogen *Leptosphaeria maculans* (Gout et al. 2006).

Mycosphaerella graminicola (Fuckel) J. Schröt. in Cohn causes septoria tritici blotch of both hexaploid bread wheat (*Triticum aestivum* L.) and tetraploid durum wheat (*T. turgidum* L. (Thell.) subsp. *durum* L.) species, and shows both host species and cultivar specificity (Kema et al. 1996a, 1996b; Kema and van Silfhout 1997). Although nuclear markers do not reveal any clear distinction between isolates of *M. graminicola* infecting bread wheat as opposed to those infecting durum wheat, isolates of *M. graminicola* are generally host species-specific, so that when a field isolate is sampled from a bread wheat cultivar, it will not be virulent on any durum wheat cultivar, and *vice versa*. In addition, isolates can be cultivar-specific, rendering them virulent on some but avirulent on other bread wheat or durum wheat cultivars. Although the existence of cultivar specificity has been debated in the past (see also Chapters 3 and 4), genetic studies eventually proved cultivar-specific gene-for-gene interactions in the *M. graminicola*-wheat pathosystem (Kema et al. 2000, 2002; Brading et al. 2002). In these studies, a single locus (hereby tentatively named *MgAvrStb6*) controlled avirulence in the bread wheat-derived isolate IPO323 that triggered resistance in bread wheat cultivars carrying the resistance gene *Stb6*, which was mapped on the wheat chromosome 3A (Brading et al. 2002; Chartrain et al. 2005).

The importance and existence of specificity in the wheat-*M. graminicola* pathosystem have been discussed for over 25 years, and only recently fungal genetics studies resolved the genetic basis for cultivar specificity (Kema et al. 1996c, 2000). The existence of host species

specificity has been widely reported based on pathogenicity testing of pathogen isolates on host species in both seedling and adult plant experiments (Eyal et al. 1973, 1985; Kema et al. 1996a; Saadaoui 1987; van Ginkel and Scharen 1988; Zhan et al. 2004). However, genetic analysis to identify genes involved in determining host species specificity requires crosses on host species which are resistant to one of the parental pathogen isolates, an occurrence that has not been previously reported.

In this study, we explore the possibility of generating crosses between virulent and avirulent *M. graminicola* isolates to address host species specificity as well as cultivar specificity. Hence, we performed crosses between bread wheat-derived and durum wheat-derived isolates of *M. graminicola* on both bread wheat and durum wheat cultivars to study host species specificity, as well as crosses between avirulent and virulent isolates on either cultivars of bread wheat or durum wheat to study cultivar specificity. Because we use an *in planta* crossing protocol, we were also interested to study the dynamics of colonization and biomass development during pathogenesis, and therefore competition experiments to simultaneously monitor two isolates within the same leaf tissue over time in comparison to individual inoculations were conducted.

We show that it is consistently possible to obtain large and viable sexual progenies from crosses between avirulent and virulent isolates of *M. graminicola*, irrespective of whether these are due to host species or cultivar specificity. Our data challenge the current paradigm of strong selection against avirulent isolates leading to the classical boom-and-bust cycles in other cereal pathogens (Kolmer 1996; McIntosh and Brown 1997; Person 1957). In contrast, *M. graminicola* effectively maintains avirulence genes in natural populations. As a result, population shifts toward virulence on new host genotypes are gradual, and this consequently affects durability of resistance in wheat to *M. graminicola* populations.

MATERIALS AND METHODS

Isolates, cultivars, and inoculation methods

In this study, we used eight isolates of *M. graminicola* from various origins which have different specificities and mating type idiomorphs (Table 1). These were used in crossing assays on nine wheat cultivars and one barley cultivar (Table 2). Growth conditions of plants and fungi, inoculum preparation, and inoculation methods were as described previously (Kema et al. 1996c).

Table 1. Summary information about the isolates of *Mycosphaerella graminicola* used in this study.

Isolate	Year	Origin	Host	Virulent on	Mating Type
IPO001	unknown	Netherlands	Bread Wheat[1]	Bread Wheat	*mat 1-1*
IPO323	1981	Netherlands	Bread Wheat	Bread Wheat	*mat 1-1*
IPO87019	1987	Uruguay	Bread Wheat	Bread Wheat	*mat 1-2*
IPO88004	1988	Ethiopia	Durum Wheat	Durum Wheat	*mat 1-2*
IPO94269	1994	Netherlands	Bread Wheat	Bread Wheat	*mat 1-2*
IPO95054	1995	Algeria	Bread Wheat	Bread Wheat	*mat 1-2*
IPO95050	1995	Algeria	Durum Wheat	Durum Wheat	*mat 1-1*
IPO95052	1995	Algeria	Durum Wheat	Durum Wheat	*mat 1-2*

[1]derived from a hexaploid derivative of a cross between bread wheat and wild emmer wheat (*T. dicoccoïdes*, AABB, 2n=28).

Table 2. Attempted crosses between isolates of *Mycosphaerella graminicola* on both bread wheat and durum wheat cultivars and on barley. V = virulent; A = avirulent; + = progeny; - = no progeny

Isolates Crossed	Cultivar	Species	Progeny
bread wheat-derived x bread wheat-derived			
IPO323 (V) x IPO94269 (V)	Obelisk	Bread Wheat	+
IPO323 (V) x IPO94269 (V)	Taichung 29	Bread Wheat	+
IPO323 (A) x IPO94269 (V)	Shafir	Bread Wheat	+
IPO323 (A) x IPO94269 (A)	Volcani 447	Durum Wheat	-
IPO323 (A) x IPO94269 (A)	Topper 33	Barley	-
IPO001 (V) x IPO94269 (V)	Obelisk	Bread Wheat	+
IPO001 (V) x IPO94269 (A)	Lakhish	Bread Wheat	+
IPO001 (A) x IPO94269 (V)	Clement	Bread Wheat	+
IPO323 (A) x IPO87019 (A)	Kavkaz-K4500	Bread Wheat	-
IPO323 (A) x IPO88004 (A)	Veranopolis	Bread Wheat	-
durum wheat-derived x durum wheat-derived			
IPO95050 (V) x IPO95052 (v)	Volcani 447	Durum Wheat	+
IPO95050 (A) x IPO95052 (A)	Obelisk	Bread Wheat	-
bread wheat-derived x durum wheat-derived			
IPO323 (A) x IPO95052 (V)	Inbar	Durum Wheat	+
IPO323 (V) x IPO95052 (A)	Obelisk	Bread Wheat	+
IPO323 (A) x IPO95052 (A)	Shafir	Bread Wheat	-
IPO94269 (A) x IPO95050 (V)	Inbar	Durum Wheat	+
IPO94269 (V) x IPO95050 (A)	Obelisk	Bread Wheat	+
IPO95054 (A) x IPO95050 (V)	Inbar	Durum Wheat	+
IPO95054 (V) x IPO95050 (A)	Obelisk	Bread Wheat	+

Growth and competition on resistant and susceptible cultivars

We studied the ability of isolates with host species- and cultivar- specific avirulences to accumulate biomass on resistant hosts when inoculated individually and in mixtures with virulent isolates. To monitor the biomass of multiple fungal isolates in wheat leaves over time, experiments were performed using three *M. graminicola* isolates with different genotypes on resistant and susceptible hosts. Two bread wheat-derived isolates, IPO323 and IPO94269, were inoculated individually and in mixtures on bread wheat cvs. Shafir and Taichung 29. Both isolates are virulent on cv. Taichung 29, but IPO323 has a specific avirulence to cv. Shafir (Brading et al. 2002; Kema et al. 2000, 2002). In addition, the bread wheat-derived isolate IPO323 and the durum wheat-derived isolate IPO95052 were inoculated individually and in mixtures on the bread wheat cv. Taichung 29 and the durum wheat cv. Volcani 447. Due to their host species-specificities, IPO323 is avirulent on cv. Volcani 447 and IPO95052 is avirulent on cv. Taichung 29. Leaves were collected immediately after inoculation and subsequently at 5-day intervals (0-20 days post inoculation, dpi) and were immediately frozen in liquid nitrogen and then stored at -80° C until lyophilization, subsequent DNA extractions, and TaqMan® analyses. Disease severity was also visually estimated using the percent leaf area covered by pycnidia that were recorded before each leaf sample was taken. All inoculations were performed in duplicate (2 pots per inoculation). After 21 dpi, re-isolations were made from single pycnidia from leaves inoculated with mixtures, and the resulting isolates were stored for further analysis to determine from which of the initially-inoculated isolate(s) they were produced.

Crossing experiments

The crossing protocol, including the discharge of ascospores, was according to the *in planta* procedure described previously (Kema et al. 1996c). A total of 19 co-inoculations of virulent and avirulent isolates of *M. graminicola* on various resistant and susceptible cultivars of wheat and one cultivar of barley were performed to generate sexual progenies (Table 2). Attempts to discharge ascospores began six weeks post inoculation and continued through 12 weeks. Success of crosses was determined by ascospore morphology and germination on water agar and subsequent phenotypic growth on both potato dextrose agar and in liquid yeast-glucose medium. The populations that were obtained from the crosses between IPO323 and IPO94269 on the bread wheat cvs. Obelisk and Shafir and the populations that were obtained from the crosses between IPO323 and IPO95052 on both the bread wheat cv.

Obelisk and the durum wheat cv. Inbar were studied in more detail using four molecular markers.

DNA isolations and PCR screens

We developed a quantitative TaqMan® PCR method to monitor and quantify the amount of fungal biomass of a specific genotype in inoculated wheat leaves over time (see Chapter 5). This method uses probes that will only fluoresce in the presence of a specific DNA target sequence. *M. graminicola* is a heterothallic fungus, which requires isolates with opposite mating-type idiomorph sequences for mating to occur. Hence, every isolate will have either the idiomorph for mating type 1 (*mat 1-1*) or the idiomorph for mating type 2 (*mat 1-2*) (Table 1). We developed probes that are specific for each mating-type idiomorph, enabling simultaneous *in planta* detection of fungal biomasses of two isolates with opposite mating types. For TaqMan® standards, dilution series' of known concentrations of pure fungal DNA from isolates IPO323, IPO94269 and IPO95052 were used in each TaqMan® run (100, 10, 1, 0.1, and 0.01 ng per µl) to determine the unknown amounts of fungal DNA in wheat leaves.

Extraction of genomic DNA of *M. graminicola* from cultures was from 50 mg lyophilized spores using the Wizard® Magnetic DNA Purification System for Food (Promega, Madison, WI, USA), with a final elution with 100 µl sterile double-distilled (sdd) water. Extractions of genomic DNA from plant material inoculated with *M. graminicola* were from 10 mg lyophilized leaves from each inoculated pot that were homogenized into a powder to represent each dpi. We used the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), with a final elution of 2 x 50 µl sdd water (100 µl final volume). Dilutions of the DNA samples were run on 1.2% agarose gels along with dilutions to 10, 25, 50, and 100 ng made from standard λ DNA (Roche, Almere, Netherlands) for precise quantification. Independent DNA isolations were performed for each of the duplicate leaf samples, and DNA from each of these resulting extractions was run twice in quantitative PCR experiments (total of 4 readings per sample). The specific *mat1-1* and *mat1-2* idiomorph TaqMan® probe and primer combinations for quantitative PCR were used in non-multiplexed 25-µl reactions according to the components and thermal cycling conditions described elsewhere in this thesis (see Chapter 5). Quantitative real-time amplifications were performed on an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Results were analyzed using Sequence Detection Software version 1.2.3 (Applied

Biosystems). The standard curves had very high R^2 values (0.990-0.996) for all data points from 3 pg to 30 ng and hence, the CT values within this range were reliable.

Nuclear segregations in ascospore progenies obtained from the crosses between isolates IPO323 and IPO94269 on cvs. Obelisk and Shafir and from crosses between isolates IPO323 and IPO95052 on cvs. Obelisk and Inbar were characterized by PCRs for mating type, a microsatellite marker, and a marker co-segregating with the avirulence locus *MgAvrStb6* (Kema et al. 2000, 2002). Mating-type PCR conditions were as described previously (Waalwijk et al. 2002). Primers for the microsatellite marker ag-0006 were described previously by Goodwin et al. (2006, *in press*), and PCR conditions and analysis of products were as described elsewhere in this thesis (see Chapter 3). Non-public primers were developed at Plant Research International (PRI) to generate a marker, Avr, that completely co-segregates with avirulence conferred by *MgAvrStb6* in IPO323 (Kema et al. 2002). Thermal cycling was as follows: cycle 1; 96° C for 2 mins., cycle 2 (repeated 12x); 96° C for 30 secs., then 55° C for 30 secs., then 72° C for 1 min., cycle 3: 72° C for 5 mins., followed by a cooling-off step to 10° C. In addition to nuclear DNA segregations, mitochondrial segregations in the aforementioned ascospore progenies were determined by PCRs to detect a microsatellite marker, mt SSR, which amplifies a region containing the repeat TTTAA using the forward primer mt SSR F (5'-3' CTCAGTTCAAGTCTGAGTGC) and the reverse primer mt SSR R (5'-3' GACGCACGCATTTCCACTCTA). PCR conditions and analysis of products was the same as for marker ag-0006.

RESULTS

As mentioned before, isolates of *M. graminicola* display a strong host species specificity when inoculated onto sets of bread wheat or durum wheat cultivars. The major question we wanted to address was whether it would be possible to generate sexual progeny from crosses between such host species-specific *M. graminicola* isolates using an *in planta* protocol that, as a consequence of the host species specificity, would utilize cultivars of either bread wheat or durum wheat that are resistant to one of the parental pathogen isolates.

Crossing experiments

Most interestingly, all crosses between two *M. graminicola* isolates were successful as long as one of the isolates was virulent on its wheat host, irrespective of whether this was a durum wheat or a bread wheat cultivar (Table 2). Evidently, avirulence did not stop *M. graminicola* from sexual exchange, regardless of whether the avirulence was due to host species specificity or cultivar specificity. As expected, all crosses between two virulent isolates on susceptible wheat cultivars were successful. As a general observation, crosses in which both parental isolates were virulent yielded larger progenies, although the numbers of ascospores generated from virulent x avirulent parental isolates were more than adequate to collect mapping populations. Only crosses between two avirulent isolates failed both on wheat cultivars as well as on the non-host barley cv. Topper 33 and hence, no progenies were obtained in these attempts.

The nuclear DNA segregations in the IPO323 x IPO94269 progenies obtained from cv. Obelisk (n=74 after two removed) and cv. Shafir (n=87 after six removed) and in the IPO323 x IPO95052 progenies obtained from cv. Obelisk (n=99 after four removed) and cv. Inbar (n=58 after two removed) were tested using polymorphic mating-type (Waalwijk et al. 2002), avirulence (Kema et al., *unpublished data*) and microsatellite markers (Goodwin et al. 2006, *in press*) (Figures 1 and 2). Isolates were removed from the analysis when there was no clear result from PCRs for at least one of the four molecular markers. Results of all three nuclear DNA PCRs supported regular Mendelian inheritance of these markers (1:1 segregation ratios; χ^2=0.054-1.707, P<0.05) in all four progenies obtained from all three cultivars. In contrast, the mitochondrial DNA segregations of these progenies deviated significantly different from 1:1 (χ^2=13.517-99.000, P<0.0001; Figures 1 and 2). In the IPO323 x IPO94269 progeny obtained from cv. Obelisk, 74% inherited the mitochondrial type of IPO94269, whereas 100% of the progeny had this mitochondrial type when the cross was made on cv. Shafir. In the IPO323 x IPO95052 progeny obtained from cv. Inbar, 74% inherited the mitochondrial type of IPO323, whereas 100% of the progeny had this mitochondrial type when these isolates were crossed on cv. Shafir.

Growth and competition on resistant and susceptible wheat cultivars

We were also interested in monitoring the dynamics of biomass development of individual isolates during pathogenesis and the onset of the sexual cycle, and we therefore used TaqMan® technology. In addition, asexual spores were re-isolated from pycnidia in leaves inoculated with mixtures of two isolates. Isolate IPO323 is completely avirulent on

A

Marker	IPO323	IPO94269	Progeny crossed on bread wheat cv. Obelisk n = 74	χ^2, *P*<0.05	Progeny crossed on bread wheat cv. Shafir n = 87	χ^2, *P*<0.05
Avr	present	absent	42:32	1.351	48:39	0.931
Mat	*Mat 1-1*	*Mat 1-2*	41:33	1.108	47:40	0.563
ag-0006	B	A	36:38	0.054	52:35	3.322
mt SSR	B	A	19:55	17.514*	0:87	87.000*

B

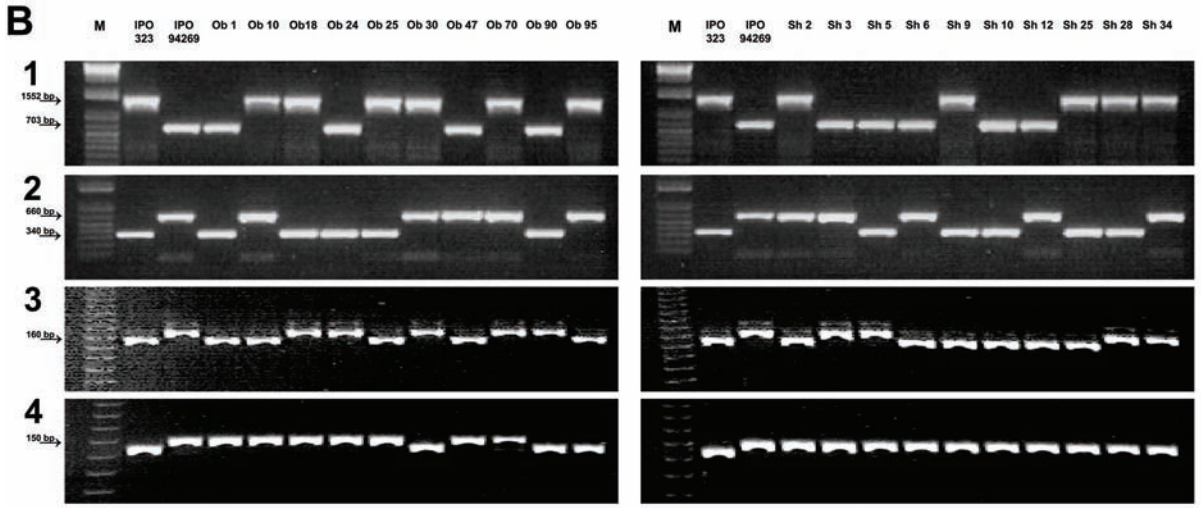

Figure 1. Segregations of molecular markers in populations that were derived from *in planta* crosses between *Mycosphaerella graminicola* isolates IPO323 and IPO94269 that were crossed on the bread wheat cvs. Obelisk and Shafir. **A,** Summary of segregation ratios for the nuclear markers for avirulence (Avr), mating type (Mat), and the microsatellite ag-0006, as well as the mitochondrial microsatellite marker mt SSR. Gray highlighted cells represent segregation ratios that differ significantly from a 1:1. *(P<0.0001) **B,** Examples of segregating markers. Left, Progeny from a cross made on the bread wheat cv. Obelisk. Right, Progeny from a cross made on the bread wheat cv. Shafir. 1, Avr. Upper band = presence, lower band = absence. 2, Mat. Upper band = *Mat 1-1*, lower band = *Mat 1-2*. 3, ag-0006. 4, mt SSR.

A

Marker	IPO323	IPO95052	Progeny crossed on bread wheat cv. Obelisk n = 99	χ^2, *P*<0.05	Progeny crossed on durum wheat cv. Inbar n = 58	χ^2, *P*<0.05
Avr	present	absent	53:46	0.495	28:30	0.690
Mat	*Mat 1-1*	*Mat 1-2*	55:44	1.222	31:27	0.276
ag-0006	B	A	43:56	1.707	28:30	0.690
mt SSR	B	C	99:0	99.000*	43:15	13.517*

B

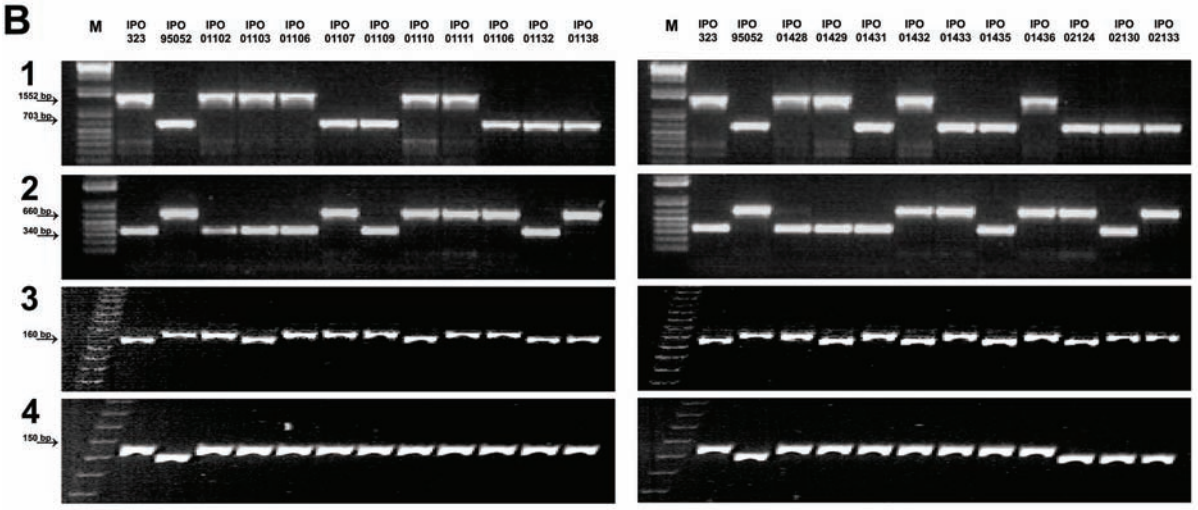

Figure 2. Segregations of molecular markers in populations that were derived from *in planta* crosses between *Mycosphaerella graminicola* isolates IPO323 and IPO95052 that were crossed on both the bread wheat cv. Obelisk and the durum wheat cv. Inbar. **A,** Summary of segregation ratios for the nuclear markers for avirulence (Avr), mating type (Mat), and the microsatellite ag-0006, as well as the mitochondrial microsatellite marker mt SSR. Gray highlighted cells represent segregation ratios that differ significantly from a 1:1. *(P<0.0001) **B,** Examples of segregating markers. Left, Progeny from a cross made on the bread wheat cv. Obelisk. Right, Progeny from a cross made on the durum wheat cv. Inbar. 1, Avr. Upper band = presence, lower band = absence. 2, Mat. Upper band = *Mat 1-1*, lower band = *Mat 1-2*. 3, ag-0006. 4, mt SSR.

cv. Shafir but develops substantial biomass on this cultivar, and remarkably IPO323 even outcompetes the virulent isolate IPO94269 on cv. Shafir (Figure 3). On cv. Volcani 447, however, the biomass of IPO323 did not significantly increase and it was not successful in competing with the virulent durum wheat-derived isolate IPO95052 (Figure 3). A similar observation was made for the latter isolate in the bread wheat cv. Taichung 29, in which its biomass did not increase when individually inoculated and significantly decreased in the mixed inoculations, which was also the case for all other combinations (Figure 3). Despite the minimal biomass of the majority of the avirulent strains in the combined inoculations, this procedure, which is part of the *in planta* crossing protocol, eventually resulted in numerous viable progeny isolates. Interestingly, high amounts of biomass were not always correlated with the pycnidial production in the septoria tritici blotch symptoms (Figure 3).

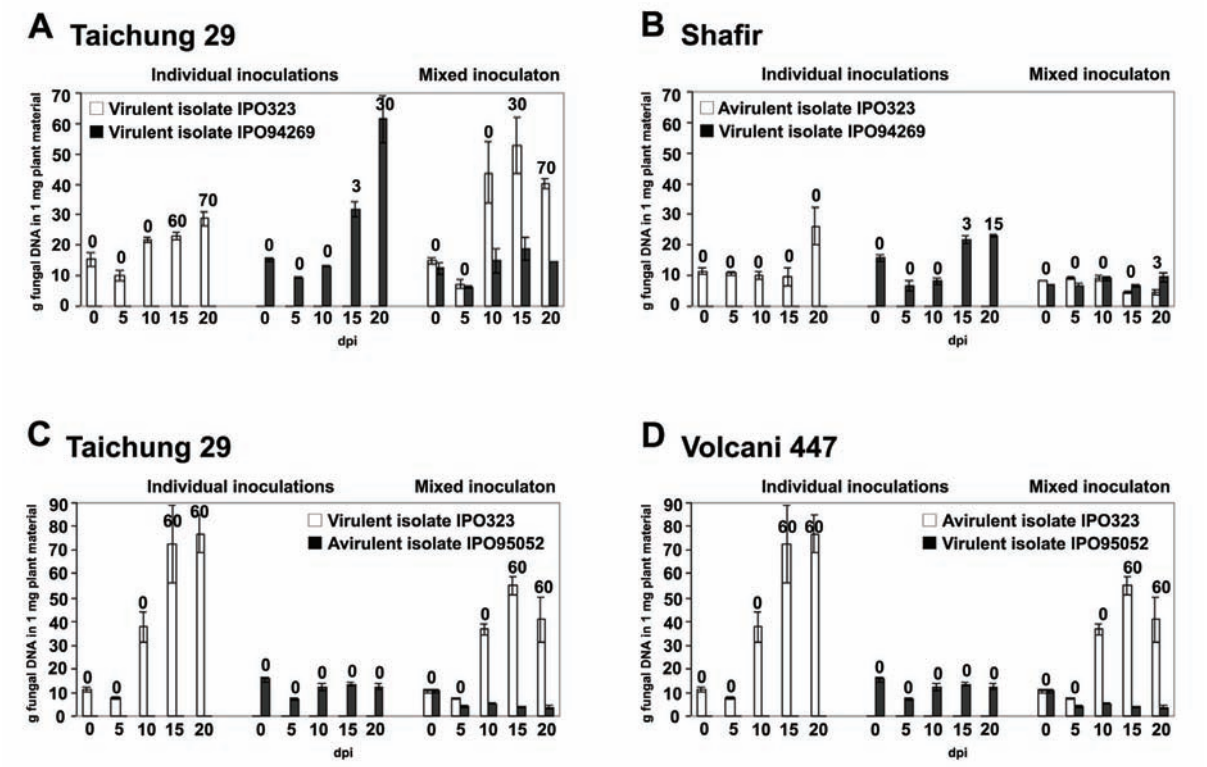

Figure 3. Quantitative fungal biomass detection of *Mycosphaerella graminicola* isolates IPO323 and IPO94269 on bread wheat cvs. Shafir and Taichung 29 **(A,B)** and isolates IPO323 and IPO95052 on bread wheat cv. Taichung 29 and durum wheat cv. Volcani 447 **(C,D)** at 0, 5, 10, 15, and 20 dpi (bars) and percent leaf area covered by pycnidia at each time point (numbers above each bar).

SSR genotyping of re-isolations from these asexual fructifications, which developed in the plants that were inoculated with two isolates, showed that only the virulent strain was able to produce pycnidia (Figure 4). Only isolate IPO323 was recovered from cv. Taichung 29 when mixed with isolate IPO94269, even though both isolates are virulent on this cultivar (Figure 4).

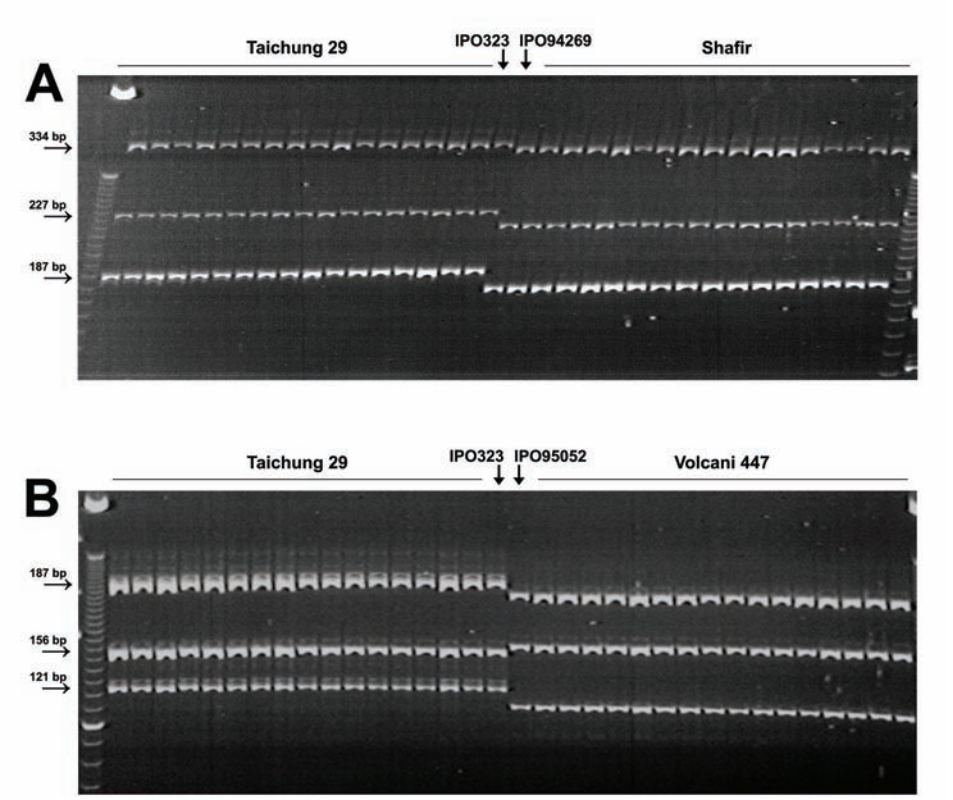

Figure 4. SSR genoyping of re-isolations of asexual pycnidial isolates from wheat leaves that were co-inoculated with *Mycosphaerella graminicola* isolates. **A,** From mixtures of IPO323 and IPO94269 using markers (from top to bottom) ac-0001, ggc-0001, and caa-0002. **B,** From mixtures of IPO323 and IPO95052 using markers (from top to bottom) ag-0003, ag-0006, and ac-0007.

DISCUSSION

The current paradigm of the gene-for-gene interaction is strongly linked with a hypersensitive response (HR), which is an early defense response by the plant that causes necrosis and cell death to restrict the growth and spread of a pathogen throughout the plant (van der Biezen and Jones 1998). When an HR occurs, an avirulent isolate is unable to multiply, and the consequence is that none of the avirulence gene(s) of this isolate will be propagated in future generations (van der Plank 1982; Zadoks and Schein 1979). Although

an HR is usually expected in gene-for-gene interactions, inoculations of avirulent strains of *M. graminicola* on resistant cultivars do not cause this response. Indeed, in our studies we confirmed that avirulent *M. graminicola* isolates can accumulate substantial biomass in resistant cultivars and even outcompete virulent isolates. However, these avirulent isolates cannot multiply asexually, as re-isolations revealed only propagules from the co-inoculated virulent isolates. Isolate IPO323 doubled its biomass in the resistant cv. Shafir during the first 20 days after inoculation, but this cultivar remains completely free of disease symptoms, oblivious to the fact that it is being extensively colonized.

Despite the lack of asexual propagation of avirulent isolates in resistant cultivars, all of them successfully partake in sexual propagation. Although it was assumed that avirulent isolates on resistant hosts would not survive or at least not multiply, our data showed that all crosses were successful except those between two avirulent isolates. We conclusively showed that neither host species specificity nor cultivar specificity can prevent the generation of sexual progeny of any mating as long as one of the parental strains is virulent. The importance of this finding cannot be overestimated and has intriguing population genetic and technical implications. The genetic structure of natural *M. graminicola* populations is complex and comprises numerous different genotypes as revealed by extensive analyses (Zhan et al. 2003), resulting in multiple genotypes infecting the same leaves and even the same lesions (McDonald and Linde 2002). It is not far-fetched to consider that natural populations also maintain a tremendous qualitative and quantitative genetic variation for virulence. Our data show that in such cases, mating between isolates with contrasting pathogenicity phenotypes does take place, and hence transmission of avirulence factors to next generations occurs as indicated by the genetic analyses of such progenies using molecular markers for nuclear genes as well as phenotypic characterization (see also Chapters 3 and 4). Clearly, biomass development is not a requirement, as minimal biomass is sufficient for successful mating and airborne dissemination. Hence, avirulence genes are not quickly removed from the gene pool, which consequently has effects on durability of resistance in the wheat-*M. graminicola* pathosystem. Thus far, there has only been one documented case in which a resistant cultivar of wheat rapidly became susceptible to *M. graminicola* (Cowger et al. 2000). It is the usual situation that a gradual decrease in resistance of cultivars is observed (Kema et al. 1999). We propose that this gradual decrease is due to the maintenance of avirulence genes throughout natural *M. graminicola* populations via sexual crosses between virulent and avirulent isolates. In this pathosystem, host selection exists when an avirulent isolate is alone on a resistant cultivar, but this selection may not be

very effective, because the population size is so large and diverse that the chance of mating with a virulent isolate is likely, particularly since mating takes place throughout the season (Eriksen et al. 2001; Hunter et al. 1999; Kema et al. 1996c).

To our knowledge, there are no other studies similar to ours in which sexual crosses on resistant cultivars were successful on which one of the parents was avirulent, and the possibility for this to occur has not been addressed in epidemiology, either in textbooks (van der Plank 1982; Zadoks and Schein 1979) or in mathematical modeling. Most existing models for co-infection only allow adaptive responses to hard selection and predict that low genetic relatedness among intraspecies pathogens co-infecting the same host will lead to the evolution of and selection for high pathogen virulence (Eshel 1977; Levin and Pimentel 1981; Bremermann and Pickering 1983; Sasaki and Iwasa 1991; Frank 1994, 1996; May and Nowak 1994, 1995; Nowak and May 1994; Lenski and May 1995; van Baalen and Sabelis 1995; Gandon 1998; Mosquera and Adler 1998). However, our results from the cross between IPO323 and IPO95052, combined with the common occurrence of crosses between avirulent and virulent isolates on hosts resistant to one of the parental isolates, plus proof of biomass maintenance and increase of avirulent isolates in resistant hosts, do not support predictions by the above models. Instead, our data suggests that co-infections of *M. graminicola* isolates lead to evolution of low pathogen virulence. Although the possibility for sexual recombination involving avirulent isolates was not considered, a collaborating prediction was modeled by Chao et al. (2000), in which both hard and soft selection were taken into account compared with an evolutionarily stable strategy (ESS) (Maynard Smith and Price 1973; Maynard Smith 1982) to show that co-infections will favor evolution of low pathogen virulence. Sex of an avirulent isolate on a resistant host is the ultimate example of soft selection. Gandon et al. (2002) applied the ideas of Chao et al. (2000) to a model in which host resistance was allowed to co-evolve with pathogen virulence according to gene-for-gene interactions (Flor 1956), a biologically realistic situation for the *M. graminicola*-wheat pathosystem. This model allows for two components to the cost of parasitism, which are (i) infectivity (the ability to infect the host) and (ii) virulence (the deleterious effect on the host). When co-infections occur, this model predicts that the presence of resistant hosts in the population decreases virulence in the pathogen population. Indeed, the *M. graminicola* population has an effective way to pass on avirulence genes through sexual recombination by combining isolates that are infective yet not virulent with isolates that are both infective and virulent to balance the overall cost of virulence within the population with the need to maintain hosts that can be exploited for nutrients.

In an organism with a heterothallic bipolar lifestyle, sexual reproduction in a random mating setting is considered to result in equal distributions of individual markers (see also Chapter 5). The same holds for markers on the mitochondrial genome under the assumption that male and female contributions to sexual reproduction are equal for both isolates in the applied crossing procedure (Coenen et al. 1996; Robinson et al. 2002). However, in our study the mitochondrial segregation ratios in progenies from two parental sets always significantly differed from the expected 1:1 ratio. The generation of progenies on one and the same wheat cultivar resulted in contrasting selection of female contributions to sexual reproduction. When isolates IPO323 and IPO95052 were crossed on cv. Obelisk, IPO323 was the exclusive female parent, but when IPO323 was crossed with IPO94269 on this cultivar, IPO94269 prevailed as the female parent. We found two aspects of mitochondrial inheritance to be true. First, interactions between each set of two parental isolates can result in a preferred mitochondrial type in progeny. Second, when the preferred father (25% mitochondrial type in progeny) is on a resistant host and that host is susceptible to the other isolate, the preference will be completely shifted to 0% for the preferred father. These data indicate that *M. graminicola* isolates with avirulence to host cultivars or species increasingly behave as paternal donors in sexual reproduction. A bias for mitochondrial type based on the cultivar for crossing is consistent with a selective sweep hypothesized to occur in the Mediterranean Basin (Zhan et al. 2004). Similar to our results in this chapter, we have also discovered skewed mitochondrial segregation ratios in progenies that were generated *in planta* under fungicide stress (see Chapter 5).

The intriguing technical implication from this study is that we are now for the first time able to study the genetic basis of host species specificity in *M. graminicola*. Mapping populations derived from crosses between two bread wheat-derived isolates and a cross between a bread wheat- and a durum wheat-derived isolate revealed that the genetic distance between the former is surprisingly larger than in the latter (see Chapter 3). The alignment of these maps with the genomic sequence of *M. graminicola* that was recently released, will aid in the identification of genes involved in pathogenicity and specificity, which will lead to an improved understanding of this important pathogen.

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CHAPTER 3

High-density maps of the fungus *Mycosphaerella graminicola* using Diversity Arrays Technology (DArT) reveal frequent loss of chromosomes

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ABSTRACT

Diversity Arrays Technology (DArT) was used to construct two high-density genetic linkage maps of the haploid wheat pathogen *Mycosphaerella graminicola*. Isolate IPO323 that originates from a Dutch bread wheat field was crossed *in planta* with either another Dutch bread wheat-derived isolate, IPO94269, or the Algerian isolate IPO95052 that was isolated from durum wheat. The segregation of markers was followed in the progenies. Because *M. graminicola* shows host species specificity, the generation of progeny from the *in planta* cross between IPO323 and IPO95052 was unexpected, because these parental isolates are avirulent to durum wheat and bread wheat, respectively. In total, 1,801 DArT markers, 258 AFLP markers, and 25 SSR markers could be mapped. The maps of the individual crosses showed absolute co-linearity to a constructed bridge map, with the exception of eight markers that appear to originate from translocations between IPO95052 and IPO94269. Graphical genotyping enabled the identification of dispensable chromosomes for 15-20 % of the progenies that were present in both parental isolates. We demonstrate that this is due to aberrations during meiosis caused at least in part by non-disjunction. The loss of chromosomes did not seem to affect viability or pathogenicity. This is the first report on detailed meiotic events using high–density mapping in a haploid filamentous fungus. We show that DArT is a highly efficient and robust marker technology for genetic mapping. The DArT markers can be readily sequenced and are currently being deployed in the assembly of the 9X *M. graminicola* IPO323 genome sequence.

INTRODUCTION

Fungi provide attractive model systems to study processes that occur during meiosis for two main reasons. First, they remain haploid after meiosis for a large part of their life cycles, which simplifies genetic studies. Second, tetrad analysis allows the complete recovery of the products of meiosis. *Aspergillus nidulans* and *Neurospora crassa* have been particularly instrumental in answering fundamental questions concerning meiosis (Davis and Perkins 2002; Geiser et al. 1996; Raju 1980). Here we describe genetic studies on another filamentous ascomycete, *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn (anamorph *Septoria tritici* Roberge in Desmaz.). *M. graminicola* causes septoria tritici blotch (STB) in wheat, a disease characterized by necrotic blotches on the foliage, which contain both asexual and sexual fructifications (respectively, pycnidia and pseudothecia). The sexual cycle is an important driver of STB epidemics (Kema et al. 1996a, 1996c; Zhan et al. 2003) and results in high genetic diversity of *M. graminicola* populations in the field. *M. graminicola* infects both hexaploid bread wheat (AABBDD, 2n=42) and tetraploid durum wheat (AABB, 2n=28) species, but does show clear host species specificity, as bread wheat-derived isolates do not generally cause disease on durum wheat, and *vice versa* (Eyal et al. 1973; Kema et al. 1996b; Saadaoui 1987; van Ginkel and Scharen 1988). In addition, a gene-for-gene interaction between *M. graminicola* isolate IPO323 and the bread wheat cvs. Shafir, Kavkaz and Veranopolis was demonstrated (Brading et al. 2002; Kema et al. 2000, 2002).

Molecular analysis of a cross between two Dutch bread wheat-derived isolates of *M. graminicola*, IPO323 x IPO94269, resulted in the first genetic linkage map of a *Mycosphaerella* species (Kema et al. 2002). The map was based on 223 Amplified Fragment Length Polymorphism (AFLP) and 57 Random Amplification of Polymorphic DNA (RAPD) markers, as well as mating type (Mat) and avirulence (Avr) markers (Kema et al. 2002; Waalwijk et al. 2002). Recently, 23 simple sequence repeat (SSR) markers were integrated into this map (Goodwin et al., *in press*).

Although the first map was a major milestone, the anonymous AFLP and RAPD markers complicated integration of genetic data sets. Furthermore, the number of markers and the map resolution were not adequate to assess the difficulties anticipated during meiosis due to chromosome length polymorphisms and the differences in chromosome numbers observed in *M. graminicola* isolates (Kema et al. 1999; McDonald and Martinez 1991; Zolan 1995). In addition, we were interested to study the genetics of host species specificity. However, thus far only *in planta* crossing protocols have been successful (Kema et al. 1996c,

2000), which consequently requires crosses to be made on a host species on which only one of the isolates is virulent. Nevertheless, we succeeded in crossing the bread wheat-derived isolate IPO323 with the Algerian durum wheat-derived isolate IPO95052 both on bread wheat and durum wheat cultivars (see Chapter 2). The resulting progeny of this cross (IPO323 x IPO95052) and the progeny of the previously studied cross (IPO323 x IPO94269) were fingerprinted using Diversity Arrays Technology (DArT).

DArT simultaneously types several thousands of DNA fragments in a genomic representation by scoring the presence versus absence of these fragments after hybridization on a microarray platform (Akbari et al., 2006; Jaccoud et al. 2001; Semagn et al. 2006; Wenzl et al. 2004; Wittenberg et al. 2005). It provides a high-throughput, cost-effective, sequence-independent tool for whole-genome profiling. In addition, DArT markers can be sequenced readily and hence are valuable in genome alignment. Here we apply DArT for the first time in the small genome (~ 40 Mbp) of a haploid eukaryotic fungus. We show that DArT can be used to effectively create one of the most dense genetic linkage maps currently available, a result that exemplifies its potential as a generic technique for high-throughput genome profiling of other fungi.

The objective of this study was to assess to what extent translocations and differences in chromosome length and chromosome number influence recombination events during meiosis. We performed this study by genetic linkage analysis in two progenies. The parental isolates for the two crosses were selected based on the presence of chromosome length polymorphism and host species specificity for the two isolates (Kema et al. 1996a, 1999). In several cases, two or three progeny derived from the same meiotic event could be identified. High-density genetic linkage maps were generated for both mapping populations. These maps were compared and the data was carefully examined for translocations, local differences in recombination frequencies, and possible cases of non-Mendelian inheritance. We found examples of translocations, recombination suppression in chromosomal regions, and loss of chromosomes during meiosis. Finally, the high-density maps with sequenced markers will support the assembly of the recently completed 9X sequence of *M. graminicola* isolate IPO323 (http://www.jgi.doe.gov/).

MATERIALS AND METHODS

Fungal isolates and growth conditions

M. graminicola isolates IPO323 and IPO94269 were isolated from leaves that were collected in two Dutch fields planted with the bread wheat cultivars Arminda and Vivant in 1981 and 1994, respectively, while IPO95052 was isolated from leaves of an unknown durum wheat cultivar that were collected in Algeria in 1995. The pathogenicity of all three isolates has been tested intensively under greenhouse and field conditions on different wheat cultivars (Brading et al. 2002; Chartrain et al. 2004; Kema et al. 2002). F_1 individuals (n=60) from the IPO323 x IPO94269 cross were used to generate DArT markers that were integrated into the existing map (Kema et al. 2002). In addition, a set of *in planta* crosses was made between IPO323 and IPO95052 using the protocol developed by Kema et al. (1996c) on both the bread wheat cv. Obelisk and the durum wheat cv. Inbar. Ascospores were discharged onto sterile water agar plates and were left overnight at 20°C to allow germination, and a random ascospore progeny was subsequently isolated (n=163) and stored at -80° C for further analyses. Table 1 gives an overview of all progeny isolates used in this study.

DNA extraction

DNA of parents and progeny was isolated using the Promega Wizard Genomic DNA purification kit (Food Kit Promega, Madison, WI, USA) according to the protocol provided by the manufacturer starting with 10 mg of lyophilized spores. DNA from 96 progeny isolates of the IPO323 x IPO95052 crosses was extracted twice starting from the same culture.

Markers

Kema et al. (2002) generated the first genetic linkage map of *M. graminicola* for the progeny of the cross IPO323 x IPO94269. For this map, 11 *Eco*RI/*Msp*I AFLP primer combinations generated 271 markers of which 223 could be positioned on 23 linkage groups (LGs). In addition, 57 RAPD markers from a set of 104 could be incorporated. In this study, we expanded the number of markers by using DArT and incorporating a number of SSR loci.

DArT procedure

Generation of genomic representations. The DNA of *M. graminicola* isolates IPO323 and IPO95052 was used to construct two genomic representations for each isolate as described by Wittenberg et al. (2005). Genomic representations were generated by digesting 100 ng of genomic DNA with 2 units of either *Hin*dIII or *Bam*HI in combination with the frequent cutters *Mse*I and *Rsa*I (New England Biolabs; NEB, USA). Cloning adapters (Table

Table 1a: *Mycosphaerella graminicola* progeny isolates (n=76) from the IPO323 x IPO94269 *in planta* cross, which was made on the susceptible bread wheat cv. Obelisk, that were used for hybridization to the DArT arrays.

Isolate number	Used for construction of genetic linkage map	Isolate number	Used for construction of genetic linkage map
IPO323[1]	-	119	Yes
IPO94269[2]	-	124	Yes
1	Yes	125	Yes
10	Yes	126	Yes
11	-	131	Yes
12	Yes	132	Yes
14	Yes	134	Yes
16	-	136	Yes
18	-	137	Yes
22	-	139	-
23	Yes	140	-
24	Yes	142	Yes
25	-	144	Yes
27	Yes	147	Yes
29	Yes	148	-
30	-	150	-
36	-	157	Yes
40	Yes	158	Yes
46	-	160	Yes
47	Yes	164	Yes
50	Yes	167	Yes
51	Yes	173	Yes
58	-	174	Yes
62	Yes	176	Yes
68	Yes	179	Yes
70	-	180	Yes
73	Yes	182	Yes
83	Yes	183	Yes
84	Yes	184	Yes
87	Yes	192	Yes
88	Yes	193	Yes
90	Yes	197	Yes
91	Yes	198	Yes
94	Yes	200	Yes
95	Yes	202	Yes
100	Yes		
109	Yes		
110	-		
111	Yes		
115	Yes		
116	-		
117	Yes		
118	Yes		

[1]Parental isolate IPO323 was isolated from the bread wheat cultivar Arminda.
[2]Parental isolate IPO94269 was isolated from the bread wheat cultivar Vivant.

Table 1b. *Mycosphaerella graminicola* progeny isolates (n=163) from the IPO323 x IPO95052 *in planta* crosses that were made on the bread wheat cv. Obelisk and the durum wheat cv. Inbar. Sixteen isolates (highlighted in gray) were not used, leaving a total of 147 that were used in the construction of the genetic linkage map. The first two numbers indicate the year of isolation and the next three numbers the order of isolation.

Isolated from bread wheat cultivar Obelisk			Isolated from durum wheat cultivar Inbar	
IPO323[1]	01135	01171	*IPO95052*[2]	02043
01101	01136	01172	01426	02044
01102	01137	01173	01427	02045
01103	01138	01174	01428	02046
01104	01139	01175	01429	02047
01105	01140	01176	01430	02121
01106	01141	01177	01431	02122
01107	01142	01178	01432	02123
01108	01143	01179	01433	02124
01109	01144	01180	01434	02125
01110	01146	01181	01435	02126
01111	01147	01182	01436	02127
01112	01148	01183	01437	02128
01113	01149	01184	01438	02129
01114	01150	01185	01439	02130
01115	01151	01186	01440	02131
01116	01152	01187	02024	02132
01117	01153	01188	02025	02133
01118	01154	01189	02026	02134
01119	01155	01190	02027	02135
01120	01156	01191	02028	02136
01121	01157	01192	02029	02137
01122	01158	01193	02030	02138
01123	01159	01194	02031	02139
01124	01160	01195	02032	02140
01125	01161	01196	02033	02141
01126	01162	01197	02034	
01127	01163	01198	02035	
01128	01164	01199	02036	
01129	01165	01200	02037	
01130	01166	01421	02038	
01131	01167	01422	02039	
01132	01168	01423	02040	
01133	01169	01424	02041	
01134	01170	01425	02042	

[1]Parental isolate IPO323 was isolated from the bread wheat cultivar Arminda.
[2]Parental isolate IPO95052 was isolated from an unknown durum wheat cultivar.

2) were simultaneously ligated to the complementary overhangs with T4 DNA ligase (NEB) as described by Wittenberg et al. (2005). A 1-µl aliquot of the ligation product was used as a template in a 50-µl amplification reaction using primers complementary to the adapter sequences and cycling conditions described by Wenzl et al. (2004).

Table 2. The adapter and primer oligonucleotide sequences used for generation of the genomic representation (cloning) from *Mycosphaerella graminicola* isolates IPO323 and IPO95052 and hybridization to the micro-arrays (genotyping) of parental and progeny isolates.

Endonuclease and recognition site	**Used for**	**Adapter sequences[a]**	**Primer sequences (5' to 3')**
HindIII 5'-A$^{\downarrow}$AGCTT-3' 3'-TTCGA$_{\uparrow}$A-5'	Cloning	5'-CTCGTAGACTGCGTCAC-3' 3'-ATCTGACGCAGTGTCGA -5'	TAGACTGCGTCACAGCTT
	Genotyping	5'-GTGCTACAGTCGCTGAG-3' 3'-ATGTCAGCGACTCTCGA-5'	TACAGTCGCTGAGAGCTT
BamHI 5'-G$^{\downarrow}$GATCC-3' 3'-CCTAG$_{\uparrow}$G-5'	Cloning	5'-CTCGTAGACTGCGATCA-3' 3'-CATCTGACGCTAGTCTAG-5'	GTAGACTGCGATCAGATCC
	Genotyping	5'-GTGCTACAGTCGCTAGA-3' 3'-GATGTCAGCGATCTCTAG-5'	CTACAGTCGCTAGAGATCC
MseI 5'-T$^{\downarrow}$TAA-3' 3'-AAT$_{\uparrow}$T-5'	Cloning	5'-ACTCGATCCTCACACGTA AAGTATAGATCCCA-3' 3'- NH_2-TTCATATCTAGGGTAT-5'	ACTCGATCCTCACACGTA
	Genotyping	5'-AGTGCATGGTGAGAGCTA AACTATACATGGGA-3' 3'- NH_2-TTGATATGTACCCTAT-5'	AGTGCATGGTGAGAGCTA
RsaI 5'-GT$^{\downarrow}$AC-3' 3'-CA$_{\uparrow}$TG-5'	Co-digestion	-	-

[a] Adapter sequences were formed by annealing the strands whose sequences are listed. Complementary sequences are underlined.

Library construction and array preparation. A 3,072-clone library for IPO323 and IPO95052 was prepared for each of the *Hin*dIII-*Mse*I-*Rsa*I and the *Bam*HI-*Mse*I-*Rsa*I complexity reduction methods according to Jaccoud *et al.* (2001) with modifications described by Wenzl *et al*. (2004). PCR products were dried, washed once with 70% ETOH, and re-suspended in 25 µl spotting buffer (unpublished observation). The amplification products were spotted in duplicate on polylysine coated slides (Erie Scientific, Portsmouth, NH, USA) using a MicroGrid II arrayer (Biorobotics, Cambridge, UK). After printing, the

slides were processed by incubation in hot water (95°C) for 2 min., dipped in a 100 mM EDTA and 100 mM DTT solution, and dried by centrifugation.

Target preparation and genotyping. The genomic representations of individual progeny isolates to be hybridized on the array were generated by applying the aforementioned complexity reduction methods, with the exception that genotyping adapters rather than cloning adapters were used (Table 2). The products of the 50-µl PCR-reactions were concentrated 10-fold by precipitation with 1 volume of isopropanol and denatured at 95°C for 3 min. Each reaction was labeled with 0.1 µl of Cy3-dUTP using 1 µl of 500µM random decamers (Amersham Biosciences, Castle Hill, NSW, Australia) and the exo$^-$ Klenow fragment of *Escherichia coli* DNA polymerase I (NEB). In experiments for which DNA was isolated twice, a replicate target labeled with Cy5-dUTP was co-hybridized with the Cy3-dUTP labeled target to the same array. The polylinker fragment of the plasmid was used as a reference (Jaccoud et al. 2001) and labeled with 6-FAM. Labeled representations, called targets, were denatured, hybridized to microarrays overnight at 65°C, and slides were washed according to Wenzl et al. (2004).

Image analysis and polymorphism scoring. Typically, each experiment comprised 96 bar-coded slides that were scanned using a Tecan LS 300 (Grödig, Austria) confocal laser scanner. Each image pair (cy3-FAM or cy5-FAM) was stored directly in the database and subsequently analyzed with DArTSoft (version 7.4.1), a software package developed at DArT P/L. (C. Cayla, G. Uszynski, D. Jaccoud., P. Wenzl and A. Kilian, *unpublished*). DArTSoft was used to both identify and score the markers that were polymorphic within each experiment as described by Wenzl et al. (2004). The program computes several quality parameters for each clone as described by Akbari et al. (2006), and markers were selected by simultaneously applying thresholds for four of those; *i.e.* P-value, call rate, reproducibility and polymorphism information content (PIC) value. Clones with P-values ≥ 77%, call rate ≥ 80%, reproducibility ≥ 95% and PIC-value ≥ 0.3 were selected for both libraries.

SSR

Recently, 23 SSR loci were identified in 10 *M. graminicola* EST libraries that were polymorphic in the IPO323 x IPO94269 mapping population (Goodwin et al., *in press*). Twenty-one of them could be positioned on the existing linkage map along with two previously published SSR loci (Owen et al. 1998; Goodwin et al., *in press*). These SSRs and the newly-generated DArT markers were used to integrate the new IPO323 x IPO94269 map

with the existing map of that population (Kema et al. 2002). Moreover, nine of these SSRs also differentiated the *M. graminicola* IPO323 and IPO95052 parents of the second mapping population. To enable the mapping of six of these SSRs in the IPO323 x IPO95052 progeny, amplification reactions were performed according to Goodwin et al. 2006, *in press*). An overview of all SSRs can be found in Table 3.

Nomenclature of markers

AFLP markers were designated by the primer combination used for the amplification and the approximate length of the generated fragment (Kema et al. 2002). For both AFLP and DArT markers, the prefix A or B indicated the phase of the marker; those originating from parent IPO323 had the prefix A, while markers from parent IPO95052 were indicated by the prefix B. DArT markers identified in cross IPO323 x IPO94269 originating from isolate IPO95052 could get the prefix A or B, as IPO94269 was not used for the library construction. Markers segregating in both populations got the prefix C. In addition, DArT markers were designated by the enzyme combination used for complexity reduction (BMR or HMR), the 384-well plate number and the position of the fragment in that plate (i.e. AHMR_04I09). The SSR markers derived from the EST libraries are denoted by the type of repeat and locus number, whereas the two earlier described SSRs (Owen et al. 1998) are denoted by the prefix ST.

Selection of unique segregation patterns and merging of twin isolates

The binary scores of polymorphic markers were converted to the correct allelic phase based on the scores of the parents. A Perl script was written that grouped loci with identical segregation patterns disregarding unknown scores. For each group, the marker with the highest call rate (percentage of scored individuals) within that group was selected as a representative for that group. The script also calculated the call rate for each individual genotype and the global call rate for the whole dataset. Individual genotypes were incorporated in the scoring table when at least 95% of the grouped markers could be scored. In *M. graminicola*, twin progeny isolates arise from the mitotic division that follows meiosis II in the ascus, resulting in four pairs of genetically identical ascospores. Although the random ascospore progenies that resulted from the applied crossing protocol minimized the isolation of twin isolates, the large number of markers efficiently identified identical progeny. These were used to calculate the reproducibility of the different marker types and were merged before the mapping analyses.

Table 3. Forward and reverse primer sequences for polymorphic SSR markers that differentiated the *Mycosphaerella graminicola* isolates IPO323 and IPO94269 and were used for mapping. Six SSRs (highlighted in gray) that were also polymorphic between IPO323 and IPO95052 were used for construction of the bridge map. [a]Estimated sizes in base pairs. *Could not be assessed.

SSR marker	Forward primer	Reverse Primer	Repeat	Repeat number	Anneal. Temp	IPO323 bp [a]	IPO94269 bp	IPO95052 bp
ag-0003	acttggggaggtgttgtgag	acgaattgttcattccagcg	ag	15	55	252	223	223
ag-0006	taaccaacaccaggggaatg	catcagttgtcagcgaatgg	ag	7	55	156	162	162
ag-0009	gactccatttacctgtggcg	tgtgaaggacacgcaaagag	ag	7	55	196	198	198
tcc-0006	atctggacaccatccaccag	gtaggtgggagggttcatgc	tcc	6	60	168	170	170
gca-0004	taacggtaacggcaacaacc	gtgtacccttgaatcgcagc	gca	6	55	177	174	174
ST1E3	gttccgccggtcgaagtcg	gccaaggcactgctgctcc	cgg	5	60	61	63	63
ST1B3	acgttcacatctcatacc	ctctagctctaccttaat	ccg	8	50	203	200	*
ac-0007	tgctcgcaagacataaaacg	ctcttagcattggtcggtgg	ac	15	55	121	120	108
caa-0002	tctgcagagatcccgttacc	atccatcacatgacgcacac	caa	11	55	334	333	*
ag-0011	ttgagcaggttacggagagg	ccagctgggagatattcgtg	ag	11	60	175	176	175
ct-0005	tgaccactgacctccacacc	agaagtggcttggtgacagg	ct	10	55	118	120	119
gaa-0001	tacaacagaacaacggcacg	aattttcagcggatccttcc	gaa	9	55	210	228	*
tcc-0009	tcaattgccaataattcggg	agacgaggcagttggttgag	tcc	8	55	157	165	157
ggc-0001	gataccaaggtggccaagg	cacgttgggagtgtcgaag	ggc	8	55	227	220	227
tcc-0008	aaaagacatgacgcccgac	acgaggaataatcgcggaac	tcc	7	55	152	150	152
tcc-0005	acgagcaaccactcaccatc	ggagagggtgaaaggagagg	tcc	7	60	228	226	*
gaa-0004	aaatccgcctgaaatccttc	ttttccgagagggaaagagg	gaa	7	55	196	168	*
ac-0002	tgaacatcaacctcacacgc	agaagaggacgacccacgag	ac	7	55	189	191	189
ac-0001	caccacaccgtcgttcaag	cgtaagttggtggagatggg	ac	7	55	187	175	187
gga-0001	gtacgacacgggctatggag	ggcgatgacgatgaaacc	gga	6	55	154	157	152
gca-0010	gtggactccgagggtggtag	ttcccaacagcatccgtatc	gca	6	55	179	182	179
gca-0008	cgcagggagagcagtttcg	gcaagcgagaagatgctacc	gca	6	60	155	153	155
gca-0007	aacatgaccttcgatgtcgc	tcggagtcttcaccttggac	gca	6	55	166	161	166
ac-0006	cagcagacaatcagctgtgc	ttgagtgaggtcgtgtcgag	ac	6	55	232	182	*
ggc-0003	gactaatctcctcgccctcc	cgcccttactcctcttctcc	ggc	6	60	272	268	*

Map construction

Segregation ratios of all markers were analyzed with JoinMap (Stam 1993) version 3.0. Markers with segregation ratios significantly different from 1:1 ($P < 0.01$) were initially set aside, and linkage analysis using the Kosambi mapping function was performed on the remaining markers. Initial assignment to LGs was based on the logarithm of the odds (LOD) ratio for each possible marker pair. We used LOD-values in the range of 3–8, whereas the final assembly of LGs was completed using a LOD-value of 4 or higher. We used linkages with a REC < 0.4, a map LOD-value of 0.05, and a χ^2-jump threshold of 5 for inclusion into the map and for the calculation of the linear order of the markers within a LG. Finally, we tested whether the markers with a distorted segregation ratio contributed to the map using an iterative addition process from markers with little to substantial segregations distortions. After each JoinMap run, the map was inspected for changes in marker order and distance. When these were not disturbed and when synteny between the two parallel crosses (IPO323 x IPO94269 and IPO323 x IPO95052) was observed, the addition of markers with distorted segregation ratios to the map was accepted. The individual maps were inspected either in Excel or with the graphical genotyping software package GGT (Berloo 1999), which allowed detection of singletons and visualized the recombination events in all progeny.

Comparison of marker order in both crosses

The use of IPO323 in both crosses enabled the efficient generation of an integrated bridge map of the *M. graminicola* genome. The C-labeled markers that segregated in both crosses were selected and merged into groups of markers with identical segregation patterns. Loci with strong segregation distortion ($P < 0.005$) were removed, and linkage analysis was performed on the remaining markers. Groups were selected at LOD ≥5.5, and the order of the markers within the LGs was determined using the same settings as for the construction of the individual maps. The bridge map was used to compare the order of the loci in the constructed IPO323 x IPO94269 and IPO323 x IPO95052 maps. We used MapChart 2.2 (Voorrips 2002) for the graphical representation of the genetic linkage maps.

Calculation of percentage double crossovers (DCOs) and recombination frequencies

We used GGT (Berloo 1999) for the calculation of DCOs in each of the marker sets. For this analysis, we removed markers with redundant segregation patterns (not knowing whether they were identical or only co-segregating) because otherwise, DCOs adjacent to blocks of identically scored markers would have been undetectable. In addition, we

calculated the average recombination frequency in both crosses. All LGs and markers were included in the analyses, but recombination events resulting from DCOs were subtracted, as these would result in an overestimation of the actual recombination frequency. Local differences in recombination frequencies in the two progenies were detected by comparing the genetic distances of the C-labeled markers that segregated in both populations.

Evaluation of missing chromosomes

We used graphical genotyping to compare the marker scores (A or B) and the phase (A or B) of the markers, which enabled us to identify whether each marker was present in a particular progeny isolate. When a LG was constructed from both marker types and specific progeny isolates lacked these markers, we concluded that such an isolate missed that LG. Hence, *in silico* chromosome polymorphisms in progeny isolates was determined if A- and B-markers that were assigned to a specific LG were absent in such a progeny isolate.

RESULTS

Marker selection

Analysis of the progeny from a cross between the bread wheat-derived isolates IPO323 and IPO94269

We obtained 1,042 DArT markers for the progeny of the IPO323 x IPO94269 cross, and these markers were added to the 271 AFLP markers from a previous study on this progeny (Kema et al. 2002), along with 25 SSR markers and markers for mating type (*mat*) and avirulence (*avr*) (Waalwijk et al. 2002).

Kema *et al.* (Kema et al. 2002) detected seven pairs of genetically identical genotypes among 68 progeny isolates screened, leaving 61 genetically unique isolates, that were used to saturate the existing map with DArT markers. These genetically identical isolates are a result of mitosis after meiosis in an ascus. Each ascus has four pairs of genetically identical ascospores (twins). In the 61 isolates, we identified an extra twin pair that had not been detected previously. This resulted in a scoring table with 60 individual genotypes and 1,341 markers. A Perl script (see Material and Methods) grouped the markers into 473 unique segregation patterns containing 297 DArT, 165 AFLP, and 11 SSR markers (Tables 4 and 5a). The global call rate (percentage of scored individuals) we obtained for this dataset was

99.12 %. The presence of twins in the dataset enabled us to calculate the reproducibility for the DArT, AFLP and SSR markers, which was 100 %, 99.89 %, and 100 %, respectively. The previously scored RAPD markers showed lower reproducibility (95.63 %) and were not used in the current map.

Table 4. Overview of the number of markers for both crosses. Mapping was performed using the software package JoinMap 3.0.

Genetic map	Total number of markers selected	Unique segregation patterns	Segregation distortion removed ($P \leq 0.01$)	Grouped	Mapped excluding segregation distortion	Mapped including segregation distortion	Total number of markers positioned on map
IPO323 x IPO94269	1341	473 35.3 %	444	443	441	451	1317 98.21 %
IPO323 x IPO95052	1162	496 42.7 %	458	457	457	486	1144 98.45 %
Bridge	389	263 67.6 %	243	241	236	251	372 95.63 %

Table 5a. Overview of type and number of molecular markers that were scored in the progeny of the cross between *Mycosphaerella graminicola* isolates IPO323 and IPO94269 before and after grouping.

Marker type	Isolate	Complexity reduction method	Number of markers	Unique segregation patterns	Percentage of total number of unique segregation patterns
AFLP	IPO323	[1]	151	93	19.66
AFLP	IPO94269	[1]	120	72	15.22
DArT	IPO323	BMR	375	156	32.98
	IPO95052	BMR	183	33	6.98
DArT	IPO323	HMR	383	80	16.91
	IPO95052	HMR	101	28	5.92
SSR	-	-	26	11	2.33
mat and *avr*	-	-	2	0	-
Sum			1341	473	100%

[1] AFLP markers were generated using 11 EcoRI-MspI primer combinations (Kema et al. 2002).

Table 5b. Overview of type and number of molecular markers that were scored in the progeny of the cross between *Mycosphaerella graminicola* isolates IPO323 and IPO95052 before and after grouping.

Marker type	Isolate	Complexity reduction method	Number of markers	Unique segregation patterns	Percentage of total number of unique segregation patterns
DArT	IPO323	BMR	265	137	27.62
	IPO95052	BMR	258	113	22.78
DArT	IPO323	HMR	296	121	24.40
	IPO95052	HMR	335	120	24.19
SSR	-	-	6	4	0.81
mat and *avr*	-	-	2	1	0.20
Sum			1162	496	100%

Table 6. Identified twin isolates in the progenies derived from crosses between either *Mycosphaerella graminicola* isolates IPO323 and IPO94269 or IPO323 and IPO95052.

IPO323 x IPO94269		IPO323 x IPO95052	
9	10	01101	01102
22	27	01103	01126
109	110	01105	01106
111	112	01109	01112
116	117	01115	01119
133	134	01132	01133
148	150	01142	01143
156	158	01154	01162
159	160	01177	01184
174	175	01196	01197
176	178	01426	01433
		01429	01430
		01434	01437
		01435	01439
		02034	02047
		02035	02043
		02037	02046
		02041	02042
		02128	02131
		02130	02136
		02133	02138
		02137	02139
		02134	02141

Analysis of the progenies from crosses between the bread wheat-derived isolate IPO323 and the durum wheat-derived isolate IPO95052

For the crosses between IPO323 and IPO95052, 148 progeny isolates were reliably scored for both DArT libraries, including 23 pairs of twin genotypes (Table 6). The marker scores of the twins were merged, resulting in one score per marker. One isolate had a genotype call rate less than 95 % and was therefore omitted from further analysis. This resulted in a scoring table of 124 individuals and 1,162 markers. These markers consisted of 1,154 DArT markers and six SSR markers, as well as Mat and Avr markers. Grouping resulted in 496 unique segregation patterns containing 491 DArT markers, four SSR markers,

and the marker, Avr, that co-segregates with the (a)virulence locus *MgAvrStb6* (Tables 4 and 5b). The global call rate for this dataset was 98.81 %, and based on the scorings of the twins the reproducibilities for the DArT and SSR markers were 99.88 % and 100 %, respectively.

Construction of individual linkage maps

Bread wheat-derived isolate IPO323 x bread wheat-derived isolate IPO94269

Twenty-nine of the 473 (6.1%) segregation patterns in the cross IPO323 x IPO94269 exhibited a significant distortion ($P < 0.01$) from the expected Mendelian 1:1 ratio based on χ^2 tests and were therefore initially omitted for the map construction.

Using a LOD threshold of 4.0, 443 out of the 444 (99.8 %) of the markers could be grouped in 24 LGs. One marker (AHMR_07I02) was not grouped, while two AFLP markers (BEGGMpAT_439 and AEGAMpAC_114) exceeded the threshold for the χ^2 jump and therefore were not positioned. The order of the markers in all 24 LGs was determined in a single round of JoinMap, and different settings of the thresholds did not alter the marker order or distance. Subsequently, we tried to incorporate the 29 markers that showed segregation distortion, and 10 of them were positioned based on the criteria listed in Materials and Methods. In this map, 258 out of the 271 (95.2 %) AFLP markers could be positioned compared to 223 (82.3%) in the previous map (Kema et al. 2002).

The resulting genetic linkage map spans 1,854 cM and contains 1,317 markers on 451 unique map positions, with an average distance of 4.11 cM between the markers. The map is 638 cM longer compared to the previously constructed linkage map from AFLP and RAPD markers (Kema et al. 2002). The previously mapped LGs 3, 4, 5, 11, and 18 were merged with LGs 22, 17, 10, 20 and 19, respectively. However, six small new LGs were also formed, resulting in a total of 24 LGs. The order of the AFLP markers remained similar compared to the previous map.

Bread wheat-derived isolate IPO323 x durum wheat-derived isolate IPO95052

Thirty-eight of the 496 (7.7%) segregation patterns in the cross IPO323 x IPO95052 exhibited a significant distortion ($P < 0.01$) from the expected Mendelian 1:1 ratio based on χ^2 tests and were initially omitted for the map construction.

Using LOD thresholds of 4.0 (1 group), 5.0 (2 groups) and 5.5 (20 groups) all markers were grouped in 23 LGs. The order of the markers in the 23 LGs was determined in a single round of JoinMap, and again different settings of the thresholds did not alter the order or

distance of the markers. Subsequently, we tried to incorporate the 38 markers that showed segregation distortion, and 28 of them could be positioned. The resulting genetic linkage map spans 1,948 cM and contains 1,144 markers on 486 unique map positions, with an average distance of 4.0 cM between the markers.

Construction of the bridge map

We constructed a bridge map to compare the IPO323xIPO94269 genetic linkage map with the IPO323 x IPO95052 map based on the 389 markers that segregated in both crosses. The scores for these markers in both progenies were merged and resulted in a dataset of 184 individuals with 389 markers, yielding 263 unique segregation patterns. The global call rate for this dataset was 99.03 %, with only five markers that showed segregation distortion ($P < 0.005$) that were subsequently removed. At a LOD of more than 5.5, 24 LGs were formed, while two markers (CBHMR_15L18 and CBBMR_15O14) could not be grouped.

Inspection of the initial map showed that five markers were positioned on different locations in the individual maps, indicating that translocations had occurred. These markers cause tension in the bridge map, and removal of these five markers enabled the construction of all LGs in a single JoinMap round. The resulting integrated map spans 1,435 cM (~75 % of both individual maps) and contains 372 markers on 251 unique map positions.

A total of 21 of the 24 LGs could be aligned with the bridge map. The remaining LGs D, E and F showed (*i*) linkage to one distal section of LG14 at a LOD of 3.5 for the IPO323 x IPO95052 cross and at a LOD of 2.8 for the IPO323 x IPO94269 cross; (*ii*) could be joined with a distal part of LG 5+10 with a LOD of 3.6 in the IPO323 x IPO94269 cross; or (*iii*) could not be aligned with certainty in the IPO323 x IPO94269 cross, respectively.

With the exception of a few markers that appear to involve translocations, the marker order on the bridge map matches the order on the two individual genetic maps. Figure 1 shows an example of the co-linearity of the markers between the two crosses for LG 5+10. This is shown for all LGs in an Appendix to this thesis.

Comparison of the genetic linkage maps

Frequency of double crossovers

To further evaluate the quality of the map, we calculated the number of possible genotyping errors (e.g. singletons) in the dataset. Singletons can be detected by evaluation of double crossover (DCO) events after merging of the markers with unique segregation

patterns. In the cross IPO323 x IPO94269 for AFLP, DArT and SSR markers, the percentages of markers leading to DCO was 0.96 %, 0.24 %, and 0.83 %, respectively. For the cross IPO323 x IPO95052, the percentage of markers leading to DCO was 0.18 % for the DArT markers and 0.16 % for the SSRs.

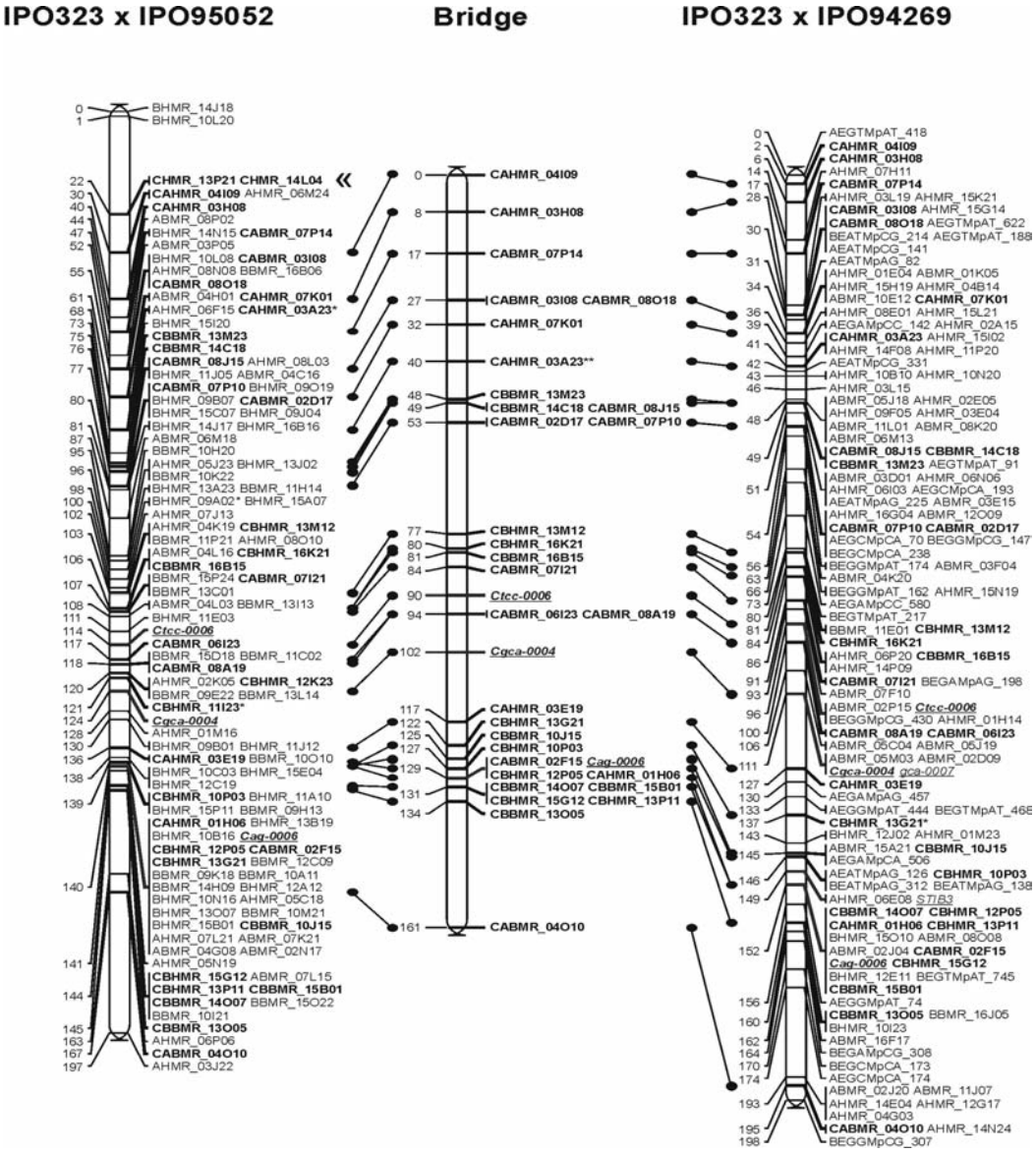

Figure 1. Co-linearity of LG 5+10 as constructed in *Mycosphaerella graminicola* mapping populations IPO323 x IPO95052 and IPO323 x IPO94269 with the bridge map that was constructed from common segregating markers. Markers segregating in both populations are shown in **bold** and SSR markers are underlined. Arrow («) indicates a translocation from LG 5+10 in IPO95052 to LG 3+22 in IPO94269. Segregation distortion of the markers is indicated with * (P<0.05) or ** (P<0.01).

Translocations

When we compared the order of the common DArT markers on the LGs of the two crosses, eight translocations between the bread wheat-derived isolates IPO323 or IPO94269 and the durum wheat-derived isolate IPO95052 were identified. One translocation (CBBMR_14G17) was within LG 6, and the other four markers were translocated from one LG to another. In addition to these translocations, we found a translocation between the two bread wheat-derived isolates based on an SSR marker (Table 7).

Table 7. DArT and SSR markers that showed translocations between two genetic linkage maps derived from crosses between either *Mycosphaerella graminicola* isolates IPO323 and IPO94269 or IPO95052.

Markers	Position in one parent	Position in other parent
CHMR_09D04, CHMR10D17	IPO323; LG 21	IPO95052; LG 18+19
CHMR_14L04, CHMR_13P21	IPO323; LG 3+22	IPO95052; LG 5+10
CBBMR_15H01	IPO94269; LG 1	IPO95052; LG3+22
CBBMR_14G17, CBMR_14D07	IPO94269; LG 6 (7cM)	IPO95052; LG6 (79cM)
CBBMR_11N22	IPO94269; LG 7	IPO95052; LG 9
ggc-0003A/B	IPO323; LG21	IPO94269; LG 4+17

Another, possible larger, translocation was observed on the distal end of LG 11+20. The 20 cM LG F from the cross IPO323 x IPO94269 contains three common markers. Marker CAHMR_08G14 could correctly be positioned in the cross IPO323 x IPO95052 but shows no significant linkage to LG 11+20 in the cross IPO323 x IPO04269. The other two common markers (CBBMR_15O14 and CBBMR_10B23) were positioned on LG A in the cross IPO323 x IPO95052. Further study will be required to establish if this truly is a larger translocation. No other translocations were detected.

Distribution of recombination over the chromosomes

The average recombination frequencies for the IPO323 x IPO94269 (17.18 ± 3.03) and IPO323 x IPO95052 (17.88 ± 2.87) crosses did not significantly deviate. However, when the local differences in recombination frequencies between the common markers were compared for the two crosses, clear differences were observed, especially at the distal parts of the LGs (Figure 2).

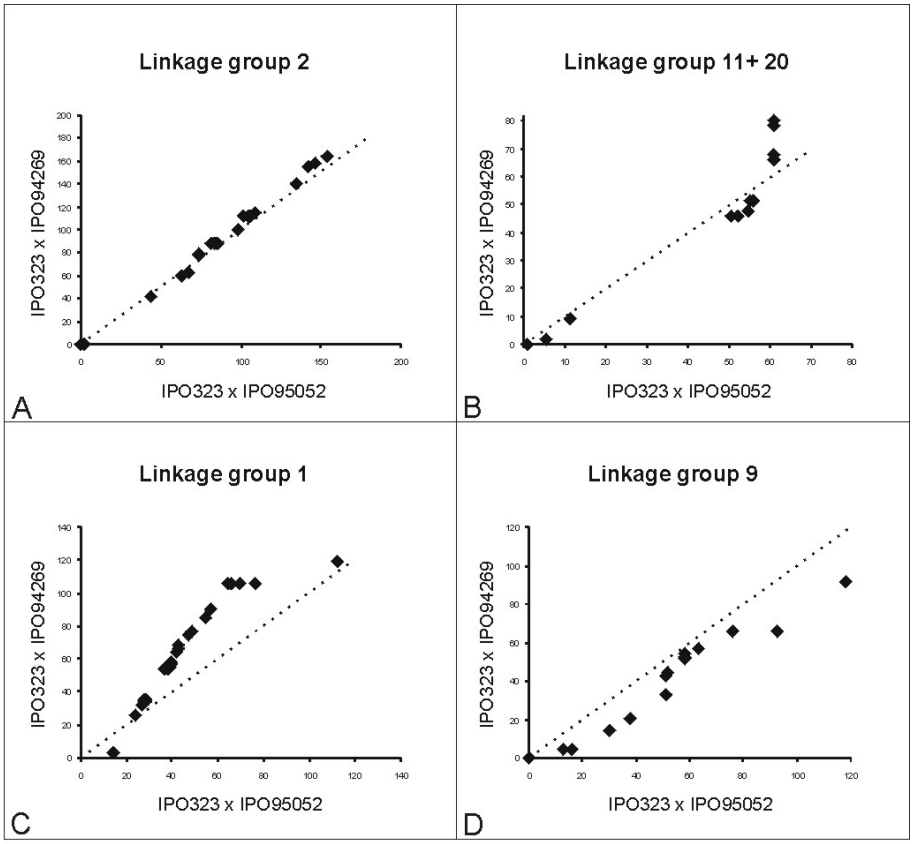

Figure 2. Local differences in recombination frequency in two *Mycosphaerella graminicola* mapping populations on four LGs. **A,** No differences in recombination frequency along the LG; **B,** Suppression of recombination in population IPO323 x IPO95052 at the distal end of the LG; **C,** Suppression of recombination in population IPO323 x IPO94269 at the distal end of LG1; and **D,** Suppression in both populations at different locations along LG 9.

In addition, we observed two large clusters of markers at the end of LGs 3+22 and 9 in cross IPO323 x IPO94269. Remarkably, all DArT markers in this cluster at LG 3+22 and most of these markers at LG 9 are cloned genome fragments from isolate IPO95052, although IPO323 and IPO94296 are the parents of the progeny in which these markers segregate. These markers also differentiate the parental isolates IPO95052 and IPO323 but do not segregate in the progeny of this cross (Figure 3). Presence versus absence of DArT clones between the parental isolates IPO323 and IPO95052 in the absence of segregation in the progeny isolates occurred in ~10 % of all clones in both libraries. Interestingly, some of these markers did segregate in the IPO323 x IPO94269 cross and could therefore be positioned.

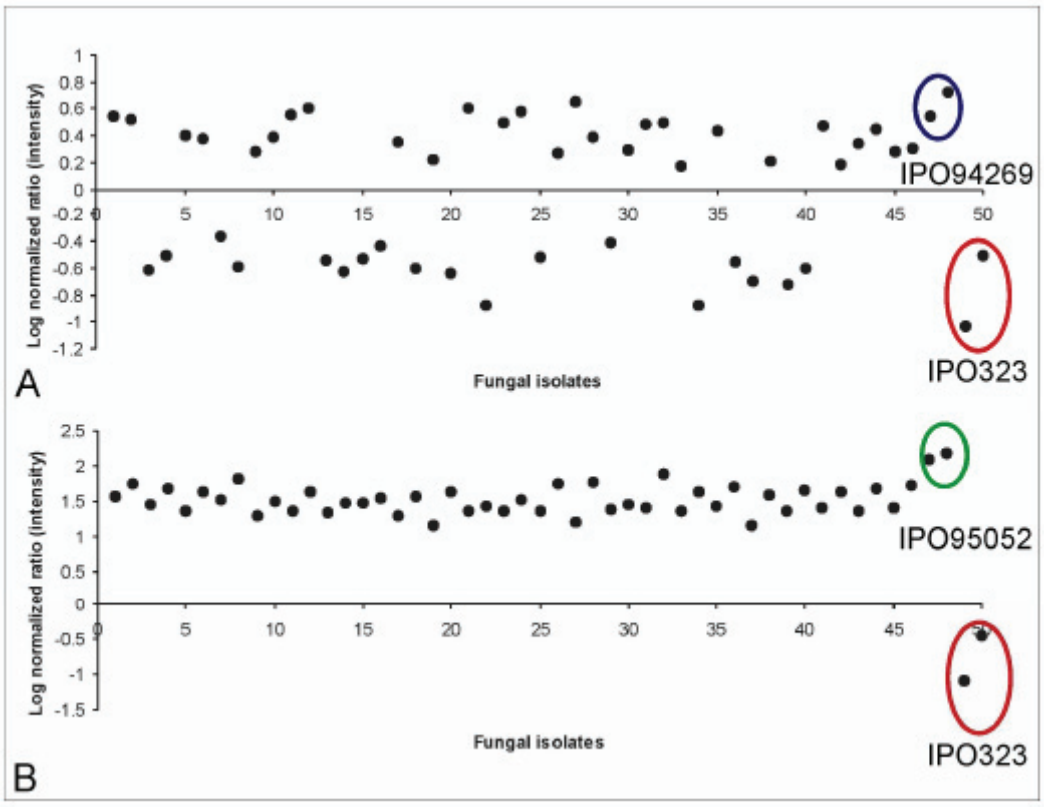

Figure 3. Normalized target ratios (Cy3:FAM) for DArT fragment BBMR_15G01 among parental and 48 *Mycosphaerella graminicola* progeny isolates derived from crosses between either isolate IPO323 and IPO94269 **(A)** or IPO95052 **(B)**. This DArT fragment is polymorphic between the parents in both crosses but only segregates in the IPO323 x IPO94269 cross. The signal intensity for parent IPO95052 is stronger compared to the intensity obtained for the progeny isolates. This may indicate the presence of dispersed multi-copy fragments in the IPO95052 genome that are absent in the IPO323 genome and occur as a single locus, possibly single copy, in the IPO94269 genome.

Absence of complete LGs

Graphical genotyping revealed that some isolates lacked one or more LGs (Table 8). An example of such a LG is shown in Figure 4. For LGs on which only IPO323-derived markers were positioned (LGs 21, B and C; cross IPO323 x IPO94269), this was not unexpected because part of the progeny would miss the complete chromosome in the absence of recombination for that chromosome during meiosis. However, for chromosomes that contain markers from both parents, such an observation is remarkable. We observed this phenomenon for LGs 8, 12, 13 and A for cross IPO323 x IPO94269 and for LGs 8, 12, 13,

15, 21, A, B and C in cross IPO323 x IPO95052. Absence of LGs 8 and 12 was confirmed by SSR markers for which neither of the parental alleles was present.

Table 8. Overview of *Mycosphaerella graminicola* F_1 isolates that lack one or more linkage groups compared to the parental isolates IPO323, IPO94269 and IPO95052.

Missing linkage group	Isolates in cross IPO323 x IPO95052	Isolates in cross IPO323 x IPO94269[a]
8	2026, 2133, 2138, 2137, 2139[b]	83, 91
12	2132, 2133, 2138	51, 62, 124, 125
13	1158	164
15	2024, 2032, 2033	-
21	1114, 1121, 1122, 1127, 1151, 1159, 1170, 1176, 1186, 1200, 2133, 2138, 2137, 2139	[c]
A	-	87, 134 and 133
B	1108, 1169, 1179, 1425, 1438, 2134, 2141	[c]
C	1128, 1139, 1179, 2030, 2132	[c]

[a] In the IPO323 x IPO94269 progeny, isolate 40 is diploid for LG13 and isolate 51 is partial diploid for LG 1.
[b] Underlined isolates are identified twins.
[c] Not assessed, as the LGs only contained IPO323 derived markers.

In contrast, we observed only one progeny isolate (no. 40) in cross IPO323 x IPO94269 that contained all markers (IPO323 and IPO94269 markers) on LG 13, indicating that this isolate was partially diploid because it inherited both parental copies of LG 13. Furthermore, we identified one progeny isolate (no. 51) in the cross IPO323 x IPO94269 that scored presence of all markers, irrespective of their parental origin, for the larger part of LG 1. This shows that isolate 51 was partially diploid for this LG, which was confirmed by SSR ac-0002 that showed that both alleles were present for this locus.

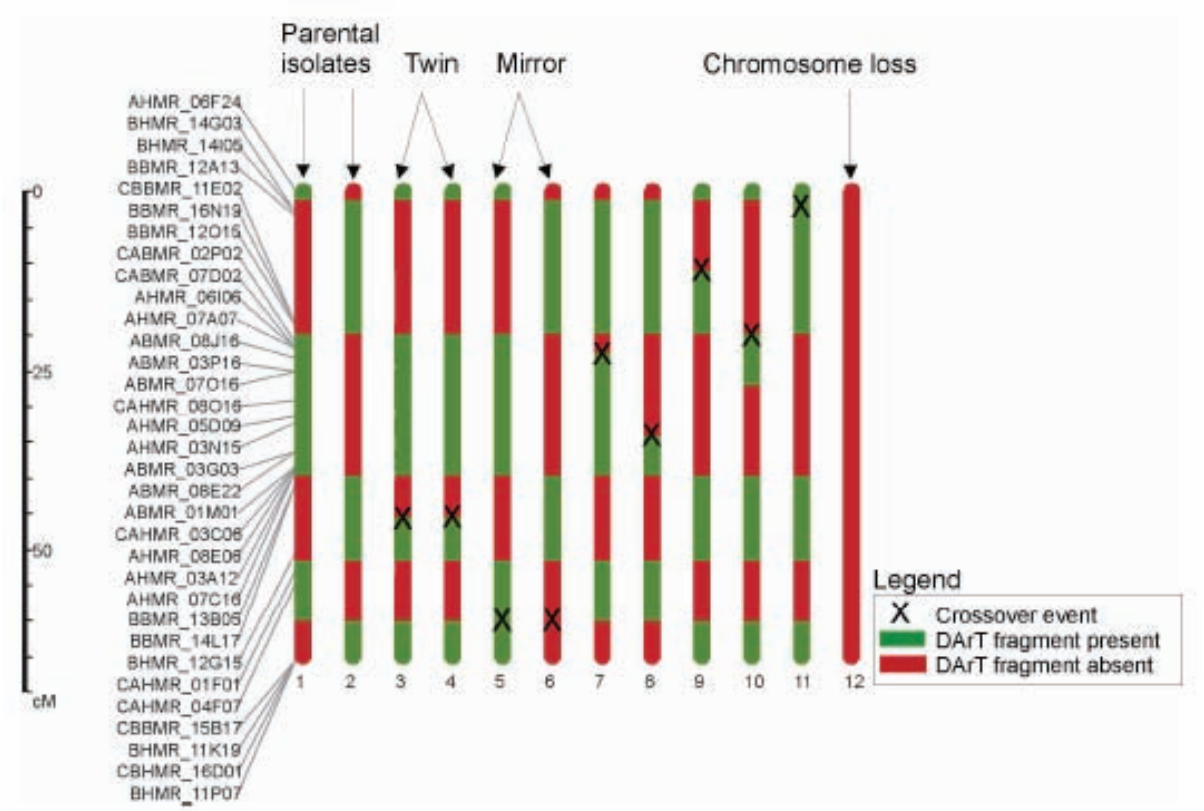

Figure 4. Graphical genotyping of LG 8 in the progeny of the cross between *Mycosphaerella graminicola* isolates IPO323 and IPO95052. The two parental isolates and 10 progeny isolates are depicted. The LG is constructed from markers derived from both parents (A and B markers). Markers can be scored present (light gray) or absent (dark gray) for a particular isolate. Positions where a crossover event had occurred are shown (X). Progeny isolates that lack all markers for this particular LG most probably lost this chromosome.

DISCUSSION

Fungi provide attractive model systems to study processes that occur during meiosis (Perkins and Maheshwari 1996). However, most of the work has been performed by cytology and targeted regions with mutant strains (Cutter 1951; Hardham and Mitchell 1998). For whole genome analysis, high-density genetic linkage maps are valuable in genetic studies, because they reveal processes that occur during meiosis. Nevertheless, the number of high density maps for fungi is limited due to the laborious process of marker generation and the necessity of high quality scoring (Hackett and Broadfoot 2003). Here, we report a high-density genetic linkage map for the plant pathogenic fungus *M. graminicola*, which contains one of the highest numbers of markers reported for a microbial genome. We used the recently-developed low-cost, high-throughput, and sequence-independent DArT technology,

which facilitates both marker discovery and routine analysis using the same hybridization-based assay. The unparalleled quality of the DArT markers allowed the detection of non-Mendelian inheritance and elucidated the processes that underly these aberrations.

Quality of different marker types

In *M. graminicola*, the mitotic division that follows meiosis II in the ascus results in four pairs of genetically identical ascospores. After analysis of the marker data, we detected a total of 31 such pairs. These pairs can be regarded as replicates, which allow evaluation of reproducibility of the marker scores for the different marker technologies. In our study, DArT and AFLP markers appeared to be more reproducible than RAPD markers. It was decided to exclude the RAPD markers, because the scoring errors of the RAPD markers would strongly increase the tension in the maps. Although the reproducibility of both DArT and AFLP was very high, the frequency of double crossovers (DCOs) was much lower for the DArT markers compared to the AFLP markers (0.24 % compared to 0.96 %), indicating that the DArT markers had the highest reliability.

Genetic linkage maps and genome coverage

The two genetic linkage maps together contain 2,080 markers comprising 1,795 DArT, 258 AFLP, and 25 SSR markers, as well as Mat and Avr markers. The grouping and the order of the markers in cross IPO323 x IPO94269 is highly similar to the previously constructed linkage map based on AFLP and RAPD markers (Kema et al. 2002). However, both new maps span a considerably larger part of the genome. Finally, 98.21 % (IPO323 x IPO94269) and 98.45 % (IPO323 x IPO95052) of the markers could be positioned reliably. Therefore, it is likely that the current genetic linkage map covers the complete genome. As expected, some of the smaller LGs observed in the previous study could now be joined with other LGs. In addition, a number of new relatively small LGs were identified. LG 22, on which the markers that perfectly co-segregated with avirulence to cv. Shafir (Kema et al. 2002) were mapped, now joined LG 3. LG 16, on which the mating type locus is mapped, expanded from 35 cM in the previous map to 100 cM in the current map. These results confirmed previous observations that these two traits reside on relatively large chromosomes based on Southern analysis of DNA separated by pulsed-field gel electrophoresis (PFGE) (Kema et al. 2002; R. Mehrabi, *unpublished*).

Differences in chromosome numbers in *M. graminicola*

The number of chromosomes varies in *M. graminicola* isolates (Kema et al. 1999; McDonald and Martinez 1991). The nature of these differences and the way in which they evolved are unclear. Despite the fact that two LGs in the cross IPO323 x IPO94269 span less than 2 cM, they contain a large number of different markers (AFLP, SSR and DArT) i.e. 49 for LG 21 and 36 for LG C. Interestingly, all these markers are derived from the parental isolate IPO323. This provides genetic evidence that these two LGs are present in IPO323 but not in IPO94269 and confirms earlier results obtained from karyotyping experiments in which IPO323 was shown to contain a least two additional chromosomes compared to IPO94269 (R. Mehrabi, *unpublished*). Due to the absence of a homologous partner, these chromosomes cannot pair during meiosis and consequently no recombination takes place. This is exactly what was observed for two of the three LGs. In contrast, in the cross between IPO323 x IPO95052 all LGs contain markers derived from both isolates. This indicates that each chromosome of the bread wheat-derived isolate IPO323 has a homologous partner in durum wheat IPO95052, and *vice versa*. In this respect, the homology between the Dutch bread wheat-derived isolate and the Algerian durum wheat-derived isolate appears to be higher than between the two Dutch bread wheat-derived isolates. This is remarkable, as it is likely that homology facilitates genetic exchange between isolates from durum wheat and isolates from bread wheat. Based on virulence data which strongly suggests host species specificity among isolates in nature, such genetic exchange of virulence and avirulence factors seems unlikely to occur. However, progeny from this mapping population were indeed found to have such recombinant factors (see Chapter 4).

In addition to these differences in karyotype between the parental strains IPO323 and IPO94269, we noticed that both parents have chromosomes that are not always transmitted to the progeny. Approximately 20 % of progeny isolates lack one or more small LGs that were present in both parents. Interestingly, for both crosses the same LGs seem to be dispensable. If a LG was absent in one genotype of a twin, it was also absent in the other genotype. Since the twins are the product of the first mitosis after meiosis, this demonstrates that these chromosomes were not lost by mitotic instability but that they were lost in meiosis I or meiosis II. For the ascomycete *Leptosphaeria maculans*, twin genotypes were also always identical according to the presence or absence of a dispensable chromosome (Leclair et al. 1996), but this was not the case for *Magnaporthe oryzae* (Chuma et al. 2003). The loss of chromosomes may be a consequence of non-disjunction in which case two chromosomes are pulled to one pole and end up in one gamete. If non-disjunction occurs during meiosis I, two pairing chromosomes are both pulled to one gamete, leading to loss of that chromosome in

another gamete. One haploid *M. graminicola* isolate would become then diploid heterozygous for one chromosome. If non-disjunction occurs during meiosis II, then the two sister chromatids are not divided among the two gametes, but are pulled both to one gamete, leading to two identical copies of the chromosome in that gamete and loss of that chromosome in the other gamete. We found only one case in which both complementary chromosomes were present in spite of the haploid nature of the fungus. This may indicate that the non-disjunction might preferentially have taken place during meiosis II. We found one isolate in which part of a chromosome was diploid heterozygous, which may indicate recombination during meiosis I followed by non-disjunction during meiosis II. For further evidence of non-disjunction during meiosis II, it would have been interesting to discriminate between one and two identical chromosome copies in an isolate. However, this would have required quantitative measures of markers, while our markers did not allow this reliably.

Apparently, LGs 8, 12, 13, 15, 21, A, B and C are dispensable for germination and growth on agar. Although variation in karyotype is also a common feature in several other pathogenic fungi (Cooley and Caten 1991; Covert 1998; McCluskey et al. 1994), this is the first time that it is demonstrated in segregating populations based on linkage analysis. Different terms are used for the presence of these extra chromosomes e.g. supernumerary, dispensable, mini chromosomes and B-chromosomes (Covert 1998). These chromosomes can carry functional genes and, in at least two fungal species (Miaot et al. 1991; van Etten et al. 1994), genes on such chromosomes play important roles in host-pathogen interactions. The *M. graminicola* isolates from our study that lack LGs will be tested for obvious decreases in pathogenicity (see Chapter 4), and a more detailed analysis of the apparently redundant genes located on these LGs in *M. graminicola* will be performed as soon as the whole genome sequence of isolate IPO323 becomes available.

Translocations

In the cross between the two bread wheat-derived isolates, one SSR locus (ggc-0003) segregated in a diploid fashion and showed either the individual bands as well as both or no bands in a 1:1:1:1 ratio (χ^2 = 1.25, 025 < P <0.75). This indicated a translocation between IPO323 and IPO94269, and we were able to map this locus on LG 21 and 4+17, respectively. By comparing the two maps, we found eight DArT markers that were translocated between IPO95052 and IPO94269. The translocated DArT markers are all derived from isolate IPO95052. All of the translocations we observed were on relatively small chromosomal fragments, because translocations of large numbers of clustered markers were not found.

Sequence information from these DArT fragments may help us to verify whether these translocations are indeed caused by transposons.

In addition to the above described translocations, we observed two large clusters of markers at the distal ends of LGs 3+22 and 4+17. The DArT markers in these clusters differentiated the parents and segregated in the progeny of the IPO323 x IPO94269 cross, but not in progeny of the IPO323 x IPO95052 cross. These results might be caused by recent expansion and dispersion of (a) multicopy fragment(s) in the durum wheat-derived isolate IPO95052. This dispersion in the genome of IPO95052 prevents mapping these fragments in this cross. However, the fragment is found as a single locus, possibly single copy, in isolate IPO94269 and is absent in IPO323, allowing the positioning of this marker on the genetic linkage map.

Three other translocations have been described so far in *M. graminicola*. Two of them were identified by hybridization of an RFLP probe (McDonald and Martinez 1991; Chen and McDonald 1996) and one by a RAPD probe (Kema et al. 1999). One transposable element, pSTL70, has been characterized in more detail and is likely to be active during meiosis (Goodwin et al. 2001). Analysis of pSTL70 revealed that it contained the 3'end of a reverse transcriptase sequence plus 29- and 79-bp direct repeats. The element appears to be active during both sexual an asexual reproduction. However, movement at a high rate was observed in only one of the parents (Goodwin et al. 2001). A possible explanation for this could be that the element is only active in certain genetic backgrounds, as is the case for the *Ac/Ds* elements in maize (Fedoroff 1989). A similar explanation could be the case for the results found here, where IPO95052 recently acquired an active transposable element that dispersed itself throughout the genome.

Sexual compatibility of isolates

Because the average recombination frequency between the two crosses is not significantly different, we can conclude that the isolate from bread wheat is as sexually compatible with the isolate from durum wheat as with the other isolate from bread wheat. This is remarkable, as durum wheat was regarded as a non-host for isolates from bread wheat, and bread wheat as a non-host for isolates from durum wheat (Eyal et al. 1973; Kema et al. 1996b; Saadaoui 1987; van Ginkel and Scharen 1988). In spite of that, the isolates are fully sexually compatible, both on durum wheat and on bread wheat. Although IPO323 is avirulent on durum wheat, it appeared to be able to grow on this host and even mate with IPO95052 and pass on its genetic material to the progeny. The same holds for IPO95052 on

bread wheat. This suggests that virulence to bread wheat and virulence to durum wheat could be accumulated in the progeny, giving rise to some progeny isolates that would be virulent to both wheat species, which indeed was recently observed (see Chapter 4).

Local suppression of recombination in either of the two crosses could be observed in some LGs (Figure 2). A possible explanation is that the chromosomes of isolates IPO94269 and IPO95052 do find a homologue in the commonly-used isolate IPO323 but do not line-up over the whole length of the chromosome, e.g. because of a very large insertion or deletion (InDel). This would result in local suppression of recombination in one cross but not in the other. Chromosome length polymorphisms can be caused by many other mechanisms as well, as described by Zolan (1995).

We have shown that *M. graminicola* strains isolated from bread and durum wheat are sexually compatible, and they result in the generation of viable offspring. The genetic linkage map presented here is one of the most dense genetic maps for a haploid fungus currently available. We demonstrated that DArT is suitable to generate a large number of markers in organisms with a relatively small genome size. The maps reveal translocations and chromosome length polymorphisms between the two crosses. Furthermore, we have genetically confirmed that isolates of *M. graminicola* differ in chromosome number, confirming our earlier results (R. Mehrabi, *unpublished*). We have evidence that these differences are caused by aberrations during meiosis, partly involving non-disjunction. The DArT markers will be sequenced, enabling us to assist in the genome assembly of isolate IPO323 currently sequenced by a shot-gun sequence approach (http://www.doe.jgi.org). This will further develop *M. graminicola* as a model species and give us insight into the differences between genetical and physical distances. The genetic map and genome sequence will be used to identify underlying genes determining host and cultivar specificity in the wheat-*M. graminicola* pathosystem.

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CHAPTER 4

Quantitative trait loci determine specificity to bread and durum wheat cultivars in the fungal wheat pathogen *Mycosphaerella graminicola*

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ABSTRACT

Host species specificity of the plant pathogenic fungus *Mycosphaerella graminicola* towards hexaploid bread wheat and tetraploid durum wheat is well- documented. Although *M. graminicola* has an important sexual cycle and in some parts of the world durum wheat and bread wheat are grown side by side, in nature most isolates are pathogenic to either bread wheat or durum wheat. We crossed an *M. graminicola* isolate derived from bread wheat (IPO323) with an isolate derived from durum wheat (IPO95052) and performed genetic studies on the host species specificity using their progeny (n=163). Virulence of the progeny was tested on four representative bread wheat cultivars and three representative durum wheat cultivars. All progeny isolates grow normally *in vitro*, but in contrast to crosses performed with two bread wheat isolates, nearly 50% were unable to cause disease on any of the generally susceptible bread wheat and durum wheat cultivars. Interestingly, other progeny caused disease on either bread wheat cultivars or durum wheat cultivars or both wheat species. This proves that the observed host species specificity was lost in a single generation. In addition, some progeny were able to cause disease on the bread wheat cv. Shafir even though both parents are avirulent to this cultivar. Detailed analyses allowed the identification of quantitative trait loci (QTLs) that determine specificity, which could be positioned on a high density genetic linkage map containing 1,144 molecular markers. In total, we identified nine QTLs on seven linkage groups. Remarkably, no locus was identified that explains the host species specificity to either durum or bread wheat. However, a locus with a very high LOD value had a major effect on specificity towards the tested durum wheat cultivars, but was previously identified as a locus involved in cultivar specificity. To our knowledge, this is the first comprehensive QTL analysis on virulence performed on a plant pathogenic fungus. We conclude that specificity in *M. graminicola* is controlled by many genes and that the long-accepted notion of species specificity among isolates of *M. graminicola* may in fact be a mix of genetically inherited factors, whereby the distinction between host species specificity and cultivar specificity is not clear-cut.

INTRODUCTION

Mycosphaerella is the largest genus in the family Mycosphaerellaceae and of the order Dothideales, having more than 3,000 names (Aptroot 2006). In addition, at least 40 anamorph genera (with no known sexual stage) are associated with *Mycosphaerella*, including over 3,000 species of *Cercospora* (Crous and Braun 2003) and over 1,000 species of *Septoria* (Crous and Braun 2003; Kirk et al. 2001). Most of these species are defined on the host from which they were isolated and are usually assumed to be host species-specific (Crous and Groenewald 2005). Many *Mycosphaerella* and *Septoria* species are plant pathogenic and cause diseases on an extremely wide diverse range of hosts, including eucalypt trees, bananas, citrus fruits, and cereals (Farr et al. 1995). Diseases caused by these species result in major economic losses in terms of both fungicide control and reductions in yield (Marín et al. 2003; Timmer et al. 2000).

Septoria tritici blotch (STB) of wheat is caused by *M. graminicola* (Fuckel) J. Schröt. in Cohn (ananmorph *Septoria tritici* Roberge in Desmaz). STB is currently the most important disease on wheat in Europe and is a major disease in wheat-growing areas around the globe (Eyal and Levy 1987; Garcia and Marshall 1992; Halama 1996; Hardwick et al. 2001; Jorgensen et al. 1999; Madariaga 1986; Scott et al. 1988), which is one of the reasons that the US Department of Energy – Joint Genome Institute (DOE-JGI) decided to sequence the *M. graminicola* genome (http://www.jgi.doe.gov). Inocula of *M. graminicola* include both asexual, splash-dispersed conidia and sexual, wind-dispersed ascospores. *M. graminicola* has a very active sexual stage that includes several sexual cycles within a single growing season that contribute to the high genetic diversity of this pathogen (Hunter et al. 1999; Kema et al. 1996c; Zhan et al. 2003).

M. graminicola infects both hexaploid bread wheat (*Triticum aestivum* L., AABBDD, 2n=42) and tetraploid durum wheat (*T. turgidum* L. (Thell.) subsp. *durum* L., AABB, 2n=28) species. Nevertheless, isolates of *M. graminicola* exhibit both host species specificity and cultivar specificity (Kema et al. 1996a, 1996b; Kema and van Silfhout 1997). Host species specificity refers to avirulence to all cultivars within wheat species, whereas cultivar specificity refers to avirulences on particular cultivars of either bread wheat or durum wheat. The most drastic experimentally reported interactions have been host species-specific, as in general, bread wheat-derived isolates found in nature are avirulent to durum wheat cultivars, and durum wheat-derived isolates are avirulent to bread wheat cultivars (Eyal et al. 1973; Kema et al. 1996a; Saadaoui 1987; van Ginkel and Scharen 1988). A gene-for-gene

interaction for cultivar specificity in *M. graminicola* to bread wheat has been proven (Brading et al. 2002; Kema et al. 2002), but the genetic basis for host species specificity has thus far not been addressed. We therefore were interested in identifying loci controlling host species specificity, in which the responsible genes can either encode the production of host selective toxins or proteins (called effectors) involved in gene-for-gene interactions.

Many resistance (*R*) genes have been cloned in plants, largely based on homology, as *R* genes primarily encode several but distinct classes of proteins (Dangl and Jones 2001). Compared to resistance genes, avirulence (*Avr*) genes are much more polymorphic, making their identifications based on homology very difficult (Chisholm et al. 2006). Map-based technologies have so far only resulted in the cloning of effector loci from three plant pathogenic fungi (Dodds et al. 2004; Gout et al. 2006; Sweigard et al. 1995; Valent 1997).

Here we report the genetic analyses of an *in planta*-generated progeny from a cross between *M. graminicola* isolates showing host species specificity. We mapped nine quantitative trait loci (QTLs) involved in host species and cultivar specificity in this haploid plant pathogenic fungus. Our data demonstrate that a previously-mapped locus for cultivar specificity (Brading et al 2002; Kema et al. 2000, 2002) is also a major determinant for host species specificity, thereby challenging the overall concept of specificity in *M. graminicola*.

MATERIALS AND METHODS

Parental isolates and crosses

Maintenance of isolates, preparation of inocula, and generation of ascospores were as previously described (Kema et al. 1996c). We used parental isolates IPO323 and IPO95052 for *in planta* generation of F1 progeny on both the bread wheat cv. Obelisk (n=103) and the durum wheat cv. Inbar (n=60) (see also Chapters 2 and 3). IPO323 was isolated in 1981 from the commercial bread wheat cultivar Arminda in the Netherlands, and IPO95052 was isolated in 1995 from an unknown durum wheat cultivar in Algeria. These isolates were chosen because IPO323 is highly and differentially virulent on many bread wheat cultivars, and IPO95052 is highly virulent on many durum wheat cultivars, but not *vice versa* (Kema and van Silfhout 1997; G.H.J. Kema, *unpublished*).

Inoculations and phenotyping

We studied the abovementioned progeny on an international set of both bread wheat and durum wheat cultivars. Phenotyping was done on seedlings of the bread wheat cvs. Taichung 29, Gerek 79, Obelisk, and Shafir (highly susceptible to most bread wheat-derived isolates), as well as on the durum wheat cvs. Volcani 447, Zenati Bouteille, and Bidi 17 (highly susceptible to most durum wheat-derived isolates) (Kema et al. 1996a, 1996b; G.H.J. Kema, *unpublished*). Isolate IPO95052 is avirulent on all bread wheat cultivars and virulent on the aforementioned durum wheat cultivars, whereas IPO323 is avirulent on all durum wheat cultivars and is virulent on all of the above bread wheat cultivars except cv. Shafir (Figure 1). Although both isolates are avirulent on cv. Shafir, this cultivar was included in the phenotyping. We were interested to compare the gene action of IPO95052, potentially encoding host species specificity to bread wheat cultivars, with the determined cultivar specificity to cv. Shafir in IPO323, which is conferred by an avirulence locus that we tentatively name *MgAvrStb6* (Kema et al. 2000; 2002). The marker that co-segregates with this locus is absent in the avirulent isolate IPO95052.

Due to the size of the experiment (163 isolates and seven cultivars), it was necessary to divide the inoculations over time. Each experiment usually consisted of 40 progeny isolates plus the two parents as controls tested one pot per isolate-cultivar. Ultimately, each isolate was tested at least twice on all seven cultivars, and many isolates were tested more (up to five) times in order to level out variation caused by environmental factors. Greenhouse conditions for plant growth both before and after inoculation (30 ml of 10^7 spores ml^{-1}) were as described previously, with relative humidity of 85% or higher, day/night rhythm of 16/8 hours, and temperature 22°C (Kema et al. 1996c). At 20-21 days post inoculation (dpi), the percent leaf area covered by pycnidia was averaged over 6-10 seedlings/pot to create a continuous scoring scale from 0-100%. Histograms with frequency distributions of progeny using log-transformed pycnidial data were generated for each cultivar using bins (classes) in intervals of 0.1 after log-transformation to evaluate segregation distributions. To confirm results of seedling experiments, a subset of isolates was tested on adult plants using the aforementioned conditions.

A

B

	Durum wheat cultivars			Bread wheat cultivars			
	Volcani 447	Zenati Bouteille	Bidi 17	Taichung 29	Obelisk	Gerek 79	Shafir
IPO323	0.08±0.09	0.08±0.09	0.83±0.87	70.83±6.36	60.00±6.17	55.83±5.22	0
IPO95052	62.08±6.79	49.17±5.67	53.75±6.00	0.21±0.15	0	0.08±0.09	0

Figure 1. Virulence phenotypes of the parental *Mycosphaerella graminicola* isolates IPO323 and IPO95052 on three durum wheat and four bread wheat cultivars. **A,** With examples of phenotypes of four progeny isolates. **B,** Percent leaf area covered by pycnidia averaged over 11 experiments, with standard deviations. Gray highlighting indicates aviruelent isolates on resistant cultivars.

It has been hypothesized that the development of pycnidia and the development of necrosis are controlled by different genes (Kema et al. 1996a, 1996b; Kema and van Silfhout 1997). Therefore, the degree of necrosis in the leaves was also scored at 20-21 dpi in combinations of G (green), C (chlorosis), and N (necrosis). A continuous scoring scale was made based on visual assessments of all available isolate-cultivar repetitions; 0 = always completely green, 1 = green and chlorotic, 2 = green, chlorotic, and necrotic or green and necrotic, 3 = chlorotic and necrotic, and 4 = always completely necrotic.

Mapping

The scores for percent leaf area covered by pycnidia and degree of necrosis from 147 out of the 163 progeny were used to map QTLs on a genetic linkage map previously generated with this progeny (see Chapter 3). This subset includes 23 twin pairs of isolates for which pycnidial data were merged after averaging. These twin pairs are genetically identical isolates resulting from mitosis after meiosis in an ascus leading to four pairs of genetically identical ascospores. Because histograms did not reveal normal distributions of virulence data, QTL mapping based on a continuous scale of percent leaf area covered by pycnidia was necessary. Mapping with the average of log-transformed pycnidial data (log[average percent pycnida]+1) from each isolate-cultivar result, versus mapping with the average raw pycnidial data, yielded higher LOD-values, so we therefore continued with log-transformed data. Furthermore, necrosis data were also mapped using the 0-4 classes.

The software program MapQTL 5.0 (van Ooijen 2004) was used to detect QTLs with both the interval mapping method (Lander and Botstein 1989) and the MQM mapping method (Janssen 1994). First, interval mapping was performed to detect QTLs. During the next step, co-factors were determined using the automatic co-factor selection option, which was followed by MQM mapping of the same trait with the selected co-factor(s) to identify new QTLs. The logarithm of the odds (LOD) profiles and the percentage of explained variance were obtained with the MQM mapping approach when co-factors were selected. When only one QTL was detected, the LOD-profile of the interval mapping procedure was shown. For declaring a QTL significant, permutation tests were performed, which resulted in a genome-wide significance threshold of LOD 3.0 for all traits. LOD-profiles were graphically displayed using MapChart version 2.2 (Voorrips 2006), including the LOD minus 1 / LOD minus 2 support interval to approximate a 95 % confidence interval (van Ooijen 1992). Finally, we tested for interactions between the identified QTLs using a generalized

linear model based on a binomial distribution of percentages yielding an analysis of deviance (GenStat 8.11)

Dispensable chromosomes and (a)virulence

During analysis of the genetic linkage map of IPO323 and IPO95052, entire linkage groups were found to be missing in 30 progeny isolates (including 3 twin pairs =27 unique genotypes, see Chapter 3). In the current study, a comparison was made between loss of such linkage groups and observed disease levels (average back-transformed percent leaf area covered by pycnidia) to identify whether this would affect severity on one or more wheat cultivars.

RESULTS

Phenotyping

The obtained IPO323 x IPO95052 progeny (n=163) showed normal *in vitro* growth on water agar, potato dextrose agar and in liquid yeast-glucose medium. Hence, production of inoculum was standard and yielded sufficient spores for inoculations and DNA extractions (Chapter 3). In compatible interactions, disease symptoms developed regularly with chlorosis starting around 10 dpi and progressed to necrosis at 14 dpi that coalesced in large blotches starting from 16 dpi, while increasing numbers of asexual fructifications were formed in them until scoring was performed at 21 dpi. In an average of 11 experiments, IPO323 produced less than 1 % pycnida on the durum wheat cvs. Volcani 447, Zenati Bouteille, and Bidi 17, while 71%, 60% and 56 % of the leaf area were covered with pycnida on bread wheat cvs. Taichung 29, Obelisk, and Gerek 79, respectively. No pycnidia (0%) were observed on the resistant bread wheat cv. Shafir. In the same 11 experiments, IPO95052 produced average pycnidia of 62%, 49%, and 54 % on durum wheat cvs. Volcani 447, Zenati Bouteille, and Bidi 17, respectively; less than 1 % on bread wheat cvs. Taichung 29 and Gerek 79; and no pycnidia (0%) on bread wheat cvs. Obelisk and Shafir (Figures 1 and 2).

Clearly, effector genes reassorted in the progeny resulting in a wide variation for host species and cultivar specificity. As expected, the progeny of the cross also varied quantitatively in the disease levels (Figure 2). Moreover, in most cases it was difficult to distinguish clearly-defined groups. On cvs. Shafir, Obelisk and Gerek 79, there were very

low numbers of highly virulent (greater than 10% pycnidia) isolates (n=9, 4 and 1, respectively), which also made discrimination of groups difficult (although a clear group with very low pycnidial percentages was observed in cv. Gerek 79). Therefore, it was necessary to map for QTLs by using a continuous scale rather than discrete scores for virulence and avirulence.

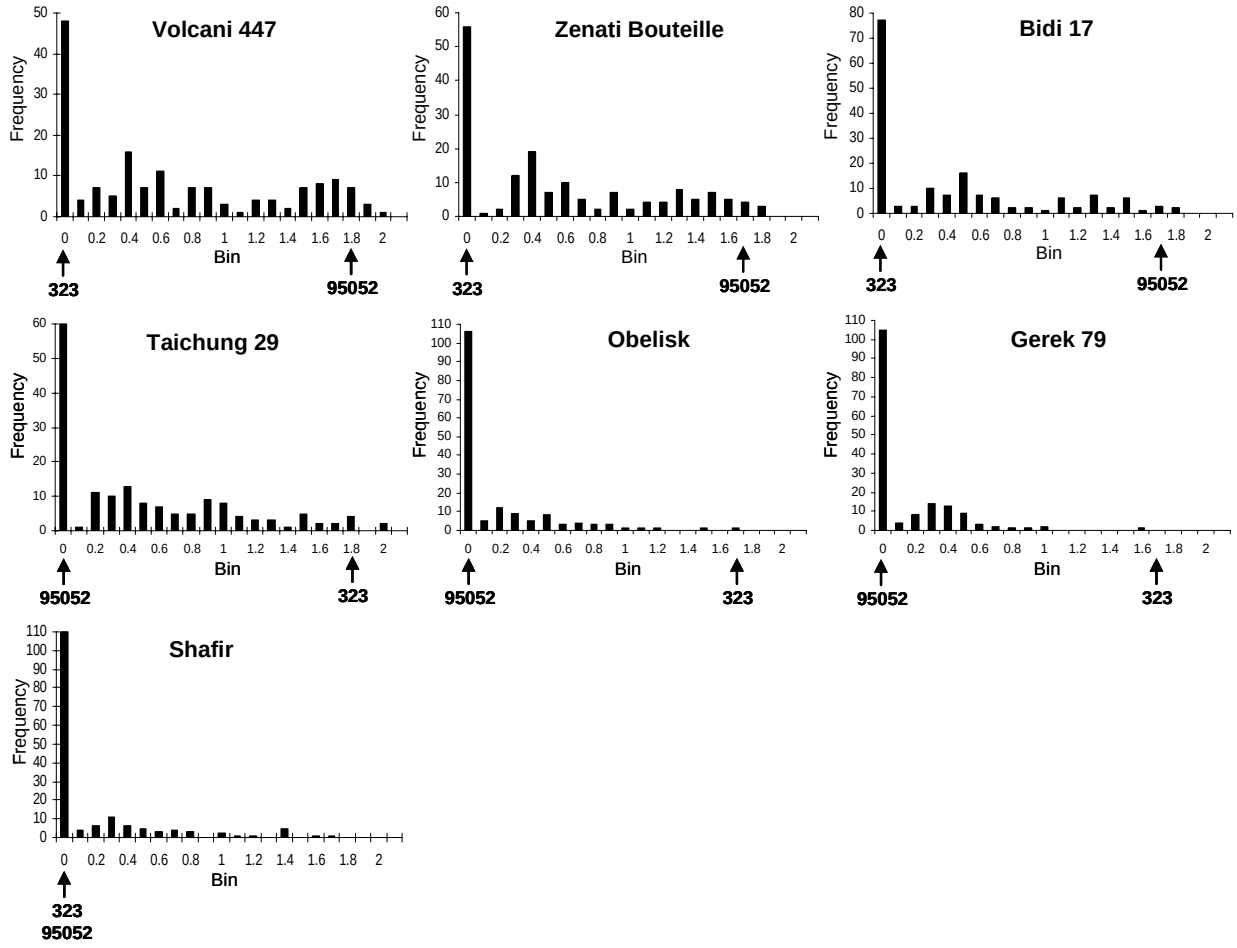

Figure 2. Histograms of the number of progeny isolates of *Mycosphaerella graminicola* in each bin spanning 0.1 after log-transformation of average percent pycnidia of individual isolates observed at 20-21 days after inoculation on durum wheat cvs. Volcani 447, Zenati Bouteille and Bidi 17 and bread wheat cvs. Taichung 29, Obelisk, Gerek 79 and Shafir. Examples of backtransformed scores to raw pycnidial scores are as follows: 0.5=2%, 1.0=9%, 1.5=30%, and 2.0=100%. Arrows indicate average parental responses on each cultivar.

Thirteen parental phenotypes were observed (8%) among 163 progeny, and all were like the durum wheat parent IPO95052. Therefore, 150 progeny represented recombinant phenotypes (Figure 1 for examples). Using the average log-transformed scores, 86 isolates (53%) had scores of less than 1 (corresponding to less than 9% pycnidia) across all seven cultivars tested. Of isolates with average scores of at least 1, 20 (12%) and 47 (29%) were exclusively virulent on bread wheat and durum wheat (at least one cultivar), respectively, and 10 isolates (6%) were virulent on both bread wheat and durum wheat cultivars. Several of the isolates that were virulent on both bread wheat and durum wheat cultivars were inoculated onto mature wheat plants to rule out the possibility that mature plants selected for host-specific isolates, but results on mature plants were similar to those obtained on seedlings (results not shown). Nine isolates (5%) were virulent on bread wheat cv. Shafir, even though neither parent was virulent on this cultivar. Finally, we determined that the number of avirulent isolates on each of the tested cultivars in the progenies originating from the *in planta* cross on bread wheat cv. Obelisk or durum wheat cv. Inbar were not significantly different (χ^2=3.8; P<0.001) and therefore considered all progeny as one population for mapping.

Necrosis phenotyping resulted in a majority of isolate-cultivar combinations that were scored within the class green/chlorotic/necrotic (Class 2). However, leaves of the bread wheat cvs. Obelisk and Shafir remained green more often than leaves of the other five cultivars when rated at 20-21 dpi. We observed sufficient variation for QTL mapping of potential loci involved in the induction of necrosis.

Mapping

Based on pycnidial data, nine loci involved in specificity were mapped on seven linkage groups (LGs). A major QTL on LG 3+22 controls specificity to the durum wheat cvs. Volcani 447, Zenati Bouteille, and Bidi 17 (LOD=14.83-17.12), as well as the bread wheat cv. Shafir (LOD=7.2). This QTL alone explained up to 47% of the observed variation on these cultivars. All other QTLs had LOD-values between 3.06 and 7.64) and were positioned on LG 1, LG 2, LG 3+22 (second position), LG 4+17 (two positions), LG 6, LG 7, and LG 16 (Table 1, Figure 3). Five of the eight newly mapped QTLs control specificity for bread wheat cultivars and three for durum wheat cultivars. Bread wheat cultivar Gerek 79, like Taichung 29, showed a QTL on LG16 with interval mapping. However, there is a 31 cM gap between these markers under which the QTL is estimated to be located. For the bread wheat cv. Taichung 29, the LOD-value is significantly above the determined threshold.

Therefore, we decided to also show the LOD-profile for cultivar Gerek 79. Although the LOD-value for cv. Bidi 17 was just below the threshold of significance, the QTL has been included. This is supported by data from the durum wheat cv. Zenati Bouteille, where a QTL could be located at the same position on LG 4+17.

Table 1. Mapped quantitative trait loci involved in (a)virulence in progeny isolates of *Mycosphaerella graminicola* from a cross between isolates IPO323 and IPO95052 tested for virulence on the durum wheat cvs. Volcani 447, Zenati Bouteille and Bidi 17 and the bread wheat cvs. Taichung 29, Obelisk, Gerek 79 and Shafir. *different position than the other locus on LG 3+22. ° different position than the other locus on LG 4+17.

Species	Cultivar	Linkage group	LOD-value	Explained overall variance (%)
Durum	Volcani 447	3+22 1	16.60 7.64	46.0 13.3
	Zenati Bouteille	3+22 4+17	14.83 3.89	42.4 7.7
	Bidi 17	3+22 7 4+17	17.12 3.33 2.91	47.0 6.2 5.4
Bread	Taichung 29	16 6	7.61 5.70	28.3 15.1
	Obelisk	3+22* 4+17°	4.80 3.76	16.7 10.9
	Gerek 79	16	3.06	14.1
	Shafir	3+22 2	7.2 3.22	23.5 8.6

We also tested for interactions between the determined QTLs (Table 2) and observed that the QTL on LG3+22 involved in specificity to all three durum wheat cultivars, as well as the bread wheat cv. Shafir has a significant overruling effect. Addition of any other QTL with specificity to the durum wheat cultivars never resulted in significant disease reductions, although these QTLs (for cv. Volcani 447 on LG1 and for cvs. Zenati Bouteille and Bidi 17 on LG4+17) individually reduced disease severity significantly. Alternatively, addition of the QTL on LG3+22 always had an additional effect to the reduction of disease severity when one of the other QTLs was present. Similar analyses for interaction between QTLs involved in specificity for the bread wheat cultivars cvs. Taichung (LG16 and LG6) and Obelisk (LG3+22 and LG 4+17) indicated that the absence of these QTLs decreased disease severity, suggesting that these loci are involved in virulence. Individual action of these QTLs did not

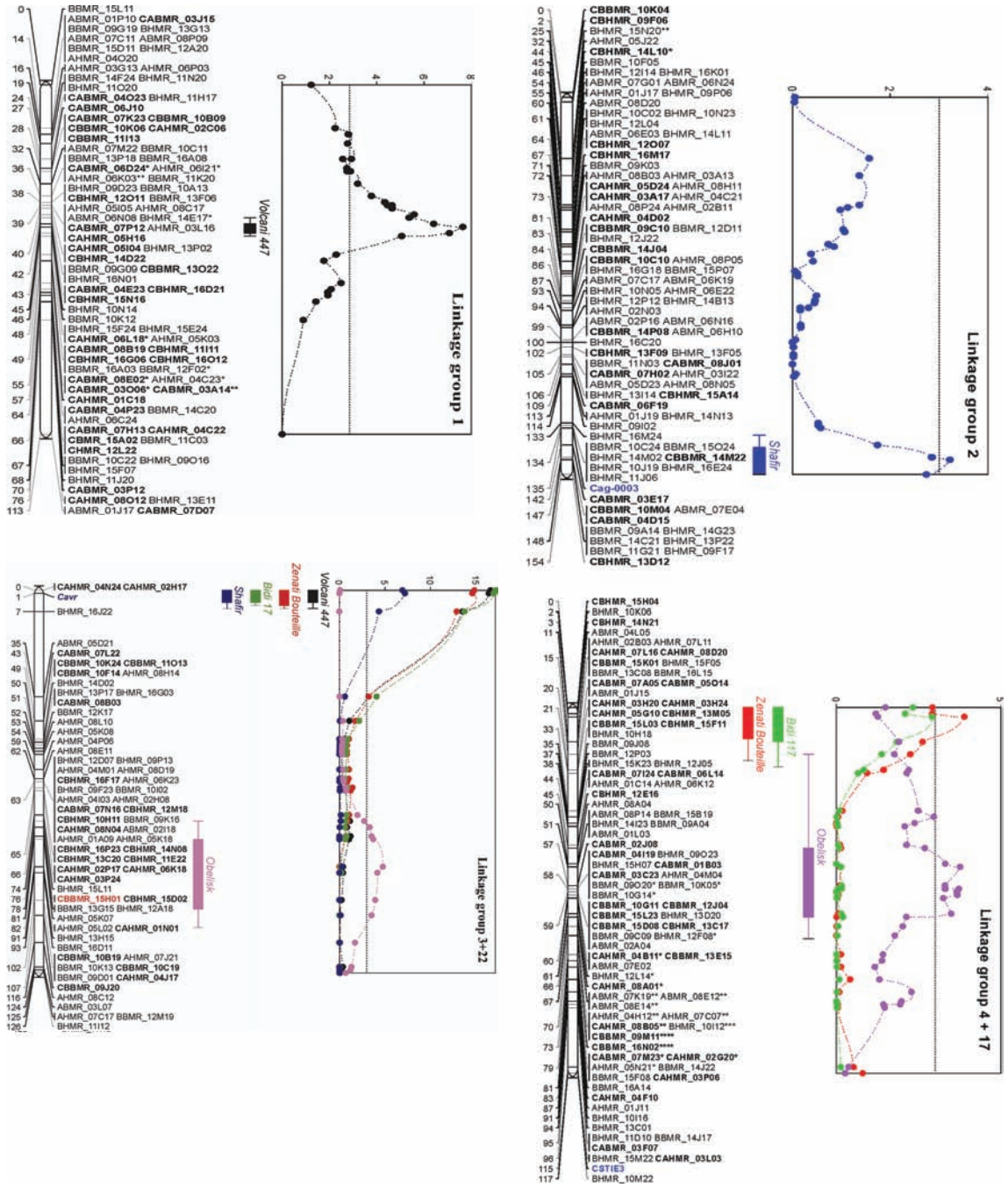

Figure 3A. Mapped quantitative trait loci involved in (a)virulence of *Mycosphaerella graminicola* to seven cultivars of wheat.

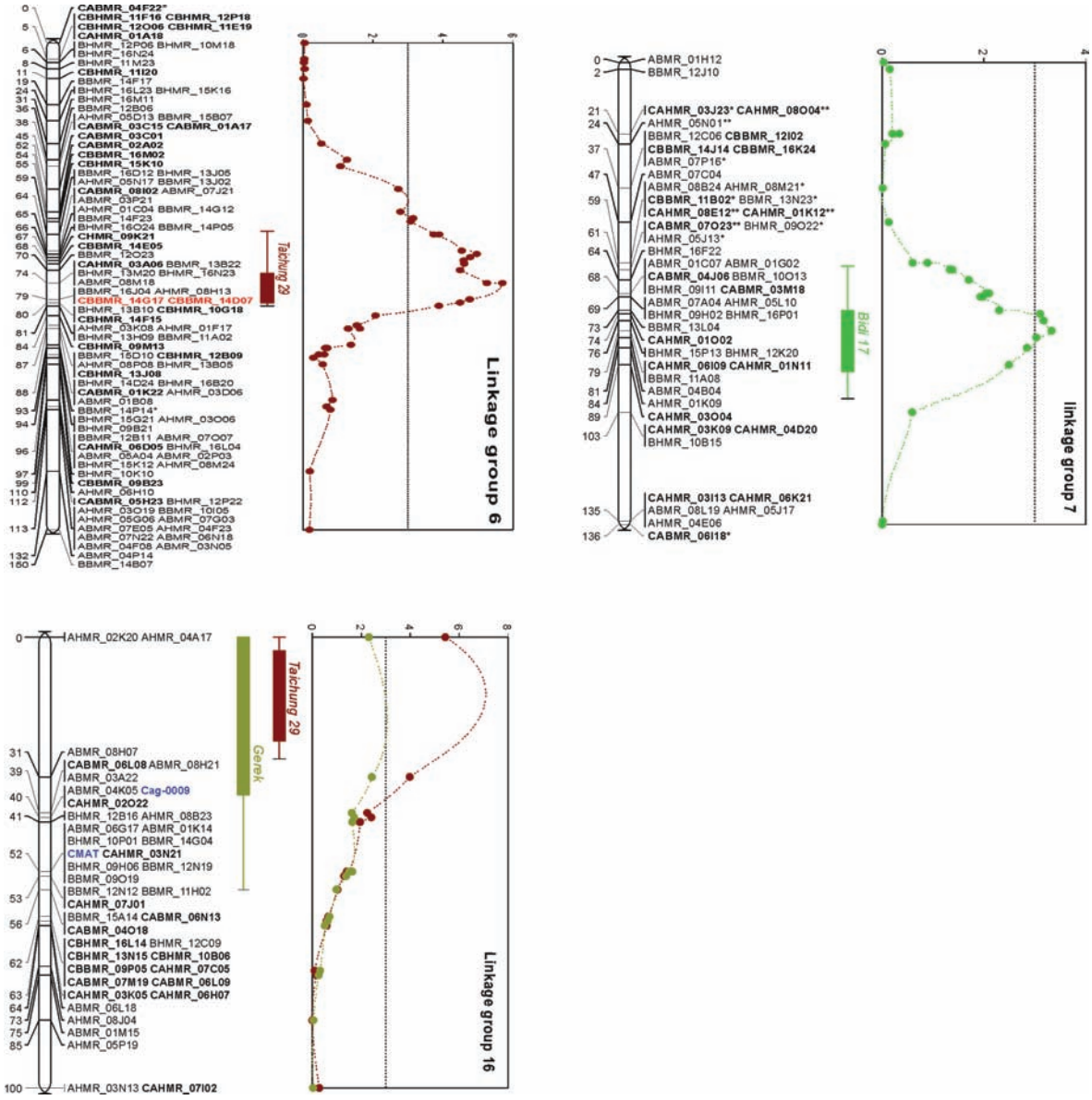

Figure 3B. Mapped quantitative trait loci involved in (a)virulence of *Mycosphaerella graminicola* to seven cultivars of wheat.

significantly increase disease severity, whereas their combined effect did. The interaction between the QTL on LG2 and the major QTL on LG3+22 for specificity to cv. Shafir was difficult to explain and requires additional analysis of more progeny, particularly since the fraction of virulent progeny on this and the other bread wheat cultivars was unexpectedly low.

Table 2. Pairwie comparisons of QTL interactions associated with specificity to cultivars using a generalized linear model (GLM) based on a binomial distribution of percentages of leaf area covered by pycnidia yielding an analysis of deviance.

Specificity to durum wheat			**Specificity to bread wheat**		
cv. Volcani 447	**+ QTL LG 3+22**	**- QTL LG 3+22**	**cv. Taichung 29**	**+ QTL LG 16**	**- QTL LG 16**
+ QTL LG 1	0.43±0.36[1]	10.25±1.73	**+ QTL LG 6**	14.61±2.15	3.29±1.24[4]
- QTL LG 1	2.06±0.86[1]	38.62±3.31	**- QTL LG 6**	2.17±0.99[4]	0.99±0.64[4]
cv. Zenati Bouteille	**+ QTL LG 3+22**	**- QTL LG 3+22**	**cv. Obelisk**	**+ QTL LG 3+22**	**- QTL LG 3+22**
+ QTL LG 4+17	1.09±0.47[2]	5.73±1.26	**+ QTL LG 4+17**	6.07±0.91	0.28±0.15[5]
- QTL LG 4+17	0.74±0.45[2]	19.04±1.90	**- QTL LG 4+17**	0.53±0.21[5]	0.12±0.11[5]
cv. Bidi 17	**+ QTL LG 3+22**	**- QTL LG 3+22**	**cv. Shaifr**	**+ QTL LG 3+22**	**- QTL LG 3+22**
+ QTL LG 4+17	0.31±0.22[3]	3.89±0.94	**+ QTL LG 2**	0.05±0.06[6]	5.90±0.78
- QTL LG 4+17	0.23±0.23[3]	13.15±1.46	**- QTL LG 2**	0.00±0.00[6]	0.54±0.23[6]

[1]All pairwise comparisons for the QTLs with specificity to *cv.* Volcani 447 are significantly different at P<0.001, except the indicated comparison with a t-probability of P=0.099.
[2]All pairwise comparisons for the QTLs with specificity to *cv.* Zenati Bouteille are significantly different at P<0.01, except the indicated comparison with a t-probability of P=0.597.
[3]All pairwise comparisons for the QTLs with specificity to *cv.* Bidi 17 are significantly different at P<0.01, except the indicated comparison with a t-probability of P=0.799.
[4]All pairwise comparisons for the QTLs with specificity to *cv.* Taichung 29 are significantly different at P<0.001, except the indicated comparisons with t-probabilities of P=0.111-0.483.
[5]All pairwise comparisons for the QTLs with specificity to *cv.* Obelisk are significantly different at P<0.001, except the indicated comparisons with t-probabilities of P=0.124-0.420.
[6]All pairwise comparisons for the QTLs with specificity to *cv.* Shafir are significantly different at P<0.001, except the indicated comparisons with t-probabilities of P=0.079-0.701.

Although necrosis ratings were based on a rough scale when compared to the continuous fine scale for pycnidial ratings, four QTLs were mapped involving necrosis (Figure 4). Again, a QTL on LG 3+22, positioned in the proximity of the QTL controlling specificity to cvs. Volcani 447, Zenati Bouteille, Bidi 17, and Shafir based on pycnidia, had the highest LOD values (LOD=4.70-9.28) and explained up to 29% of the variation in necrosis (Table 3, Figure 4). The same region is involved in necrosis development in all three durum wheat cultivars. Similarly, a QTL involved in necrosis was found for the durum wheat cvs. Volcani 447 and Zenati Bouteille on LG7 in the same region as a QTL involved in pycnidia for the durum wheat cv. Bidi 17. Weak QTLs (below the significant threshold of LOD-value 3.0) were found at this position for necrosis for cv. Bidi 17 and for pycnidia for cvs. Volcani 447 and Zenati Bouteille, so this locus is probably involved in both pycnidial development and necrosis for all three durum wheat cultivars. Two QTLs involved in necrosis were mapped on LG 5+10 for the bread wheat cvs. Shafir and Taichung 29, but no QTLs involved in pycnidial production were mapped on this LG. Therefore, these two QTLs appear to be specific for necrosis development.

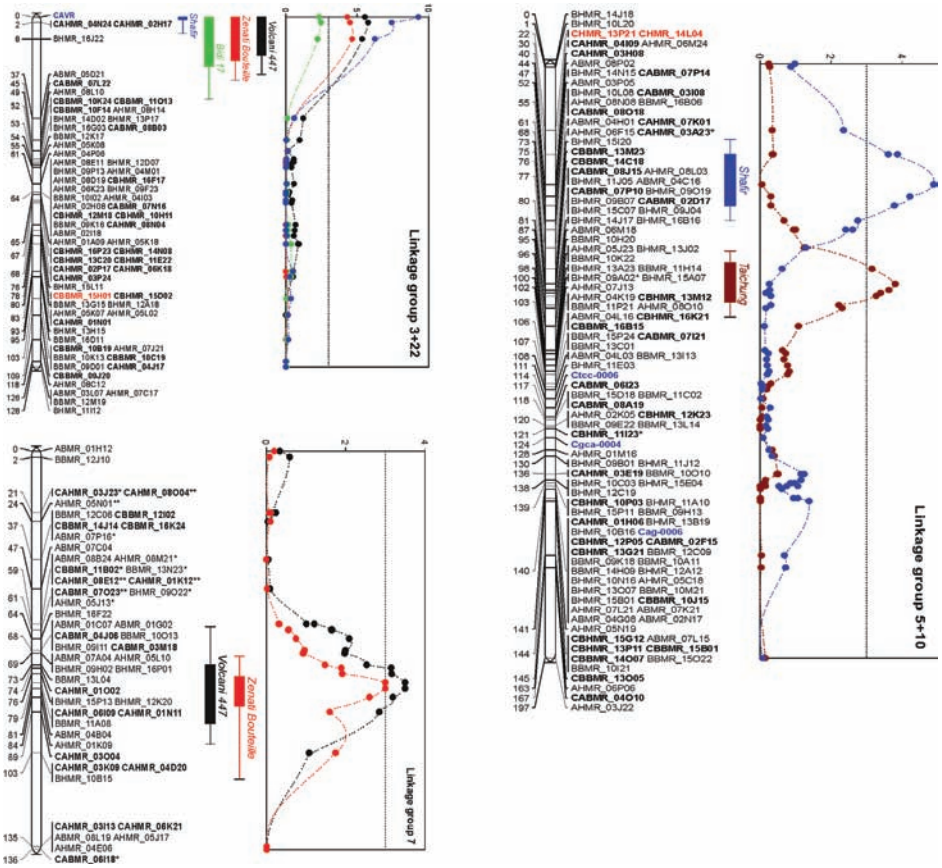

Figure 4. Mapped quantitative trait loci involved in necrosis caused by *Mycosphaerella graminicola* on seven cultivars of wheat.

Table 3. Mapped quantitative trait loci involved in necrosis in progeny isolates of *Mycosphaerella graminicola* from a cross between isolates IPO323 and IPO95052 tested for symptoms on the durum wheat cvs. Volcani 447, Zenati Bouteille and Bidi 17 and the bread wheat cvs. Taichung 29, Obelisk, Gerek 79 and Shafir. *different position than the other locus on LG 5+10

Species	Cultivar	Linkage group	LOD-value	Explained overall variance (%)
Durum	Volcani 447	3+22 7	5.76 3.57	19.2 10.2
	Zenati Bouteille	3+22 7	4.70 3.06	16.4 9.2
	Bidi 17	-	-	-
Bread	Taichung 29	5+10	3.81	13.2
	Obelisk	-	-	-
	Gerek 79	-	-	-
	Shafir	3+22 5+10*	9.28 4.90	29.2 17.1

Dispensable chromosomes and (a)virulence

No QTLs were mapped on any of the LGs found to be missing in some of the progeny (Table 4). Nevertheless, we compared the disease severity induced by these isolates on the durum and bread wheat cultivars. We conclude that LGs 8, 12, 21, B and C can be missed for pathogenicity to at least one of the wheat cvs. Volcani 447, Zenati Bouteille, Bidi 17, and Taichung 29. Isolates missing LGs 13 or 15 did not develop disease on the tested cultivars and therefore could carry unidentified effectors.

DISCUSSION

Host species and cultivar specificity of pathogens has intrigued biologists and phytopathologists for many decades (Flor 1956; Heath 1985; Nurnberger and Lipka 2005; Thompson and Burdon 1992). Throughout the last century, many pathogens have been named based on the species on which they were identified. Only during the last decade has a new nomenclature been introduced, a necessity fuelled by results obtained by using various molecular techniques. Exceptionally complicated cases of host species specificity exist in *Mycosphearella* species (Crous and Braun 2003; Crous and Groenewald 2005).

Table 4. Comparative analysis of progeny from a cross between *Mycosphaerella graminicola* isolates IPO323 and IPO95052 that miss one or more entire linkage groups and disease severity (average back-transformed percent leaf area covered by pycnidia) after inoculation of these isolates onto four durum wheat and three bread wheat cultivars.

Missing LG	Isolate	Volcani 447	Zenati Bouteille	Bidi 17	Taichung 29	Obelisk	Gerek 79	Shafir
8	02026	2	2	1	31	6	6	1
8	02133*1	79	57	40	0	0	0	1
8	02138*	60	37	18	0	0	0	1
8	02137+	0	0	0	3	0	1	0
8	02139+	1	4	0	17	0	1	0
12	02132	1	2	2	7	2	1	0
12	02133*	79	57	40	0	0	0	1
12	02138*	60	37	18	0	0	0	1
13	01158	2	4	2	1	0	0	0
15	02024	0	1	1	8	0	1	0
15	02032	0	0	0	0	1	0	0
15	02033	0	0	0	1	0	0	0
21	01114	28	11	2	1	0	0	0
21	01121	1	0	0	7	0	0	0
21	01122	44	4	1	0	0	1	0
21	01127	8	3	1	15	0	0	2
21	01151	0	1	0	1	0	0	0
21	01159	0	0	0	35	0	0	0
21	01170	1	2	1	2	0	1	0
21	01176	1	0	0	0	0	0	0
21	01186	77	20	28	0	0	0	5
21	01200	1	6	4	2	0	1	0
21	02133*	79	57	40	0	0	0	1
21	02138*	60	37	18	0	0	0	1
21	02137+	0	0	0	3	0	1	0
21	02139+	1	4	0	17	0	1	0

Table 4 (continued).

Missing LG	Isolate	Volcani 447	Zenati Bouteille	Bidi 17	Taichung 29	Obelisk	Gerek 79	Shafir
B	01108	0	0	0	0	0	0	0
B	01169	0	1	0	2	0	0	0
B	01179	0	0	0	0	0	0	0
B	01425	74	3	2	1	0	1	1
B	01438	0	0	0	0	0	0	0
B	02134°	2	3	2	2	0	1	0
B	02141°	0	0	0	0	0	0	0
C	01128	0	0	0	25	0	0	0
C	01139	1	0	0	0	0	0	0
C	01179	0	0	0	0	0	0	0
C	02030	7	0	0	0	0	0	0
C	02132	1	2	2	7	2	1	0

[1] Matching superscripts represent twin pairs of isolates.
Gray highlighting indicates that the corresponding linkage group is not required for pathogenicity.

Here we describe detailed genetic studies of specificity in *M. graminicola*, which infects both hexaploid bread wheat (*Triticum aestivum* L., AABBDD, 2n=42) and tetraploid durum wheat (*T. turgidum* L. (Thell.) subsp. *durum* L., AABB, 2n=28) species. Despite the fact that specificity of *M. graminicola* for these host species represents among the most drastic experimentally reported isolate-cultivar interactions (Eyal et al. 1973; Kema et al. 1996a; Saadaoui 1987; van Ginkel and Scharen 1988), we were routinely able to generate progeny from *in planta* crosses between such strains (see Chapter 2). This confirms sequence information of the internally transcribed spacer region of the ribosomal DNA (ITS), as well as the β-tubulin, actin, and calmodulin genes that did not support any clear distinction between isolates of *M. graminicola* infecting bread wheat as opposed to those infecting durum wheat (Groenewald and Crous, *unpublished*).

We generated progeny between *M. graminicola* isolates with host species specificity to bread wheat and durum wheat and used 147 isolates to study the genetic basis of this specificity. This same progeny set was used recently to construct a high-density genetic

linkage map using Diversity Arrays Technology (DArT) (see Chapter 3) in which 1,144 nuclear DNA markers from both parents segregated according to regular Mendelian inheritance.

Detailed QTL analysis with the aforementioned high-density genetic linkage map resulted in the identification of nine different QTLs involved in specificity. One major QTL (LOD-values from 14.83-17.12) is positioned on LG 3+22 and is a major effector controlling specificity for all three tested durum wheat cultivars. Intriguingly, the same locus also controls cultivar specificity to cv. Shafir (LOD=7.20), carrying resistance gene *Stb6*, which showed a gene-for-gene interaction with this locus (Kema et al. 2000, 2002; Brading et al. 2002). Apart from that, additional QTLs were mapped for disease severity on the bread and durum wheat cultivars using both pycnidia and necrosis data. This observation challenges the concept of specificity in *M. graminicola*, as many alternative specificities were generated in one sexual generation, which emphasizes the tremendous genetic variation observed in natural populations of this pathogen (Zhan et al. 2003). We also mapped shared QTLs for necrosis and pycnidia on LG 7 and LG 3+22, which seems logical because pycnidia always develop in necrotic tissue. Nevertheless, two QTLs on LG 5+10 (LOD-values of 3.81 and 4.90) specifically control development of necrosis, which supports previous results indicating that genes conferring necrosis and pycnidial development can be under separate genetic control (Kema et al. 1996a, 1996b; Kema and van Silfhout 1997). The QTL locus on LG 5+10 that causes necrosis in cv. Shafir does not cause necrosis in cv. Taichung 29, and *vice versa*, indicating cultivar specificity for necrosis development in these two bread wheat cultivars.

There was past debate on specificity in the wheat-*M. graminicola* pathosystem, which was fuelled by the absence of boom-and-bust cycles, the presence of quantitative virulence, and the lack of hypersensitive responses (Eyal et al. 1973, 1985; Johnson 1992; Parlevliet 1993; van Ginkel and Scharen 1988). This debate was brought to an end by formal genetic proof (Kema et al. 2002; Brading et al. 2002) showing that *MgAvrStb6* in IPO323 corresponds with the resistance gene *Stb6* that was later identified in 50% of a worldwide collection of bread wheat cultivars and lines (Chartrain et al. 2005). The significant effect of the QTL on LG3+22 that maps on the same chromosomal region as *MgAvrStb6* suggests that *Stb6* is also present in durum wheat cultivars, which is conceivable because this gene is located on wheat chromosome 3A that is shared by both wheat species (Brading et al. 2002). Hence, the current study supports our earlier finding, but in addition we found that this chromosomal region also controls host species specificity to all three durum wheat cultivars

tested. We found that some progeny (6%) derived from crosses on both wheat species were able to cause disease on both bread wheat and durum wheat cultivars, but the majority of progeny isolates that were able to cause disease could do so only on either bread wheat or only durum wheat cultivars. Therefore, if these isolates were sampled from pycnidia in a field, they would likely only be virulent to bread wheat or durum wheat cultivars and therefore be "host-adapted". The small percentage of isolates that are virulent to both bread wheat and durum wheat cultivars seems to be merely a reflection of the simple inheritance of different combinations of many cultivar- and possibly host species-specific effectors. In this current study, recombination resulted in avirulence in 53% of the progeny to all seven wheat cultivars tested and left the large remainder of isolates with specific avirulences to either bread wheat or durum wheat cultivars. A minority of the isolates had accumulated virulence to both species. One of the reasons that host species specificity is commonly reported could be explained by too narrow surveys of isolates in previous studies. However, a small percentage (6%) of isolates in natural populations were identified that were able to cause disease on both bread wheat and durum wheat cultivars in an international sampling of 110 single pycnidial isolates from locations where both wheat species were grown (Kema et al. 1999; Zhan et al. 2004).

Several loci determining host species or cultivar specificity were isolated using a map-based strategy and were found to reside in regions close to the telomere (Gout et al. 2006; Lee et al. 2001; Orbach et al. 2000). Interestingly, several of the QTLs that we mapped, including the QTL on LG 3+22 with the high LOD-value, are also located at the end of linkage groups. Indeed, functional analyses of *MgAvrStb6* has been so far been unsuccessful (Mehrabi et al. 2002). However, we now have markers on both flanking regions of this locus (see Chapter 3), and the high density genetic linkage map that will be aligned with the 9X genome sequence of *M. graminicola* isolate IPO323 will facilitate future map-based cloning strategies to further unravel specificity in *M. graminicola*. In addition, this sequence will be instrumental in the identification of genes on the dispensable LGs that apparently can be missed without a penalty on pathogenicity.

Our results demonstrate the genetic tractability of *M. graminicola* and the importance of its frequent sexual recombination to generate diversity and to efficiently circumvent resistance in wheat species and cultivars, which may explain its success as a pathogen.

ACKNOWLEDGEMENTS

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CHAPTER 5

Strobilurin fungicides cannot prevent sex and cause preferential mating in the wheat septoria tritici blotch pathogen *Mycosphaerella graminicola*

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ABSTRACT

Over the past three years, resistance to strobilurin fungicides has rapidly evolved in the foliar wheat pathogen *Mycosphaerella graminicola*. This resistance is conveyed by a point mutation in the cytochrome b gene in the mitochondrial genome. In order to study the inheritance of strobilurin resistance, we crossed resistant and sensitive isolates of *M. graminicola* on wheat seedlings that were both untreated and preventively treated with azoxystrobin (Amistar™, dose rates: 0, 3.125, 6.25, 12.5, 25, 50, 100, and 200%). Forty-two *in planta* crosses were made using three parental pairs at each concentration. All crosses were successful under all preventive applications of Amistar™, and ratios of resistant to sensitive progeny were determined by comparing germinations of over 25,000 ascospores after discharge onto water agar and water agar amended with 1 ppm azoxystrobin. These ratios were independently confirmed by strobilurin-sensitivity PCRs on a subset of ~2,000 isolates that was discharged onto unamended water agar. Preventative rates from 3.125-200% Amistar™ resulted in completely resistant progeny populations, and hence we conclude that sensitive isolates overcome the disruption of mitochondrial respiration and participate in sexual reproduction even under high fungicide pressure. Moreover, fungicide stress induces or results in preferential mating in *M. graminicola*, which could be the driving force behind the rapid spread of strobilurin resistance in *Mycosphaerella* species such as *M. graminicola* on wheat and *M. fijiensis* on banana.

INTRODUCTION

Strobilurins belong to the newest classes of fungicides controlling plant pathogenic fungi. The first strobilurin fungicides, azoxystrobin and kresoxim-methyl, were released in 1996 (Bartlett et al. 2002). Just three years later, azoxystrobin was the biggest-selling fungicide in the world and is now registered for over 80 crops in 85 countries (Bartlett et al. 2002; http://www.syngentacropprotection-us.com). In addition to azoxystrobin and kresoxim-methyl, seven other strobilurin fungicides have been registered; namely trifloxystrobin, metominostrobin, picoxystrobin, pyraclostrobin, fluoxastrobin, oryzastrobin and dimoxystrobin (Bartlett et al. 2002; http://www.frac.info). However, resistance to strobilurins in the field rapidly evolved and was first observed in the wheat powdery mildew pathogen, *Blumeria graminis* f. sp. *tritici*, in Germany in 1998 (Felsenstein 1999). To date, resistance has been reported for at least 21 pathogens on many economically important hosts, including *Plasmopara viticola* on grape (Heaney et al. 2000), *Venturia inequalis* on apple (Steinfeld et al. 2002), *Glomerella cingulata* on strawberry (Ishii 2004), and *Mycosphaerella fijiensis* on banana (Sierotzki et al. 2000a).

The mode of action of strobilurins is based on binding to the quinone outside (Q_o) binding site located in the mitochondrial cytochrome bc1 protein complex (Complex III) to thereby inhibit mitochondrial respiration (Wiggins and Jager 1994). Because all fungi require mitochondrial respiration for the majority of their ATP production, it is not surprising that strobilurins are effective against a broad range of plant pathogenic fungi, including ascomycetes, basidiomycetes, and deuteromycetes, as well as plant pathogenic oomycetes. Resistance to strobilurins is conveyed by a single nucleotide substitution that prevents binding at the Qo site (Sierotzki et al. 2000a, 2000b; Kim et al. 2003). In most fungi, guanine is replaced by cytosine to convert the amino acid glycine to alanine at position 143 (G143A) of the cytochrome b protein. This mutation prevents binding of strobilurins to the Q_o site, hence restoring the ability of the fungus to carry out mitochondrial respiration. The threshold of resistance conveyed by the G143A mutation enables resistant isolates to survive strobilurin treatments at concentrations ~100 times higher than sensitive isolates.

The septoria tritici blotch disease of wheat is caused by *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn. *M. graminicola* has a heterothallic, bipolar mating system, and ascospores play an important role in the epidemiology of the disease (Shaw and Royle 1989, 1993; Chen and McDonald 1996; Kema et al. 1996). Rapid development and spread of fungicide resistance is atypical for *M. graminicola*, yet resistance to strobilurins evolved soon

after their introduction in Western Europe (Gisi et al. 2005). A very low frequency of strobilurin-resistant *M. graminicola* isolates was first discovered late in the growing season of 2002. Despite low to moderate disease pressure due to dry weather, the levels and incidence of resistance rapidly increased in different geographical regions all over Europe in the 2003 season (Gisi et al. 2005), which suggests many independent mutation events.

Because the target site for strobilurins is in the mitochondrial genome, we were interested in the inheritance of strobilurin resistance, since these organelles are almost always exclusively maternally inherited (Birky 1995; Russell 1996). We developed a marker system to track this process and hypothesized that sensitive isolates might escape from fungicide stress by entering into sexual reproduction. Since mitochondria are maternally inherited, preferential mating or selection would result in progenies with an excess of resistant isolates. We therefore crossed resistant and sensitive isolates of *M. graminicola* on wheat plants that were preventatively treated with strobilurin and monitored the rate of mitochondrial inheritance by determining strobilurin resistance in the progenies. Interestingly, sensitivity to strobilurins did not prevent mating in *M. graminicola*. Indeed, we discovered that sensitive *M. graminicola* isolates have an excessive paternal mating behavior on wheat seedlings preventatively treated with azoxystrobin (Amistar™), resulting in progenies that are predominantly resistant to strobilurins. This process is independent of fitness differences and regular Mendelian segregation of nuclear genes.

MATERIALS AND METHODS

Fungal isolates, maintenance, and inoculum preparation

After an initial screening of 19 isolates of *M. graminicola*, six isolates were selected for crosses and competition experiments based on mating type, strobilurin sensitivity, and pathogenicity results (Table 1, Figure 1). In addition, two reference isolates, IPO323 and IPO94269, were used (Table 1). Ascospore progenies from 42 crosses resulting from three parental pairs were collected and stored for further analyses. Maintenance of isolates and preparation of inocula were as previously described (Kema et al. 1996). In cases where sensitive isolates developed disease symptoms in infection assays on wheat seedlings preventatively treated with Amistar™, pycnidial isolates were recovered and maintained at -20° C for further characterization.

Table 1. Isolates of *Mycosphaerella graminicola* used in this study.

Pycnidial Isolate	Sensitivity to strobilurins	Location	Year	Strobilurin sprayed?	Mating type
IPO03001 (BCS3R)[1]	Resistant	Germany	2003	unknown	*mat 1-1*
IPO03002 (BCS8S)	Resistant	Germany	2003	unknown	*mat 1-2*
IPO03003 (BCS16S)	Sensitive	Germany	2003	unknown	*mat 1-2*
IPO03005 (BCS17S)	Sensitive	Germany	2003	unknown	*mat 1-1*
IPO04001	Sensitive	Biddinghuizen, Netherlands	2004	No	*mat 1-1*
IPO04011	Resistant	Lelystad, Netherlands	2004	No	*mat 1-2*
IPO323 (reference)	Sensitive	West Brabant, Netherlands	1981	No	*mat 1-1*
IPO94269 (reference)	Sensitive	Kraggenburg, Netherlands	1994	No	*mat 1-2*

[1]Isolate code from Bayer CropScience

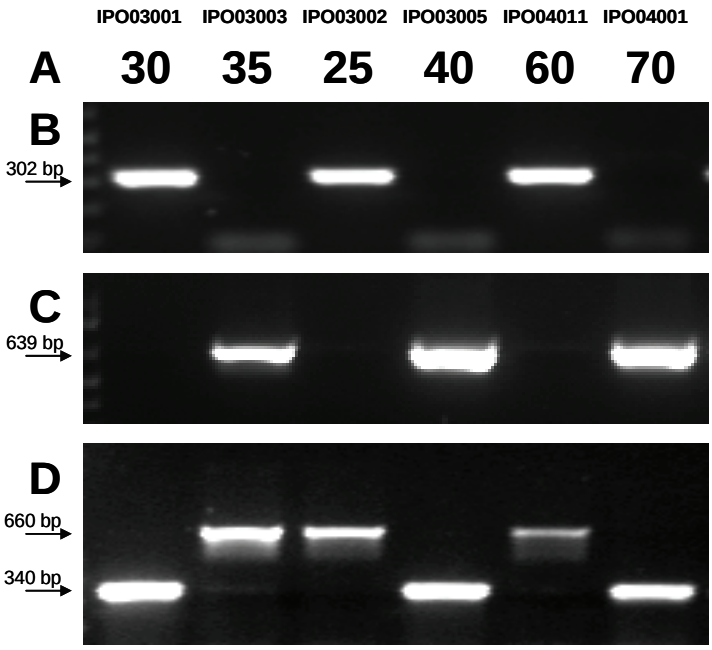

Figure 1. Characterization of *Mycosphaerella graminicola* isolates. **A,** Percent leaf area covered by pycnidia on untreated cv. Taichung 29, 20 days after inoculation. **B**, Amplicons in strobilurin-resistant isolates. **C**, Amplicons in strobilurin-sensitive isolates. **D**, Amplicons for mating types. Upper band indicates *mat 1-2*, Lower band indicates *mat 1-1*.

DNA isolation, quantification, and PCR screens

Extraction of genomic DNA of *M. graminicola* from cultures was from 50 mg lyophilized spores using the Wizard® Magnetic DNA Purification System for Food (Promega, Madision, WI, USA), with a final elution with 100 µl sterile double-distilled (sdd) water. For quantitative PCR (TaqMan®), extractions of genomic DNA from inoculated plant material were made from 10 mg lyophilized leaves using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), with a final elution of 2 x 50 µl sdd water (100 µl final volume). Dilutions of the DNA samples were run on 1.2% agarose gels along with dilutions to 10, 25, 50, and 100 ng made from standard λ DNA (Roche, Almere, Netherlands) for precise quantification. A dilution series was then made for each of the six parental isolates at 100,

10, 1, 0.1, and 0.01 ng per µl, and these series were included in each TaqMan® run to quantify unknown concentrations of *M. graminicola* DNA within leaves.

We developed a 96-well format (Hoffman-LaRoche thermocycler plates, Roche, Almere, The Netherlands) method for colony extraction of DNA to enable rapid screening of the large number of ascospore progeny isolates. Sterile toothpicks were used to transfer barely visible amounts of either spores (preferably) or mycelia to individual wells containing 30 µl TE buffer, which were closed with re-usable rubber lids (Perkin Elmer, Milano, Italy) that were secured by placing four rubber bands around the perimeter of the plate. These plates were subsequently subjected to microwave boiling (1200 W for 5 min.) with a layer of water on the microwave plate to prevent melting of the PCR plate. After boiling, samples were immediately put on ice (5 min.) and centrifuged (5 seconds at 6,000 rpm) to spin down cell debris. Each sample was diluted 5X by transferring 3 µl supernatant to a new plate containing 12 µl sdd water in each well.

We developed qualitative PCR screens for strobilurin sensitivity by using a mismatch amplification mutation assay (MAMA) (Cha et al. 1992) on a part of the cytochrome b gene to rapidly determine strobilurin sensitivity or resistance among generated *M. graminicola* ascospore progenies. Primers were designed with a mismatch on the penultimate nucleotide of the 3' end of the forward primer, in which the ultimate nucleotide was at position 143 of the cytochrome b gene (Table 2). The primer set to specifically amplify a DNA fragment in sensitive isolates used a sense primer StrobSNP2fwd [5'-3' (404-428)] with a mismatch of T instead of G at nucleotide 427 of the cytochrome b gene and an antisense primer StrobSNP1rvs [5'-3' (1024-1043)]. The primer set to specifically amplify a DNA fragment in resistant isolates used an antisense primer StrobSNPrcF7 [5'-3' (428-453)] with a mismatch of T instead of G at nucleotide 429 and a sense primer StrobSNPrcR1 [5'-3' (152-173)] (Table 2). This resulted in a single nucleotide mismatch on the sequence of the sensitive isolates and a double nucleotide mismatch on the sequence of the resistant isolates and vice versa, for each primer set. One and 0.5 µl of DNA were used for the strobilurin and mating-type PCR, respectively. Primers for the mating type PCR and thermal cycling conditions were as previously described (Table 2, Waalwijk et al. 2002). Amplicons were analyzed on 1.2% agarose gels using 25 µl aliquots of the PCR products.

PCRs to amplify simple sequence repeats (SSRs) were in 20 µl containing 20 ng DNA, 2 µl 10X PCR buffer with $MgCl^{2+}$, 2 µl each forward and reverse primers (2 µM), 0.8 µl dNTPs (5 mM), 0.2 µl Taq DNA polymerase (5U/ µl), and x µl sdd water. Thermal cycling was as follows: cycle 1; 94° C for 2 mins., cycle 2 (repeated 12x); 94° C for 30 secs.,

Table 2. Probes and primers for *Mycosphaerella graminicola* used in this study.

Name	Sequence (5' to 3')	Location
MAT1-1 F	CCGCTTTCTGGCTTCTTCGCACTG	*mat 1-1*
MAT1-1 R	TGGACACCATGGTGAGAGAACCT	*mat 1-1*
MAT1-2 F	GGCGCCTCCGAAGCAACT	*mat 1-2*
MAT1-2 R	GATGCGGTTCTGGACTGGAG	*mat 1-2*
StrobSNP2fwd	CTTATGGTCAAATGTCTTTATGATG	*cytochrome b*
StrobSNP1rvs	GGTGACTCAACGTGATAGC	*cytochrome b*
StrobSNPrcF7	CAATAAGTTAGTTATAACTGTTGCGG	*cytochrome b*
StrobSNPrcR1	CTATGCATTATAACCCTAGCGT	*cytochrome b*
Mmat1P3	FAM- CGCAGTCTGCTTTGAATGAGAAGTTATC –Darquencher	*mat 1-1*
Mmat1F3	CGTGCGTCGCTGACTTGT	*mat 1-1*
Mmat1R3	TGCCGCGAGCGACAC	*mat 1-1*
Mmat2P4	YY- CCTCGCAAGCCATCGGAGA -Darquencher	*mat 1-2*
Mmat2F7	GCATCCGGGATACCAGTA	*mat 1-2*
Mmat2R7	CTTGGTCATGCGACGTT	*mat 1-2*
ag-0003 F	ACTTGGGGAGGTGTTGTGAG	LG 2[a]
ag-0003 R	ACGAATTGTTCATTCCAGCG	LG 2
gca-0004 F	TAACGGTAACGGCAACAACC	LG 5+10
gca-0004 R	GTGTACCCTTGAATCGCAGC	LG 5+10
tcc-0008 F	AAAAGACATGACGCCCGAC	LG 4+17
tcc-0008 R	ACGAGGAATAATCGCGGAAC	LG 4+17
ag-0006 F	TAACCAACACCAGGGGAATG	LG 5+10
ag-0006 R	CATCAGTTGTCAGCGAATGG	LG 5+10
ag-0009 F	GACTCCATTTACCTGTGGCG	LG 16
ag-0009 R	TGTGAAGGACACGCAAAGAG	LG 16
tcc-0006 F	ATCTGGACACCATCCACCAG	LG 5+10
tcc-0006 R	GTAGGTGGGAGGGTTCATGC	LG 5+10

[a]LG = linkage group (Wittenberg et al. 2006, Chapter 3)

then 66° C for 30 secs. minus 1° C per cycle, then 72° C for 30 secs., cycle 3 (repeated 27x); 94° C for 30 secs., then 53° C for 30 secs., then 72° C for 30 secs. and cycle 4; 72° C for 7 mins., followed by a cooling-off step to 10° C. Fragments were separated on a Mega-Gel Dual High-Throughput Vertical Electrophoresis Unit (CBS Scientific, Del Mar, California, USA) with 6% non-denaturing acrylamide gels stained with ethidium bromide during the run.

To monitor biomass of isolates in crossing and infection assays, we designed specific TaqMan® probe/primer combinations for quantitative PCRs based on the *mat1-1* and *mat1-2* idiomorph sequences of the two reference isolates IPO323 and IPO94269. Primers that specifically amplify DNA fragments in *mat1-1* isolates were Mmat1F3/Mmat1R3, with a FAM-fluorescent probe IP3, and primers to specifically amplify DNA fragments in *mat1-2* isolates were Mmat2F7/Mmat2R7, with a YY-fluorescent probe 2P4 (Table 2). Both quantitative real-time amplifications were performed in a single PCR on an Applied

Biosystems 7500 Real-time PCR System (Foster City, CA, USA). Total reaction volumes were 25 µl, including 3 µl DNA, 12.5 µl Premix Ex Taq™ (2X) (TaKaRa, Shiga, Japan), 1 µl each forward and reverse primers (6 µM), 0.67 µl for each probe (5 mM), 0.5 µl ROX Reference Dye II (50x), and 8.33 µl ultraPURE™ nuclease-free water (Gibco, Paisley, Scotland). Thermal cycling was as follows: cycle 1; 50° C for 2 mins., cycle 2; 95° C for 10 mins., cycle 3 (repeated 39x); 95° C for 15 secs., then 60° C for 20 secs. Results were analyzed using Sequence Detection Software version 1.2.3 (Applied Biosystems). Standard curves from serial dilutions of known concentrations of pure fungal DNA of all six parental isolates plus the DNA from the reference isolates gave highly similar results in CT values. Therefore, serial dilutions of DNA from IPO323 and IPO94269 were included in each TaqMan® PCR run to calculate the unknown concentrations of fungal DNA in inoculated wheat seedlings. The standard curves had very high R^2 values (0.990-0.996) for all data points from 3 pg to 30 ng and, therefore, CT values within this range were reliable (data not shown).

Strobilurin sensitivity determination

We used strobilurin-amended potato dextrose agar (PDA) plates and PCR assays for the initial assessment of strobilurin sensitivity in progeny (n=107) generated from a cross between *M. graminicola* isolates IPO03001 and IPO03003 to test whether both methods were congruous. The minimal inhibitory concentrations (MICs) of two different technical samples of strobilurins, kresoxim-methyl (BASF, Ludwigshafen, Germany) and trifloxystrobin (Bayer CropScience, Monheim am Rhein, Germany) were determined by spotting isolates on PDA amended at various concentrations. The concentrations for kresoxim-methyl were 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1.0 ppm, and the concentrations for trifloxystrobin were 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1 and 0.25 ppm. All isolates were spotted in triplicate in a volume of 5 µl per spot at a concentration of 4×10^5 spores ml^{-1}. As a positive control for growth, isolates were also plated on PDA amended with the strobilurin solvent (1% methanol). Plates were placed at 18 °C in the dark for 10 days, after which MIC values were assessed. MIC values for the six parental isolates were determined using Amistar™ containing azoxystrobin at 0.1, 1.0, and 10 ppm.

Infection and crossing assays

In order to determine which concentrations of strobilurin to use for infection and crossing assays, we established *in planta* dose response curves for the sensitive *M. graminicola* isolates using different preventive applications of Amistar™ (250 g L^{-1} a.i. of azoxystrobin; 50% E. C.; Syngenta, Roosendaal, Netherlands). Ten-day-old seedlings of cv. Taichung 29 were preventatively treated (48 h) with azoxystrobin using a track sprayer that was calibrated to deliver the recommended application of 1 L ha^{-1} sprayed at a rate of 250 L ha^{-1}, with the following percentages of the full recommended dose : 0, 3.125, 6.25, 12.5, 25, 50, 100 and 200%. These rates correspond to strobilurin solutions with 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, and 2 g active ingredient L^{-1}, respectively.

Subsequent inoculations with *M. graminicola* were as described previously by Kema et al. (1996). Percentages of leaf area covered by pycnidia were recorded at 20, 23, 26, and 29 days post inoculation (dpi) for dose response curve experiments, at 20 dpi for infection assays, and at 0, 5, 10, 15, 20, 25 and 30 dpi for biomass monitoring over time. In addition to these visual observations, we monitored the fungal biomass development of isolates IPO03001, IPO03003, IPO03002, IPO03005, IPO04001 and IPO04011 individually and in pairwise mixtures on untreated and preventatively treated (48h, 100% azoxystrobin) seedlings of the wheat cv. Taichung 29. Leaf samples were collected at 0, 5, 10, 15, 20, 25 and 30 dpi and were immediately frozen in liquid nitrogen before storage at -80° C until lyophilization, subsequent DNA extraction, and quantitative TaqMan® analyses. Two extractions were made from each sample, and the mean results were expressed in ng of fungal DNA per mg dry weight leaf material.

Three sets of parents with opposite mating types, contrasting strobilurin sensitivities, and approximately equal virulence (Figure 1) were selected for generation of *in planta* ascospore progenies according to the protocol described previously (Kema et al. 1996). Seedlings of cv. Taichung 29 were preventively treated (48 h) with Amistar™ at: 0 (control), 3.125, 6.25, 12.5, 25 and 50% of the full rate and were inoculated using mixtures of *M. graminicola* isolates IPO03001 and IPO03003, IPO03002 and IPO03005, and IPO04001 and IPO04011 to generate ascospore progenies (18 crosses: 3 parental sets x 6 concentrations of azoxystrobin). A second series of crosses was made using the previous concentrations as well as the full rate (100%), and the double rate (200%) (24 crosses: 3 parental sets x 8 concentrations of azoxystrobin) for further ascospore generation, isolation, and analyses. From 6 through 12 weeks after inoculation, material was harvested for ascospore collection. Ascospores were isolated as much as possible from diverse locations within a plate or within

several plates from each cross to obtain random ascospore progenies. All 42 ascospore progeny sets were randomly selected from unamended WA plates for mating-type and strobilurin sensitivity PCR screens to determine segregation ratios for these characteristics. In addition, baseline germination frequencies were determined for all 42 crosses on unamended water agar. Germination frequencies in the 24 ascospore progenies for the second series of crosses were also determined on WA amended with 1 ppm active ingredient azoxystrobin Amistar™, and these frequencies were expressed as percentages relative to the mean of the control germination frequencies on unamended water agar.

Genotyping of ascospores

We used SSR markers and mating-type primers to confirm that collected ascospores resulted from crosses of the co-inoculated parental isolates of *M. graminicola* in subsets of the 24 ascospore progenies from the second set of crosses. For each parental set, three polymorphic SSR markers were selected from a pre-determined list of primer sets for *M. graminicola* (Goodwin et al. 2006, *in press*). Multiplex PCRs of three primer sets were as follows; for IPO03001 and IPO03003: ag-0003, gca-0004, and tcc-0008; for IPO03002 and IPO03005: ag-0006, ag-0009, and tcc-0008; and for IPO04001 and IPO04011: ag-0003, tcc-0006, and tcc-0008 (Table 2). These primer sets were also used to confirm the identity of re-isolated sensitive strains from inoculation experiments.

RESULTS

Validation of methodologies for strobilurin sensitivity determination

The MIC values determined in the *in vitro* screens for kresoxim-methyl and trifloxystrobin were 0.5 ppm and 0.1 ppm, respectively (results not shown). Progeny (n=107) from a cross between IPO03001 and IPO03003 (no strobilurin pressure) segregated for strobilurin resistance (no difference between PDA agar plates amended with kresoxim-methyl and trifloxystrobin), which matched exactly with the strobilurin sensitivity PCR screen that amplified 639 bp and 302 bp products in sensitive and resistant isolates, respectively (Figure 1). The strobilurin sensitivity PCR screen also showed complete correlation with the MIC value for azoxystrobin (1.0 ppm) in a number of genetically unrelated field isolates. We therefore used this assay in all other analyses.

Dose response curves

All isolates inoculated onto untreated seedlings produced characteristic disease symptoms that started with faint chlorosis after 10 days and necrosis after 12 days, which subsequently developed into large necrotic blotches containing numerous pycnidia that were formed from between 14 and 21 days after inoculation of untreated plants. Resistant isolates were fully pathogenic on plants preventively treated with Amistar™ (3.125-200%; Figure 2 for examples). At 20 dpi, sensitive isolates produced ≤5% pycnidia at the lowest azoxystrobin rates (3.125-6.25%), and no disease was observed with higher rates (12.5%-200). However, at 26-29 dpi, all sensitive isolates were able to produce the characteristic disease symptoms of necrotic leaves bearing the asexual fructifications (pycnidia) for all azoxystrobin rates (Figure 2 for example). This contrasts significantly with *in vitro* assays where sensitive isolates never developed at the MIC values of azoxystrobin, even after prolonged maintenance (several months, not shown). We therefore used all concentrations of azoxystrobin for preventive treatments of cv. Taichung 29 seedlings that were used for the *in planta* crossing assays.

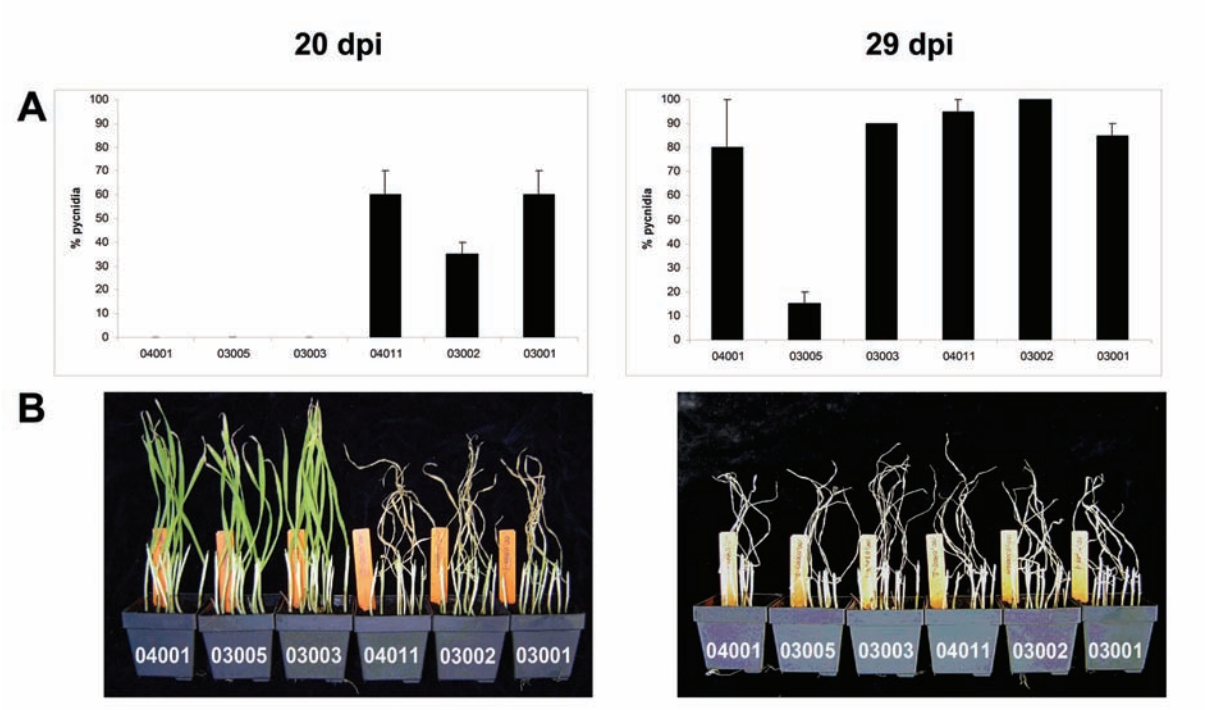

Figure 2. Responses of wheat seedlings incoculated with sensitive and resistant isolates of *Mycosphaerella graminicola* after a pre-treatment with the full recommended rate of Amistar™ at 20 dpi (left) and 29 dpi (right). **A,** Percent pycnidia based on visual observations. **B,** Overall view of seedlings.

Crossing experiments

Surprisingly, ascospores were discharged from all 42 crosses on plants treated with the full range of Amistar™ rates, and a total of 2,614 random ascospore progeny isolates were collected and stored for further analysis. Additionally, over 25,000 ascospores were visually scored for germination on either WA or WA amended with Amistar™. On WA, germination frequencies were high and ranged from 74-100% irrespective of the Amistar™ rate on the preventively treated seedlings (results not shown). The absolute mean percentages of germination of progeny on WA for IPO03001/IPO03003, IPO03002/IPO03005, and IPO04001/IPO04011were 99%, 86%, and: 89%, respectively, and these results were used to normalize germination frequencies on Amistar™-amended WA. A large variation was observed when ascospores were discharged onto Amistar™-amended WA (Table 3a). From the second series of crosses, progeny from the control crosses IPO03001/IPO03003, IPO03002/IPO03005, and IPO04001/IPO04011 on untreated plants showed germination frequencies of 93, 15, and 49%, respectively, after normalization. The germination frequency remained high in the seven additional crosses between IPO03001 and IPO003003 on plants that were preventatively treated with Amistar™ and was irrespective of the applied rate. The germination frequencies in progeny from the other two parental sets increased when crossed on plants preventively treated with doses of just 3.125-6.25% Amistar™ and remained high (nearly 100%) when crosses were made on plants preventatively treated with the remaining doses of 12.5-200% Amistar™. Resistance frequencies based on PCRs from all 42 progenies (~50 isolates from each progeny set) from both the first and second series of crosses confirmed the results obtained from germination frequencies on Amistar™-amended WA, except that resistant frequencies in crosses made on untreated plants varied considerably between both repeats (Table 3b).

Pathogenicity and biomass determination

Strobilurin-resistant and -sensitive strains of each parental set were equally fit based on the leaf area covered by pycnidia on untreated cv. Taichung 29 wheat seedlings after 20 days (Figure 1). Upon increasing concentrations of azoxystrobin, the leaf area covered by pycnidia decreased dramatically for the strobilurin-sensitive isolates but not for the strobilurin-resistant isolates (Figure 2).

Table 3. Non-Mendelian inheritance of resistance to azoxystrobin in ascospore progeny populations of *Mycosphaerella graminicola*. **A,** The percentage of strobilurin-resistant progeny was determined by monitoring the germination of 9.025 ascospores, originating from 20 *in planta* crosses between strobilurin resistant and sensitive *M. graminicola* isolates on seedlings of wheat cv. Taichung 29 that were preventively treated with seven doses of Amistar™ and then discharged onto water agar amended with 1 ppm (MIC value) Amistar™. **B,** The percentage of strobilurin resistant progeny determined by a strobilurin sensitivity PCR screen in 42 *M. graminicola* progenies.

A

IPO03001 x IPO03003		IPO03002 x IPO03005		IPO04001 x IPO04011	
Total #	Germ freq, a	Total #	Germ freq	Total #	Germ freq
630	93	856	15	2108	49
85	89	390	48	709	64
188	92	183	78	556	100
191	76	135	88	607	100
237	100	166	88	336	99
496	99	105	93	349	100
186	96	NA	NA, b	512	100
2013		1835		5177	

a) Normalized germination frequency. b) Not assessed due to limited leaf material.

B

	IPO03001 x IPO03003		IPO03002 x IPO03005		IPO04001 x IPO04011	
Percent of full rate of Amistar™	% Resistant in PCR 1st Round	% Resistant in PCR 2nd Round	% Resistant in PCR 1st Round	% Resistant in PCR 2nd Round	% Resistant in PCR 1st Round	% Resistant in PCR 2nd Round
0	100	100	0	33	100	38
3.125	100	100	98	49	96	71
6.25	100	100	100	100	65	98
12.5	100	100	100	98	100	100
25	100	100	96	98	100	100
50	100	100	92	92	100	100
100	*	100	*	100	*	100
200	*	100	*	100	*	100

*) Concentration not used in the first series of crosses.

Because of observed pycnidial development of sensitive isolates in dose response experiments, TaqMan® assessments were also used to determine the biomass of single and mixed inoculations of isolates in each parental set inoculated on preventively treated (48 h, 100% Amistar™) plants. From 0 -10 dpi (when the leaves were still green), none of the isolates increased noticeably in biomass. From 10 to 15 dpi, the resistant isolates IPO03001 and IPO04011 alone increased greatly (~600-800%) in biomass, and a similar increase was observed from 15 to 20 dpi for the resistant isolate IPO03002 (Figure 3). In the single

inoculations, the biomass of the sensitive isolates was undetected until 25 dpi, and at 30 dpi the biomass reached ~20-50% of that of the resistant isolates, which was also supported by visual observations of percent pycnidia (Figure 3). In the mixed inoculations at 15 dpi, the biomass of the resistant isolates equaled the biomass in the individually inoculated plants, but the biomass of the sensitive isolates could not be detected even at 30 dpi. Nevertheless, all mixed inoculations resulted in the generation of ascospore progenies.

Genotyping

The segregation ratios for the mating type genes were maintained at 1:1 (χ^2 between 0 and 3.63, where P=0.05) irrespective of the doses of azoxystrobin in all 24 progenies from the second replication (not shown), except for the progenies from the cross between IPO03002 and IPO03005 on untreated and 100% azoxystrobin-treated plants (χ^2=4.59 and 5.25, respectively, P=0.05).

Microsatellite markers amplified from DNA of all progenies generated by the parental all parental sets confirmed that they resulted from a sexual cross of the co-inoculated parental strains. Only the markers found in the parental isolates were found in the progeny, and they segregated in 1:1 ratios in all cases (χ^2 between 0 and 3.63, where P=0.05) (Figure 4 for examples). These results indicate that these populations underwent regular meiosis resulting in the observed Mendelian segregation for nuclear loci.

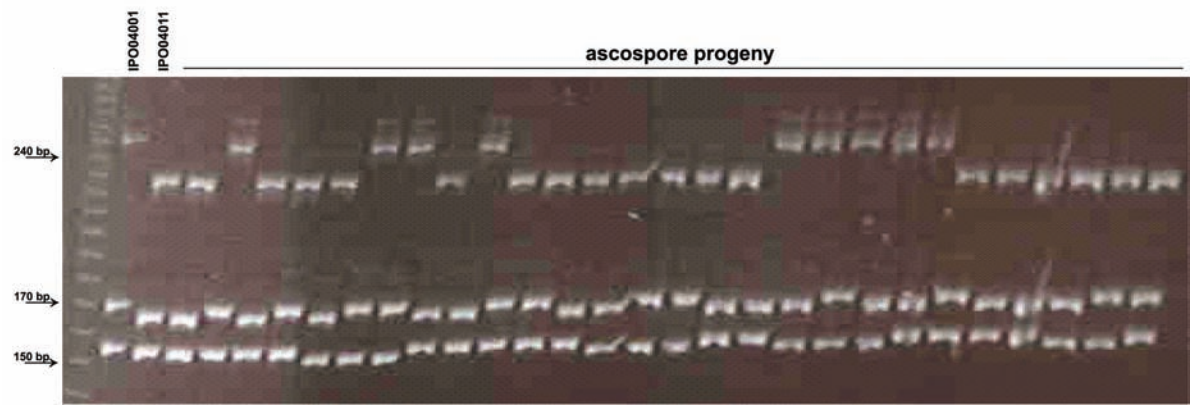

Figure 4. SSR genotypes of progeny from crosses between *Mycosphaerella graminicola* isolates IPO04001 and IPO04011 generated on wheat seedlings preventatively treated with Amistar™ in doses ranging from 0-200%. Multi-plexed PCRs using three differentiating primer sets revealed recombinant SSR patterns in progeny.

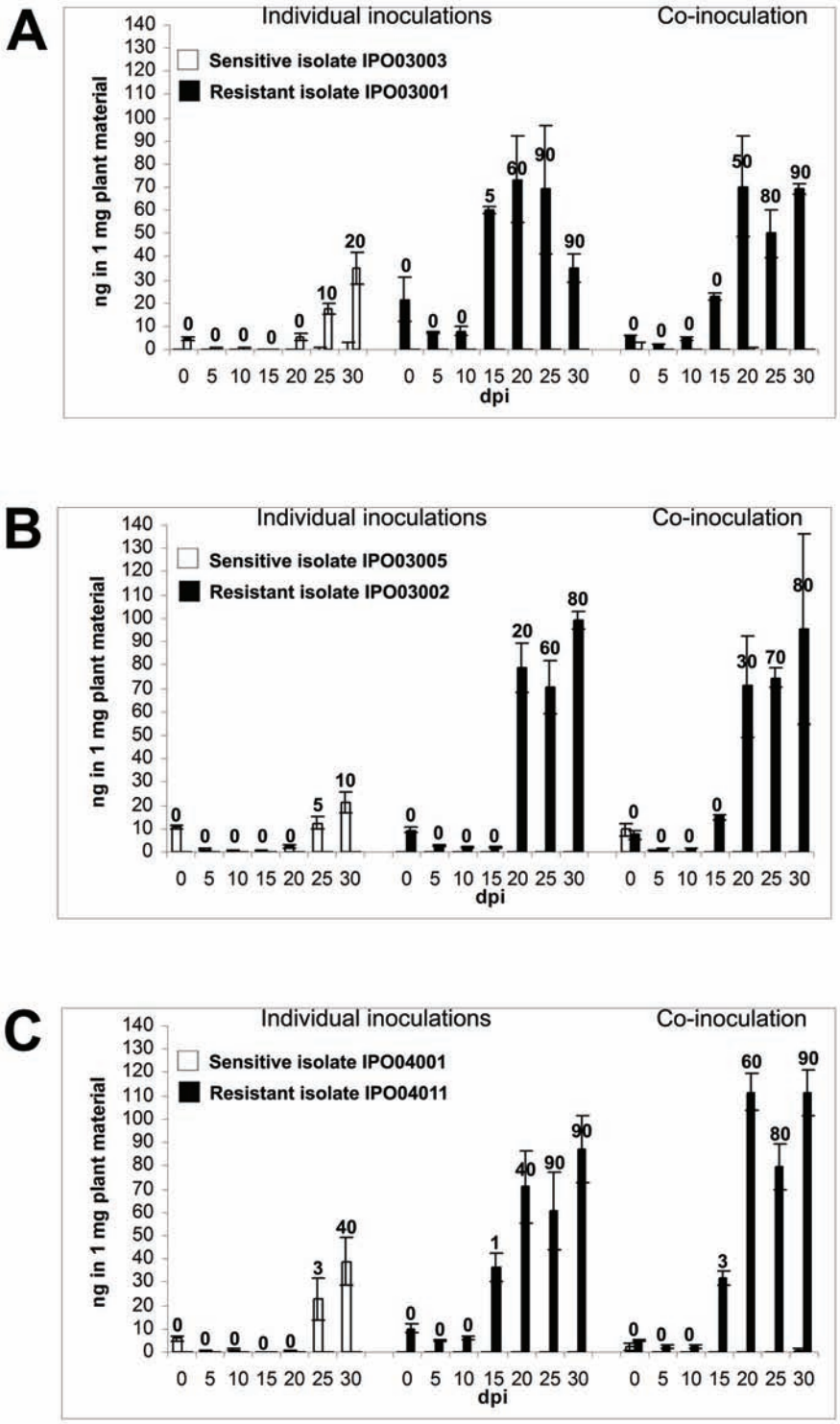

Figure 3. Fungal biomasses and percent pycnidia of parental isolates of *Mycosphaerella graminicola* inoculated individually and in mixtures on cv. Taichung 29 after preventative treatment with the full recommended field rate of azoxystrobin at 0, 5, 10, 15, 20, 25, and 30 dpi. Left to right: **A,** IPO03003 (sensitive), IPO03001 (resistant), and mixture of IPO03001 and IPO03003. **B,** IPO03005 (sensitive), IPO03002 (resistant), and mixture of IPO03002 and IPO03005. **C,** IPO04001 (sensitive), IPO04011 (resistant), and mixture of IPO04001 and IPO04011. Pycnidial percentages based on visual observations listed above each biomass quantification.

Furthermore, SSR genotyping of 12 pycnidial isolates recovered from preventively treated (48 h, 50 and 100% Amistar™) cv. Taichung 29 seedlings confirmed their identity with the inoculated isolates, indicating that sensitive isolates are able to survive and effectively colonize wheat leaves at half and full dose azoxystrobin treatments (Figure 5).

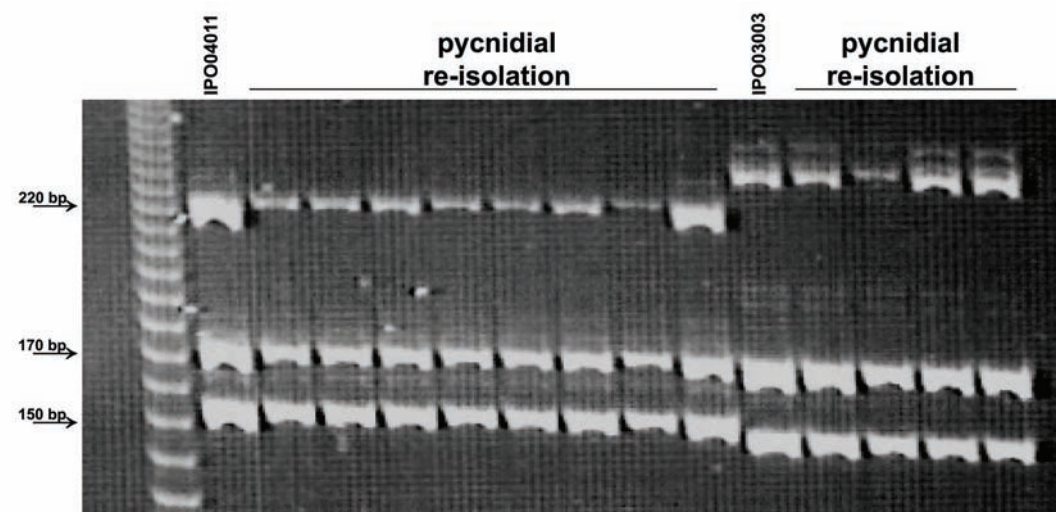

Figure 5. SSR genotypes of pycnidial isolates of *Mycosphaerella graminicola* recovered from wheat seedlings preventatively treated with Amistar™ at half and full doses. All SSR patterns are clonal like the sensitive pycnidial isolates IPO04001 or IPO03003. Multi-plexed PCRs using three differentiating primer sets revealed no recombinant SSR patterns in recovered pycnidial isolates.

DISCUSSION

The aim of this study was to investigate the role of sex in the rapid evolution and distribution of strobilurin resistance in *M. graminicola*. To our surprise, we observed that all crosses on Amistar™-treated wheat seedlings generated offspring, indicating that azoxystrobin in a wide variety of doses did not prevent sensitive strains from mating. Our data strongly suggest that strobilurin applications stimulate maternal preference in mating of the strobilurin-resistant isolate, or that, more likely, strobilurin pressures force the sensitive isolate to act as the paternal donor. This process appears to be the driving force behind the unusually rapid spread of resistance through Western European field populations. This is very plausible considering that *M. graminicola* undergoes multi-cyclic sexual propagations per season and that ascospores are airborne-dispersed. Hence, preferential mating significantly accelerates the distribution of resistance after an initial single nucleotide mutation event, which also seems to occur at high frequencies. This mechanism might also be responsible for

the rapid spread of strobilurin resistant isolates of *M. fijiensis* in Costa Rica (Knight et al. 2002; Marín et al. 2003).

In crosses between isolates of hermaphroditic fungi with bipolar, heterothallic mating systems (such as *M. graminicola*), successful sexual reproduction depends on the compatibility between the mating type idiomorphs (*mat1-1* and *mat1-2*), which is independent of the morphologically differentiated male and female gametangia that can be produced by isolates with either type of idiomorph. In the order of the Dothidiales (Schoch et al. 2006, in press) and hence in the genus *Mycosphaerella* (Crous 1998), male gametangia are spermagonia (producing spermatia) and the female gametangia are ascogonia. Thus, in heterothallic species, each strain has one mating-type idiomorph but produces both male and female gametangia and is self-sterile. Maternal inheritance in a population without selective pressure is expected to be 1:1, since each parental isolate has an equal chance to produce male and female structures and contact is considered to be a random process (Coenen et al. 1996; Robinson et al. 2002). This 1:1 segregation of mitochondrial genomes with the G143A mutation conveying strobilurin resistance was observed in progeny from resistant and sensitive isolates of *Blumeria graminis* f.sp. *tritici* crossed on untreated wheat detached leaves (Robinson et al. 2002). We assumed that susceptible wheat seedlings untreated with fungicide would provide such a control situation without selective pressure when inoculated with virulent isolates of *M. graminicola* having similar fitnesses. However, we did not generally observe the expected 1:1 inheritance of the mitochondrial genome even when isolates were crossed on untreated seedlings, and furthermore, the ratios of mitochondrial inheritances in progenies from the same crosses differed between different crossing dates. This indicates that the preferred maternal donor is already variable due to selective pressure by yet undetermined environmental factors independent of the host. Strong maternal preferences in control crosses on plants untreated with strobilurins have also been reported for resistant and sensitive isolates of the basidiomycete corn smut pathogen *Ustilago maydis* (Ziogas et al. 2002). We used 42 independent crosses on plants that were preventatively treated with different Amistar™ rates to prove that strobilurins cannot prevent sex and to uncover the parental contributions to the progenies.

Amistar™ treatments of 6-200% almost exclusively resulted in resistant progeny. We have two working hypotheses that explain these results. Consider that both male and female gametangia are produced by both resistant and sensitive parents. When the female ascogonia are produced by the resistant parent, fertilized ascogonia will exclusively contain resistant mitochondrial genomes, as will subsequently developing ascogenous hyphae, ascus mother

cells, and ascospores. In that case, successful fertilization depends on spermatia originating from the sensitive strain that apparently escape fungicide stress. However, when the female ascogonia are produced by the sensitive parent, fertilized ascogonia will exclusively contain sensitive mitochondrial genomes. In this case, post-fertilization growth stages such as growth of ascogenous hyphae, the formation of ascus mother cells, and formation of ascospores will be strongly impaired or fully inhibited by fungicide residues in the treated leaves. Indeed, the low number of strobilurin-sensitive ascospores found in progeny from leaves treated with rates of Amistar™ below the recommended rate can probably be ascribed to escapes from the toxic activity of Amistar™. In our study, most fertilizations occurred between ascogonia from the resistant isolate and spermatia from the sensitive isolate, resulting in progenies that almost exclusively contain the resistant mitochondrial genome. Hence, fungicide stress either selects against ascogonia from the sensitive strains or induces the production of spermatia.

From a biological point of view, it is remarkable that sexual crosses between the strobilurin-sensitive and -resistant isolates were observed at all, since biomass of the sensitive isolate was not found in mixed inoculations on leaves treated with the full rate of Amistar™ (Figure 3). These results indicate that minute, undetectable amounts of the sensitive isolate are sufficient to enable mating, and that stress of this isolate may even stimulate the production of male gametangia, hence favoring fertilization. Amistar™ treatments evidently can control disease development but do not preclude sex in *M. graminicola*.

These findings most likely explain why largely resistant progenies of *M. graminicola* have swept through the Western European wheat crops. Previous studies showed that 83% of the mitochondrial diversity in *M. graminicola* isolates originating from bread wheat is covered by only two mitochondrial haplotypes. Although it is unknown whether these haplotypes experience identical selection pressure from strobilurins, we hypothesize that the application of strobilurins has significantly contributed to the uniformity of the mitochondrial genome in the Western European *M. graminicola* population. Such an event is called a selective sweep, and might be similar to the one that was hypothesized on the basis of adaptation to bread wheat or durum wheat in this species (Zhan et al. 2004).

Resistance to strobilurins has been reported for at least 21 different fungal plant pathogens and has been remarkably unprecedented in speed of development and spread compared to other fungicides having targets in the mitochondrial respiratory chain, such as organotins and carboxamides that only developed resistance over 10 years after their commercial release (Giannopolitis 1978; Dirske et al. 1982; Felsenstein 1999). Cases of resistance to strobilurins in *Mycospharella* species have been reported at an explosive rate

(Fraaije et al. 2005; Sierotzki et al. 2000a, 2000b). Until now, there was no experimentally supported explanation as to why the incidence of the G143A strobilurin resistance mutation in these species has increased so rapidly and has spread so quickly over large geographical areas in Western Europe and the Carribean. A major cause for rapid resistance to strobilurins seems to include the high mutation frequency of the point mutations that convey the single-site resistance. However, neither this phenomenon nor the widespread use of strobilurin fungicides on a wide range of pathogens can explain the rapid distribution over vast areas. Recently, Fraaije et al. (2005) used ascospore trapping and quantitative PCRs to show a shift towards more resistant ascospores in *M. graminicola* in the air "immediately" following a curative strobilurin treatment but did not speculate on a potential mechanism. Through the utilization and analysis of progeny from sexual crosses using different methodologies for strobilurin-sensitivity determination and isolate identification, we have shown that maternal inheritance of strobilurin resistance in this hermaphroditic, bipolar, heterothallic fungus explains the rapid spread and frequency of strobilurin-resistant isolates of *M. graminicola* within Europe.

ACKNOWLEDGEMENTS

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CHAPTER 6

Discovery of a functional *Mycosphaerella* teleomorph in the presumed asexual barley pathogen *Septoria passerinii*

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Submitted for publication.

ABSTRACT

We studied the possibility of a teleomorph associated with the genotypically diverse barley septoria speckled leaf blotch (SSLB) pathogen, *Septoria passerinii*. A teleomorph in the genus *Mycosphaerella* had been predicted previously based on phylogenetic analyses. In experiments in the Netherlands and the United States, isolates with opposite mating types were co-inoculated onto susceptible barley cultivars, and leaves were monitored for the presence of sexual structures and for the discharge of ascospores. Characterization of a progeny population by both molecular and phenotypic analyses confirmed that a *Mycosphaerella* teleomorph of *S. passerinii* has been discovered nearly 130 years after the description of the anamorph. A previously unknown sexual cycle explains the high degree of genetic variation among isolates found in nature. The experimental identification of a predicted teleomorph for *S. passerinii* indicates that cryptic sexual cycles may be common for many other “asexual” fungi with high levels of genotypic diversity.

INTRODUCTION

Septoria passerinii Sacc. causes septoria speckled leaf blotch (SSLB) on barley (*Hordeum vulgare*) and was first discovered nearly 130 years ago in Italy (Passerini, 1879). Since then, SSLB has been reported around the globe in such areas as the Upper Midwest region of the United States, the Prairie Provinces of Canada, Northern Europe, Northern Africa, Western Asia, and Australia (Cunfer and Ueng, 1999; Mathre, 1997). Over the past decade, SSLB epidemics have increased in frequency, and SSLB has become one of the most important, albeit sporadic, foliar diseases of barley in the United States and in Canada (Mathre, 1997; Steffenson, 2003; Toubia-Rahme et al., 2003). Yield losses of up to 38% have been reported in Minnesota and North Dakota, with similar reports of losses up to 20% in Canada (Green and Bendelow, 1961; Toubia-Rahme and Steffenson, 1999). In addition to reductions in yield, SSLB can render the remaining barley grain unacceptable for malting due to reductions in both kernel size and amount of malt extract (Green and Bendelow, 1961).

Many barley cultivars are resistant to *S. passerinii* (Banttari et al., 1975; Buchannon, 1961; Green and Dickson, 1957; Koble et al., 1959; Rasmusson and Rogers, 1963; Toubia-Rahme and Steffenson, 2004). Green and Dickson (1957) reported that 50 of 126 *H. vulgare* cultivars tested were resistant to this pathogen, but these resistant cultivars were of low malting quality. Extensive breeding programs exist for barley, but there has been little attempt to incorporate resistance to *S. passerinii* into new cultivars (Toubia-Rahme and Steffenson, 2004). This is because breeding programs mainly focus on developing cultivars with high yields and high malting qualities and thus have used parents with little or no resistance to SSLB. Consequently, all of the commercially important cultivars for malt and feed in the Upper Midwest region of the United States grown over the past 25 years have been and still are highly susceptible to this pathogen, even though the major cultivars have changed throughout the years (Helm et al., 2001; Toubia-Rahme and Steffenson, 1999; Toubia-Rahme and Steffenson, 2004). Toubia-Rahme and Steffenson (2004) argued that because of the increasing importance of SSLB, there should be more invested in the development of cultivars that incorporate resistance to SSLB with high yield and malting quality characteristics. They reported that resistance could be found in cultivars from diverse geographical origins, such as North America, South America, Europe, North Africa, and East Asia.

Presently there is evidence of up to six genes controlling resistance to SSLB in barley (Buchannon, 1961; Metcalfe et al., 1970; Rasmusson and Rogers, 1963). These specific

resistance genes in the host suggest the presence of avirulence genes in the pathogen. However, such avirulence genes have not yet been identified in *S. passerinii*. Furthermore, formal genetic analysis of the pathogen is not possible due to the fact that only the imperfect stage has been reported (Cunfer and Ueng, 1999). Our previous work, however, provided lines of evidence suggesting the possibility of sexual recombination in this fungus. Despite the fact that *S. passerinii* was generally considered to be an asexual fungus (Cunfer and Ueng, 1999), we used heterologous mating-type probes from the wheat pathogen *Mycosphaerella graminicola* (Waalwijk et al., 2002) to clone the mating type genes of *S. passerinii* (Goodwin et al., 2003), based on a previously identified close phylogenetic relationship between these two species (Goodwin et al., 2001; Goodwin and Zismann, 2001). In addition, it was shown that both mating-type idiomorphs of *S. passerinii* were found commonly in natural populations on the same leaf among 22 isolates tested, suggesting that sexual recombination under field conditions was possible. This was further substantiated by combined isozyme and RAPD genotyping of these 22 isolates, which yielded 22 unique haplotypes, as expected for sexual, but not asexual, populations (Goodwin et al., 2003).

The purpose of this paper was to test the hypothesis that *S. passerinii* has a cryptic teleomorph in the genus *Mycosphaerella*. The relative ease of generating the predicted teleomorph of *S. passerinii*, which has not been noticed in nature over the past 120-plus years, has broad implications for mycology and indicates that many other fungi may be incorrectly classified as asexual.

MATERIALS AND METHODS

Isolates, crossing, and phenotyping procedures

Twelve isolates of *S. passerinii* and two isolates of *M. graminicola* were used in this study (Table 1). Crosses were made both in Wageningen, The Netherlands, and in West Lafayette, IN, USA. Inoculum preparation, inoculations, and crossing procedures were as described previously for *M. graminicola* by Kema et al. (1996c), except that spore suspensions were sprayed onto seedlings instead of using cotton. Environmental conditions for growing seedlings both before and after inoculation were as described previously (Kema et al., 1996a). Isolate combinations for crosses are listed in Table 2. *S. passerinii* crosses were made on 10-day-old seedlings of the barley cvs. Topper 33 and/ or Kindred. A cross between *S. passerinii* isolates with the same mating type was included as a negative control to

Table 1. Summary information about the isolates of *Septoria passerinii* and *Mycosphaerella graminicola* used in this study.

Species	Isolate	Collection location	Mating type
S. passerinii	P62[a]	North Dakota, USA	*mat 1-1*
	P63	North Dakota, USA	*mat 1-1*
	P64	North Dakota, USA	*mat 1-1*
	P65	North Dakota, USA	*mat 1-1*
	P66	North Dakota, USA	*mat 1-2*
	P67	North Dakota, USA	*mat 1-2*
	P68	North Dakota, USA	*mat 1-1*
	P71[b]	North Dakota, USA	*mat 1-1*
	P75	North Dakota, USA	*mat 1-1*
	P78	Minnesota, USA	*mat 1-2*
	P81	Minnesota, USA	*mat 1-2*
	P83[b]	North Dakota, USA	*mat 1-2*
M. graminicola	IPO323[b]	The Netherlands	*mat 1-1*
	IPO94269[b]	The Netherlands	*mat 1-2*

[a] The isolates of *S. passerinii* were as reported previously by Goodwin *et al*. (2003).
[b] Cultures of these isolates have been deposited into the collection of the Fungal Biodiversity Center (Centraalbureau voor Schimmelcultures) in Utrecht, the Netherlands.

Table 2. *In planta* crosses between and among isolates of *Septoria passerinii* and *Mycosphaerella graminicola*.

Isolates	Cultivars for crossing	Locations for crossing
P71 x P83	Topper 33 and Kindred	Wageningen and West Lafayette
P78 x P83	Topper 33	Wageningen
P62 x P81	Kindred	West Lafayette
P62 x P83	Kindred	West Lafayette
P62 x P81	Kindred	West Lafayette
P63 x P78	Kindred	West Lafayette
P64 x P81	Kindred	West Lafayette
P65 x P66	Kindred	West Lafayette
P68 x P67	Kindred	West Lafayette
P71 x P81	Kindred	West Lafayette
P71 x P83	Kindred	West Lafayette
P75 x P78	Kindred	West Lafayette
P63 x P67	Topper 33 and Kindred	Wageningen and West Lafayette
IPO323 x IPO94269	Taichung 29	Wageningen
P71 x IPO94269	Topper 33 and Taichung 29	Wageningen
IPO323 x P83	Topper 33 and Taichung 29	Wageningen

differentiate ascospores generated from environmental contaminants on barley from those generated by *S. passerinii*. *M. graminicola* test crosses were made on the wheat cv. Taichung 29 and served as a positive control for the crossing procedure, as a negative control to differentiate ascospores generated from environmental contaminants on wheat, and as a reference for diagnostic comparison of *M. graminicola* ascospores with those potentially produced by the *S. passerinii* teleomorph, since we speculated earlier that ascospores from these species were likely to be similar morphologically (Goodwin et al., 2003). Finally, we also performed interspecific crosses between *S. passerinii* and *M. graminicola* because of the suggested close phylogenetic relationship between these species (Goodwin and Zismann, 2001). Plants were placed on a rotating table in an inoculation cabinet, and spore suspensions (at concentrations of 10^7 per ml in a total of 30 ml, 15 ml per parental isolate) were sprayed until run-off. Incubations in Wageningen and West Lafayette were conducted as described by Kema et al. (1996c) and Adhikari et al. (2003), respectively. After symptoms developed during incubation in the greenhouse (22° C, >85% RH), seedlings were placed outside. Crosses were attempted seven times between September 2002 and May 2005. Leaf samples were collected once per week from 7-12 weeks after inoculation in the Netherlands and from 4-10 weeks in the U. S. for discharge of ascospores onto 2% water agar and for microscopical identification of the sexual structure. Proposed parental isolates and the resulting progeny were inoculated onto the susceptible barley cv. Topper 33 for phenotypic comparisons.

Comparative taxonomical analyses of discharged ascospores and of sexual structures

Because plants were outside for up to 10 weeks, there was a high probability for the development and discharge of ascospores of naturally occurring contaminant fungal species. In addition, it was impossible to know for certain what type of ascospores to expect because they had not been described previously. Therefore, all discharged ascospores were meticulously screened according to size, shape, number of cells, pigmentation, and germination pattern on 2% water agar. All non-*M. graminicola* ascospore types discharged from leaves inoculated with *M. graminicola*, as well as all ascospore types discharged from leaves that were co-inoculated with isolates of *S. passerinii* with the same mating type, were considered to be environmental contaminants. Examples of the different types of discharged ascospores were transferred as single spores to yeast-glucose broth (YGB) and then onto potato dextrose agar (PDA) for comparisons of growth with that of *S. passerinii*. Infected

leaf samples that were co-inoculated with isolates of *S. passerinii* with opposite mating types were also screened microscopically to find the associated sexual structure.

DNA extraction and analyses

In preparation for DNA extraction, isolates were grown in YGB for 10 days, at which time spores were pelletted and subsequently lyophilized. Total genomic DNA was extracted from 10 mg of lyophilized spores using the Puregene DNA isolation kit (Gentra System Inc., Minneapolis, MN, USA) and eluted with 50 µl of TE buffer (pH 8.0). All PCRs were performed in either an MJ PTC-200 Peltier (MJ Research, Watertown, MA, USA) or a Perkin-Elmer 9600 (Perkin Elmer, Foster City, CA, USA) thermal cycler. Primers and adapters used in this study are listed in Table 3.

Table 3. Primers and adapters for *Septoria passerinii* used in this study.

Name	Sequence (5' to 3')	Purpose
MT-F	CTTCTTGCCTGCGCCACAGG	*mat 1-1* and *mat 1-2* PCR
Alpha(1594)R	CGGTATGTGGATGGAAGAAAGG	*mat 1-1* PCR
HMG(849)R	TAGTCGGGACCTGAAGGAGTG	*mat 1-2* PCR
OPA-9	GGGTAACGCC	RAPD
*Eco*RI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTC	AFLP
*Mse*I adapter	GACGATGAGTCCTGAG TACTCAGGACTCAT	AFLP
E00	GACTGCGTACCAATTC	AFLP
M00	GATGAGTCCTGAGTAA	AFLP
E19	GACTGCGTACCAATTCGA	AFLP
M16	GATGAGTCCTGAGTAACC	AFLP
ITS4	TCCTCCGCTTATTGATATGC	ITS sequencing
ITS5	GGAAGTAAAAGTCGTAACAAGG	ITS sequencing

To confirm ascospores as progeny from *S. passerinii* crosses and to determine segregation ratios, parental isolates and presumed progeny were screened using mating-type PCR, Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Internal Transcribed Spacer (ITS) analysis. For the mating-type analysis, primers developed by Goodwin et al. (2003) were used. PCRs were done in 25-µl reactions, each containing 2.5 µl of 10 mM dNTPs, 2.5 µl of 10X PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 0.1 µl of 5U/µl AmpliTaq DNA polymerase (Applied Biosystems), 2.5 µl of 0.01% G-2500 Gelatin (Sigma), 1.33 µl each of 4 µM MT-F, Alpha(1594)R, and

HMG(849)R primers, 3 µl of 1 ng/µl target DNA, and 8.9 µl of sterile double-distilled (sdd) water. Thermal cycler conditions were as described previously (Goodwin et al., 2003), and the annealing temperature was 55°C. For the RAPD analysis, PCRs were done in 25-µl reactions, each containing 2.5 µl of 2 mM dNTPs, 2.5 µl of 10X PCR+ $MgCl_2$ buffer, 0.25 µl of 50 mM $MgCl_2$, 0.06 µl of 5U/µl Taq DNA polymerase (Roche), 2.5 µl of 10 ng/µl OPA9 primer (Operon Technologies), 1.5 µl of 0.5 ng/µl DNA, and 15.69 µl of sdd water. Cycling parameters were as described previously by Kema et al. (1996c). Amplicons from both RAPD and mating-type PCRs were run on 1.2% agarose gels for visualization. Fluorescent AFLP analysis was done according to the protocol described previously by Flier et al. (2003). DNA was digested using enzymes *Eco*RI and *Mse*I with primers E00 and M00 and then ligated with *Eco*RI and *Mse*I adapters. Primary amplification was with primers E00 and M00, while secondary amplification was with primers E19 (fluorescent, Cy5-labeled) and M16, each with two selective nucleotides. Amplified bands were viewed using ALFwin Evaluation software (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). For ITS sequencing, PCRs were done in 25-µl reactions, each containing 2.5 µl of 10 mM dNTPs, 2.5 µl of 10X Mango PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 1U/µl Mango Taq DNA polymerase (Bioline), 2.5 µl each of 2 µM primers ITS4 and ITS5, 1 µl of 10 ng/µl target DNA, and 12 µl of sdd water. Cycling parameters were as described previously by Goodwin and Zismann (2001). Sequencing was done with the ThermoSequenase fluorescence-labeled primer cycle sequencing kit on an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described previously (Goodwin and Zismann, 2001). Digestions of ITS regions were done with the enzyme *Sau*3AI as described previously (Goodwin and Zismann, 2001).

RESULTS

Comparative taxonomical analyses of discharged ascospores and of sexual structures

Routine test crosses between *M. graminicola* isolates IPO323 and IPO94269 that were used as positive controls for the crossing procedure discharged ascospores from eight through 12 weeks after inoculation. During weeks 11 and 12 (November 2002), we also identified a substantial number of two-celled ascospores (~80) from plants that were co-inoculated with *S. passerinii* isolates P71 and P83 that closely resembled those from *M. graminicola* in

morphology and early growth development. Ascospores of the two species were similar in their germination patterns (Figure 1). Initially, two germ tubes arose from the polar ends and grew parallel to the long axis of the spore. Additional germ tubes (1–2) arose at the ascospore septum and grew perpendicular to the long axis of the ascospore. Ascospores remained hyaline and did not develop additional septa during the initial phase of germination. We were able to isolate 17 of those as single-ascospore cultures for further analyses. Repeated attempts to cross *S. passerinii* resulted in a positive discharge of eight ascospores of the same type as mentioned above during May 2005 in West Lafayette, this time from cv. Kindred that was co-inoculated with *S. passerinii* isolates P63 and P67. One of these was isolated as a single-ascospore culture. The colonies developing from all 18 proposed progeny on PDA plates (Figure 2 for examples), as well as their morphology and growth rate in YGB cultures (not shown), were identical to those of the parental isolates.

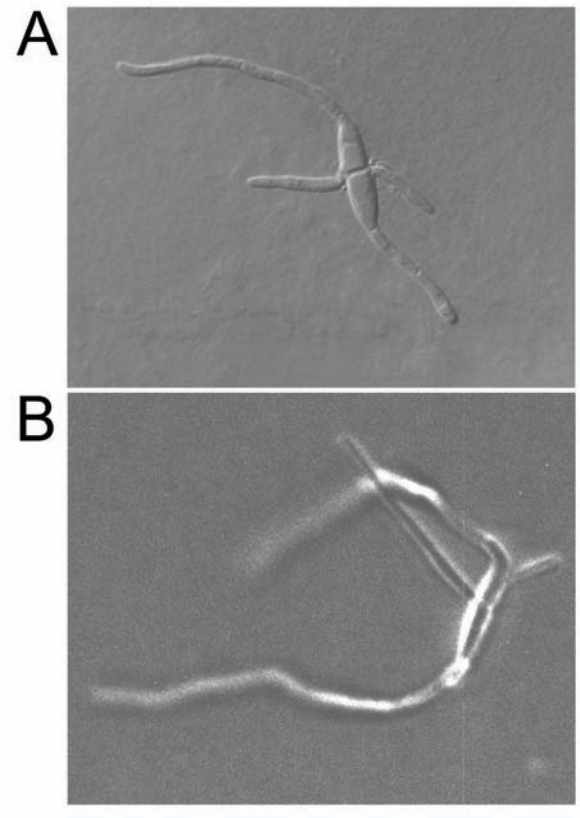

Figure 1. Microscopical comparison of the morphology of ascospores derived from crosses between isolates of *Mycosphaerella graminicola* and *Septoria passerinii*. **A,** Phase-contrast microscopical photograph of an ascospore of *M. graminicola* from a cross between isolates IPO323 and IPO94269 24 hours after discharge. **B,** Light microscopical photograph of an ascospore of the *Mycosphaerella* teleomorph of *S. passerinii* from a cross between isolates P71 and P83 15 hours after discharge.

In addition, numerous different types of ascospores were discharged from barley leaves that were co-inoculated with two *S. passerinii* isolates, including the control crosses between isolates of the same mating type, during this same time period. We monitored thousands of ascospore contaminants on barley, some of which could be identified. One species of *Didymella* with an *Ascochyta* anamorph, one species of *Leptosphaerulina*, and four species of *Paraphaeosphaeria* (including *P. michotii*) were isolated commonly. In addition, 2-celled ascospores of *Davidiella tassiana*, the teleomorph of *Cladosporium herbarum*, also were encountered regularly on older leaf material. Single-spore isolates from

a sampling of these contaminants did not show any similarity to *S. passerinii* in *in vitro* growth tests on PDA or in YGB (Figure 2).

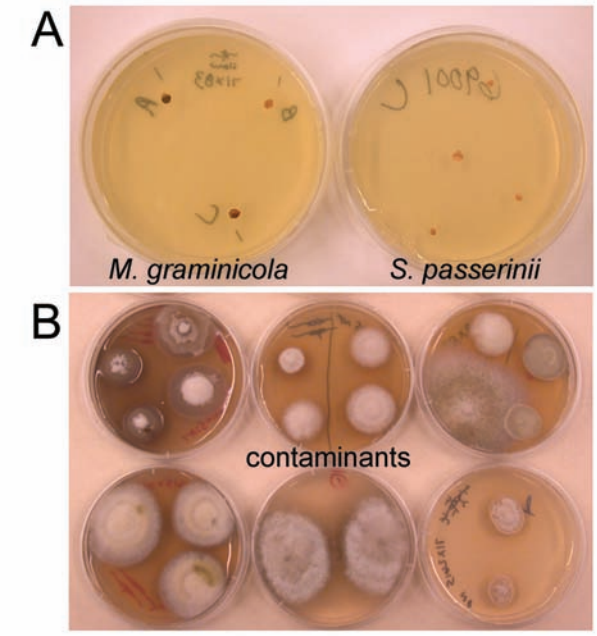

Figure 2. Growth of isolated single ascospores, which were discharged from leaves that were co-inoculated with two isolates with opposite mating types of either *Septoria passerinii* or *Mycosphaerella graminicola*, on potato dextrose agar. **A,** Left: Single-ascospore cultures of *M. graminicola* from an *in planta* cross between isolates IPO323 and IPO94269. Right: Single-ascospore cultures from the *Mycosphaerella* teleomorph generated by an *in planta* cross between *S. passerinii* isolates P71 and P83. **B,** Examples of single spore cultures from contaminant fungal species that discharged ascospores from the co-inoculated barley and wheat plants.

The interspecies crosses between *S. passerinii* and *M. graminicola* resulted in numerous ascospores (2 to 4 celled), but their growth on PDA and in YGB did not resemble that of either *S. passerinii* or *M. graminicola*. Subsequent RAPD characterization (data not shown) excluded them as interspecies hybrids, so they were considered to be contaminants.

Infected leaf samples inoculated with isolates of *S. passerinii* with opposite mating types from which *Mycosphaerella* ascospores were successfully harvested were examined microscopically to locate ripe ascomata. Despite numerous attempts over several years, only a single, partly decayed ascoma was found. Ascospores were observed to be hyaline, thin-walled, obovoid, and 10–15 × 3–4 µm. Due to the poor state of the material, the sexual stage could not be officially named, though it clearly resembled *M. graminicola* in genera morphology. We therefore propose that the *S. passerinii* teleomorph belongs to the genus *Mycosphaerella*, as is indicated by its DNA phylogeny.

Genotyping

The 17 proposed progeny from the cross between *S. passerinii* isolates P71 and P83 were genotyped based on mating-type PCR, RAPD and AFLP markers, and ITS analyses. The mating- type PCRs were positive and matched the expected 1:1 segregation ratio (*mat1-*

1:*mat1-2* = 10:7; χ^2=0.53; *P*=0.05) typical for an organism with a heterothallic, bipolar mating system (data not shown). Furthermore, the RAPD analysis showed that the majority of these progeny had recombinant genotypes based on just three markers (not shown). The parental isolates P71 and P83 had two and one unique bands, respectively, and had no additional bands in common. Three of the 17 proposed progeny isolates had the same genotype as P71, one isolate had the same genotype as P83, and the remaining 13 isolates showed recombinant patterns. The segregation ratios for the presence or absence of each parental band were 1:1, as expected for independent segregation (χ^2=0.059-0.529; *P*=0.05). None of the progeny had bands that were not present in isolates P71 and P83, indicating that these *S. passerinii* isolates were the parents of the collected offspring. Genotyping of the ascospore set using AFLP confirmed this conclusion (Figure 3). Results of the AFLP

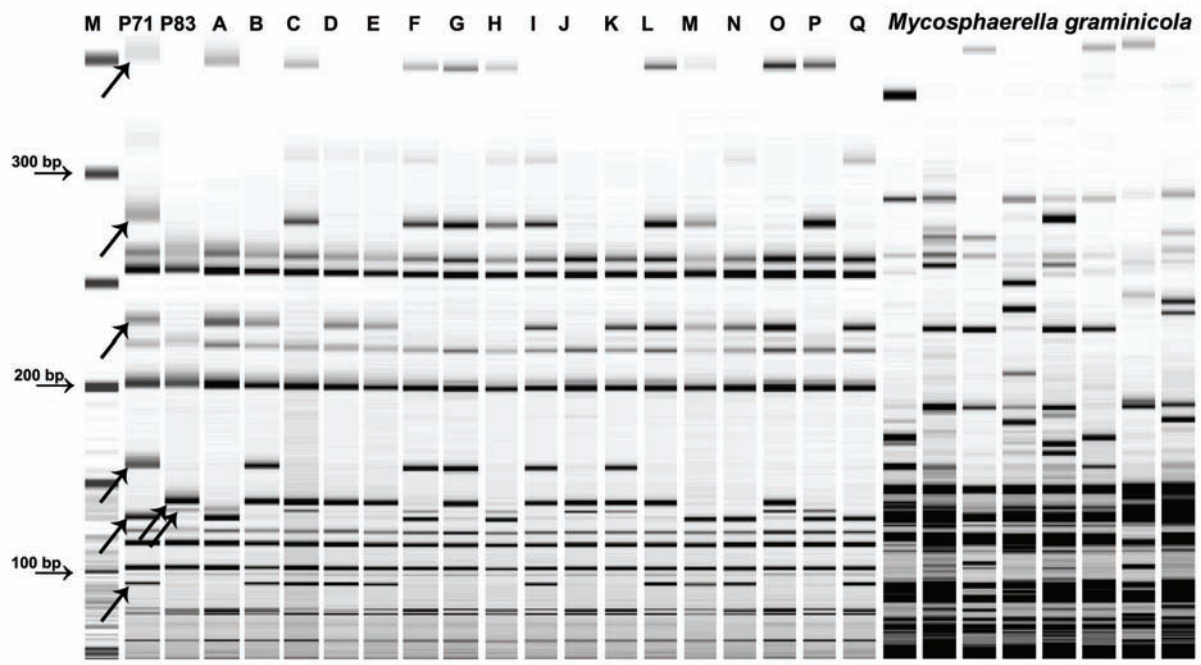

Figure 3. Genotypes of the parental *Septoria passerinii* isolates P71 and P83 compared to 17 ascospore progeny (isolates A through Q) and seven single-ascospore isolates of *Mycosphaerella graminicola* using AFLP markers. Primers *Eco*RI-GA and *Mse*I-CC were used for all isolates. Diagonal arrows indicate polymorphic bands between the *S. passerinii* parental isolates (six from P71 and two from P83). Horizontal arrows indicate reference size markers.

analysis with the primers *Eco*RI-GA and *Mse*I-CC showed that the parental isolates P71 and P83 had six and two unique bands, respectively, and had an additional 10 bands in common. All putative progeny isolates possessed these 10 shared bands and additionally displayed at

least two of the eight unique bands observed for the parental isolates P71 and/or P83. All 17 proposed progeny had recombinant genotypes except for one that had the same genotype as P83, but this one had a recombinant genotype in the RAPD analysis. None of the progeny had bands that were not present in the parents. For comparison, seven isolates of *M. graminicola* were included on the same polyacrylamide gel and using the same AFLP enzymes and primers. The vast majority of bands were not shared between the two species, and the AFLP patterns clearly distinguish *S. passerinii* from *M. graminicola*. There was at least one (at ~230 bp) and possibly more shared bands between *M. graminicola* and *S. passerinii*, which can be expected since these species are closely related, but bands having the same size do not necessarily have the same sequences.

To further distinguish the *S. passerinii* progeny from *M. graminicola*, the ITS region was digested with the enzyme *Sau*3AI. All *S. passerinii* progeny showed the same pattern as both of the parental isolates, P71 and P83 (not shown). This pattern was different than the pattern of *M. graminicola* isolates IPO323, IPO94269, and T48. In addition, the ITS regions of P71, P83, A, E, K, and M were cloned and sequenced, and sequences from all isolates were identical to one another and to archived sequences of several isolates of *S. passerinii* in a blastn search of Genbank. Isolates P71, P83, A, and E have been deposited into the culture collection of the Fungal Biodiversity Center (Centraalbureau voor Schimmelcultures) in Utrecht, the Netherlands. The one proposed progeny isolate from the cross between *S. passerinii* isolates P63 and P67 was characterized as *mat1-1*, and its ITS sequence was also identical to that of *S. passerinii*. This isolate must have been a progeny derived from isolates P63 and P67, because barley is not grown in central Indiana and *S. passerinii* has not been found on wild barley, so no source of natural inoculum exists.

Phenotyping

Plant inoculations confirmed the ability of the progeny isolates to infect barley. Inoculation of barley seedlings with spores from offspring from the cross between P71 and P83 caused the typical SSLB symptoms on barley (Figure 4) that began as small chlorotic flecks at 10 days after inoculation. These slowly developed into larger chlorotic blotches that eventually turned necrotic at ~17 days after inoculation. These lesions contained numerous pycnidia, the asexual fructifications that produce the slender multi-celled pycnidiospores typical for *S. passerinii*. In contrast, inoculations using *M. graminicola* on the barley cv. Topper 33 or *S. passerinii* on the wheat cv. Taichung 29 did not develop any symptoms, even after extended incubation periods (data not shown).

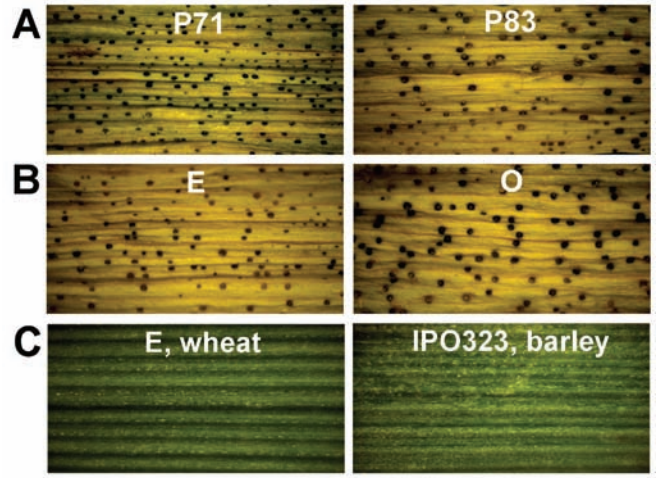

Figure 4. Symptom development of isolates of *Septoria passerinii* and *Mycosphaerella graminicola* on the barley cultivar Topper 33 or the wheat cultivar Obelisk at 21 dpi. **A,** *S. passerinii* parental isolates P71 and P83 on cv. Topper 33. **B,** *S. passerinii* progeny isolates E and O on cv. Topper 33. **C,** Negative controls. Left: *S. passerinii* progeny isolate E on cv. Obelisk. Right: *M. graminicola* isolate IPO323 on cv. Topper 33.

DISCUSSION

High genotypic diversity in natural populations, the identification of apparently intact mating-type genes, and the occurrence of both mating types within single leaves all led to the speculation that *S. passerinii* had the capacity for sexual recombination (Goodwin et al., 2003). However, there was no concrete proof of a functional teleomorph for this fungus that was hitherto considered to be asexual (Cunfer and Ueng, 1999). Therefore, we proceeded to test the hypothesis of a functional teleomorph by crossing isolates of *S. passerinii* with opposite mating types using the *in planta* protocol developed for the closely related sexual species *M. graminicola* (Kema et al., 1996c). This led to the generation of the teleomorph for *S. passerinii* both in Europe and in the United States.

Even though we have generated and characterized sexual progeny from two crosses of *S. passerinii* isolates, we cannot formally describe the sexual stage as required by the International Code of Botanical Nomenclature due to the lack of well-preserved teleomorph material. The identification of the sexual structure has been hampered by the necessity to place inoculated plants outside for approximately two months. Due in part to this, the vast majority of the ascospores discharged from the inoculated barley leaves did not originate from crosses of *S. passerinii* isolates but instead were contaminants from fungi in the environment. Likewise, the vast majority of sexual structures observed on leaves were not produced by crosses of *S. passerinii* isolates but rather by naturally occurring contaminant species. This complicated the localization of the very few ascomata generated by the teleomorph of *S. passerinii*. Furthermore, our observations suggest that the inconspicuous,

thin-walled, medium-brown ascomata degenerate quickly once ascospores are discharged, which could explain our difficulty in locating ripe ascomata on leaf sections known to harbor the teleomorph. Three species of *Mycosphaerella* have been described on *Hordeum* (barley), two on *Secale* (rye), and three on *Triticum* (wheat) (Corlett 1991), but the dimensions of their ascospores as well as their associated anamorphs indicate that they are distinct from the *Mycosphaerella* teleomorph of *S. passerinii*.

Recently, many presumably asexual fungi have been found to be sexual, such as: *Colletotrichum acutatum* (teleomorph *Glomerella acutata*), a pathogen of flowering plants (Guerber and Correll, 2001); *Phaeoacremonium aleophilum* (teleomorph *Togninia minima*), associated with Petri disease in grapevines (Mostert et al., 2006); and *Beauveria bassiana* (teleomorph *Cordyceps bassiana*), a widely used biological control agent against insects (Huang et al., 2002). Similarly, the identification of mating-type genes in *S. passerinii* has led to the current discovery of a cryptically active sexual cycle. However, mating-type genes have been identified in many other fungal species in which a sexual cycle has not yet been confirmed. One such example is the barley pathogen *Rhynchosporium secalis*. After a phylogenetic analysis showed that this pathogen probably has a teleomorph in the genus *Tapesia* (Goodwin, 2002), two groups cloned its mating-type genes using degenerate primers designed from sequences of *T. yallundae* and *Pyrenopeziza brassicae* (Foster and Fitt, 2003; Linde et al., 2003). Screening of natural populations of *R. secalis* revealed high genetic diversity and a 1:1 ratio for *mat1-1*: *mat1-2* in most populations sampled (Linde et al., 2003). Another example is *Fusarium oxysporum*, a well studied plant pathogen with a wide host range (Armstrong and Armstrong, 1981). Mating-type genes from *F. oxysporum* have been cloned by Arie et al. (2002). However, attempts to cross isolates of *F. oxysporum* with opposite mating types have not yielded sexual spores (S. Ware, unpublished), nor have these spores been found in nature, although high genotypic diversity in natural populations of *F. oxysporum* also suggests the possibility of a sexual cycle (Baayen et al., 2000; Bao et al., 2002). More recently, Paoletti et al. (2005) found evidence for sexuality in the opportunistic human pathogen *Aspergillus fumigatus*.

Almost certainly, many presumably asexual fungi are sexually recombining (see review by Taylor et al., 1999, for a parallel opinion with expanded arguments). In addition to the examples already given, a brief review of findings for the human pathogen *Cryptococcus neoformans* represents an excellent example of why the reproductive capabilities of fungi should not be underestimated. The anamorph *C. neoformans* was first described by Busse (1894) and was presumed to be asexual until the discovery of a bipolar heterothallic mating

system in 1976, which led to the naming of the teleomorph *Filobasidiella neoformans* (Kwon-Chung, 1976). Twenty years later, monokaryotic fruiting between isolates with the same mating type was reported in *C. neoformans*, but this type of reproduction was considered to be strictly mitotic and asexual based on descriptions in other fungi (Wickes et al., 1996). However, in 2005 this monokaryotic fruiting was proven to be a second sexual form of mating for this pathogen (Lin et al., 2005). Thus, major ideas on mating for *C. neoformans* have changed three times since the description of the anamorph, and even a completely new type of sexual reproduction in fungi has been discovered. Therefore, the possibility and even probability of sexual recombination for presumably asexual fungi cannot be excluded, as has been demonstrated in our study.

It is noteworthy that the success rate of crosses and the number of ascospores obtained from successful crosses are much lower for *S. passerinii* than for *M. graminicola*. Two explanations for our observed sporadic recombination are that either the sexual cycle is much less active in *S. passerinii* on barley than in *M. graminicola* on wheat, or that conditions of the crossing procedure for *M. graminicola* on wheat need to be adapted to meet the environmental requirements for formation of the teleomorph of *S. passerinii* on barley. Thus far, we do not have an indication of what these environmental requirements are, especially since ascospores were harvested from the two successful crosses during cold and wet conditions in Europe (November 2002) and during warm and dry conditions in the United States (May 2005). Other crossing procedures have been attempted for both *S. passerinii* and *M. graminicola*, including leaving the inoculated plants in the greenhouse instead of placing them outside, following the *in vitro* crossing method used for *M. citri* (Mondal et al., 2004), and others. However, only the protocol developed by Kema et al. (1996c) resulted in ascospore production in both species.

The discovery of a functional sexual cycle for *S. passerinii* has potentially important consequences for future study of this pathogen as well as for resistance breeding efforts in the host. In a comparison between *S. passerinii* and *M. graminicola*, the time lapse between the description of the anamorph and the discovery of the corresponding teleomorph is similar (127 and 130 years, respectively). *S. tritici*, the anamorph of *M. graminicola*, was first reported in 1842. The teleomorph was discovered in 1894, but it was not linked to *S. tritici* until 1972 (Sanderson, 1972). Once this link was made, the emphasis of research efforts extended from epidemiological studies (Royle and Shaw, 1986; Shaw and Royle, 1993) to studies on population genetics (McDonald et al., 1995; McDonald et al., 1999) and host-pathogen interactions (Kema et al., 1996a,b; Kema et al., 2000). The development of fungal

genetics in *M. graminicola* (Kema et al., 1996c) had an important impact on the identification of resistance genes in wheat (Brading et al., 2002). To date, at least 12 resistance genes have been identified that are currently being used in practical breeding programs (Chartrain et al., 2005). In this study, we have identified the existence of the sexual stage of *S. passerinii* and report a crossing protocol that potentially can, with some adaptation, be used to generate a mapping population of *S. passerinii* progeny to study genetics of avirulence on barley. We hypothesize that this will substantially benefit resistance breeding in barley to this economically important pathogen.

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CHAPTER 7

General discussion

Introduction

Co-evolution occurs when a pathogen has a long history of being pathogenic on a particular host. As a result, gene-for-gene interactions develop between the host and pathogen in a mutually dynamic evolutionary process in which a virulence trait of a pathogenic species evolves in response to a resistance trait in the host species, and *vice versa* (Janzen 1980). Population genetics studies deal with the processes that govern the evolution of pathogen populations which are influenced by mutation, natural selection, genetic drift, gene flow, and mating systems. These factors influence biological adaptation, which is an aspect of a pathogen that has evolved over a period of time by the process of natural selection such that it increases the expected long-term reproductive success of the organism.

Research presented in this thesis mainly focuses on genetics in the wheat pathogen *Mycosphaerella graminicola* in order to better understand host-pathogen interactions, genome organization, fungicide resistance, and the role of sexual recombination in the pathogen for host adaptation and pathogen survival. Aspects of the findings of this thesis are also addressed in relation to durable resistance in this pathosystem.

Population genetics in *M. graminicola*: expected vs. observed pathogen risk

According to mathematical modeling, pathogens that pose the greatest risk for circumventing resistance genes have a mixed reproduction system (asexual and sexual), a high potential for genotype flow, large population sizes, and high mutation rates (McDonald and Linde 2002). Based on such a mathematical model, the predicted theoretical risk for *M. graminicola* is a rating of 9 on a scale from 1 to 10. Although *M. graminicola* causes major disease of wheat, the observed risk rating is only 5 (McDonald and Linde 2002). In the following discussion, we propose some potential reasons that could account for the major discrepancy in expected and observed risk of *M. graminicola*.

Concepts of non-hosts and host specificity related to *M. graminicola*

Non-host resistance exists when all members of a plant species exhibit resistance to all members of a given pathogen species (Heath 1985, Thordal-Christensen 2003). Isolates of *M. graminicola* can cause disease on cultivars of both bread wheat and durum wheat, so accordingly bread wheat and durum wheat species cannot be considered non-hosts in this respect. Nevertheless, host specificity has been repeatedly demonstrated among isolates of

M. graminicola; isolates originating from bread wheat and durum wheat cultivars do not produce pycnidia in durum wheat and bread wheat cultivars, respectively.

Therefore, as no asexual propagation via pycnidiospore production will occur, it would seem that the life cycle cannot be completed and hence, in such a case it is assumed that genes of the avirulent isolate will be removed from the gene pool (van der Plank 1982; Zadoks and Schein 1979). However, in this thesis we show that although disease development and asexual clonal propagation are indeed prevented, sexual reproduction occurs. Hence, avirulence genes, as well as all other genes in the avirulent isolate are passed on to next generations. We generated a mapping population of progeny from a bread wheat-derived and durum wheat-derived isolate on both bread wheat and durum wheat cultivars. Progeny obtained from either host species produced pycnidia on bread wheat cultivars, durum wheat cultivars, both, or none, which is surprising, because in natural populations host species specificity is observed. Nevertheless, host species specificity for either bread wheat or durum wheat as defined by the necessity of one or the other species to complete a life cycle is not valid according to the current work, because the life cycle can be completed on both wheat species via sexual crossing and generation of ascospore progeny. Figure 1 shows a schematic representation of the aforementioned conventional versus proven situation in the *M. graminicola*-wheat pathosystem. Moreover, our analyses showed that the *MgAvrStb6* locus conferred avirulence to both the bread wheat cv. Shafir and all three durum wheat cultivars tested, which further contradicts the idea of host species specificity in *M. graminicola*.

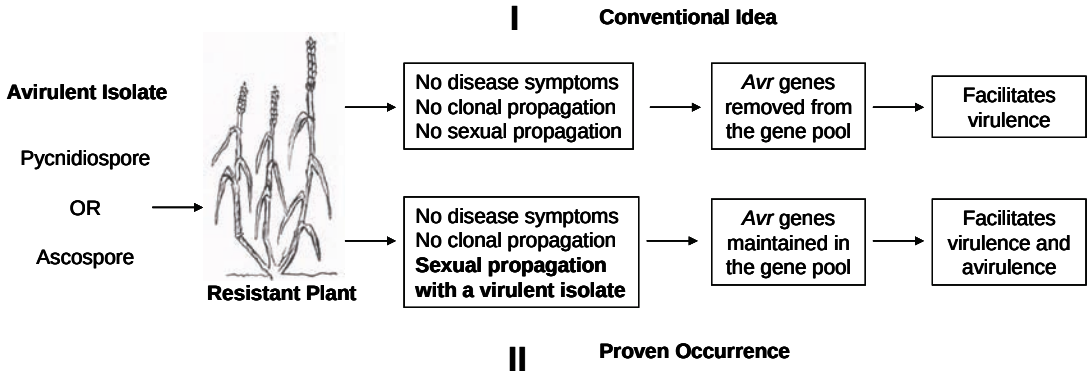

Figure 1. Schematic representation of a conventional model explaining what happens when an avirulent isolate lands on a resistant host (I) versus what is proven to occur in the *M. graminicola*-wheat pathosystem (II).

Absence of boom-and-bust in the gene-for-gene *M. graminicola*-wheat pathosystem

In some pathosystems, employment of resistant cultivars seems rather ineffective, as resistant cultivars decrease in effectiveness within a few years after release. Boom-and-bust cycles leading to circumvention of resistance have been particularly well-documented in cereals infected by rusts (Kolmer 1996, McIntosh and Brown 1997; Person 1957) and powdery mildew pathogens (Brown et al. 1997, Wolfe and McDermott 1994). However, boom-and-bust cycles seem not to occur regularly for the *M. graminicola*-wheat pathosystem, as breakdown of resistance in wheat cultivars is generally unreported (Johnson 1992; Kema et al. 1996a, 1999), except for the bread wheat cv. Gene planted in Oregon (Cowger et al. 2000).

Boom-and-bust cycles are usually associated with hosts and pathogens with gene-for-gene interactions. We have identified nine loci conferring (a)virulence in *M. graminicola* (eight in this thesis), of which *MgAvrStb6* was proven to be involved in a gene-for-gene interaction with the corresponding resistance gene *Stb6* that was mapped on the wheat chromosome 3A (Brading et al. 2002; Kema et al. 2000, 2002, this thesis). It is an interesting question why the classical boom-and-bust cycles are not obvious in this pathosystem.

Evolutionary adaptation and durable resistance in wheat to *M. graminicola*

Evolutionary adaptation of an organism evolves over a period of time by the process of natural selection that increases the long-term reproductive success of the organism. Half of our mapping population from the cross between IPO323 and IPO95052 on cvs. Obelisk and Inbar caused less than 9% leaf area covered by pycnidia when tested on 7 cultivars of bread wheat and durum wheat. Furthermore, only five individuals out of 163 progeny from this cross were able to produce more than 9% leaf area covered by pycnidia when tested on cv. Obelisk, even though the parent IPO323 was highly virulent on cv. Obelisk. One conclusion could be that the reproductive success of this cross was low. However, *M. graminicola* has co-evolved with both species and shows adaptation to remain viable when avirulent to pass on mixed, complex patterns of virulence and avirulence genes to sexual offspring to ensure long-term reproductive success of the pathogen species without depleting the host species. Although few progeny could infect cv. Obelisk, a greater proportion could infect other bread wheat and durum wheat cultivars. Furthermore, although both IPO323 and IPO95052 are avirulent on cv. Shafir, recombination on two other cultivars yielded some progeny that were highly virulent on cv. Shafir. Because avirulence genes are maintained in the gene pool as a result of its sexual behavior, *M. graminicola* favors durability of resistant

cultivars. This might explain why boom-and-bust cycles are not prevalent in the *M. graminicola*-wheat pathosystem and why the observed risk of *M. graminiciola* to overcome resistance is lower than expected.

Increased evidence for the importance of the sexual cycle in *M. graminicola*

The importance of the sexual cycle in *M. graminicola* has been widely acknowledged for over a decade (Chen and McDonald 1996; Fraaije et al. 2005; Hunter et al. 1999; Kema et al. 1996b, Shaw and Royle 1989; Zhan et al. 2003). Regular recombination yields ascospores that contribute to the high genetic diversity found in *M. gramincola* populations throughout the world (Zhan et al. 2003). Several sexual cycles can occur within a single growing season, and the ascospores generated can even serve as an inoculum to spread epidemics during the same growth season (Hunter 1999; Kema et al. 1996b; Zhan et al. 2003). In addition, ascospores have been implicated in the spread of strobilurin resistance (Fraaije et al. 2005), although no mechanism for this phenomenon had been proposed.

Results of our current work further emphasize the importance of the sexual cycle of *M. graminicola*. We found that a sexual cycle can occur under very stressful conditions. Progeny can be generated (i) when one parent is avirulent on a resistant host and no significant increase in biomass of the avirulent parent is observed, and (ii) after treatment of a plant with 200% of the full recommended dose of strobilurin (azoxystrobin, Amistar™) for which one parent is sensitive. We demonstrated that nuclear avirulence genes from avirulent isolates can be passed on in normal Mendelian segregation ratios through sexual progeny, whereas these would not be passed on without the sexual cycle. In addition, we found that mitochondrial genomes were not inherited equally from both parental isolates and that the inheritance was influenced by interactions between specific parental pairs, environmental factors (crossing date), the cultivar on which the cross was made, and the application of fungicide (strobilurin). Preference for being the paternal donor increased under the stressed conditions of being an avirulent isolate crossed on a resistant cultivar and being a strobilurin-sensitive isolate crossed in the presence of azoxystrobin. In the latter situation, the strobilurin resistance mutation was passed on to progeny along with recombined nuclear genes from the strobilurin-sensitive parent, which would likely not occur without the sexual cycle.

Many question still remain to be solved, including (i) how is biomass production on a resistant cultivar achieved in the absence of HR?, (ii) can sexual progeny be achieved on resistant cultivars also for other pathosystems?, (iii) why is host species specificity observed in natural populations of *M. graminicola,* whereas the bread wheat-derived and durum wheat-

derived isolates easily produce sexual progeny, some of which infect both species?, (v) what causes an isolate to act as the maternal donor OR what causes an isolate to act as the paternal donor?

Discovery of a sexual cycle for *Septoria passerinii*

In our current studies, we have proven for the first time that there is an active sexual cycle in the barley pathogen *S. passerinii* by generating and characterizing sexual ascospore progeny *in planta*. This work opens the door to the generation of mapping populations and genetic studies in *S. passerinii* towards effective breeding of resistant barley cultivars that are currently lacking for the malting industry. In a broader perspective, the discovery of this sexual cycle nearly 130 years after the first description of the anamorph underscores that much remains to be discovered concerning the mating capacities of fungi.

Concluding remarks

Progeny from successful crosses on resistant hosts will facilitate the identification of avirulence genes, which is the subject of future studies. Furthermore, including the sexual cycle in disease forecast models will help to explain the complex population structure of the fungus in the field and assessing the importance of both sexual and asexual spores in the epidemiology of *M. graminicola.*

M. graminicola is a fungus that is extraordinarily amenable to genetic studies. The ease of inducing successful crosses even under unexpected conditions belies the complexity of interpreting the results of such crosses. However, it underscores the complex ways in which the sexual cycle contributes to the overall success of *M. graminicola* on wheat.

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APPENDICES

Genetic Linkage Map

Summary in English

Summary in Dutch

Acknowledgements

About the author

List of publications

Education certificate of the EPS graduate school

Color figures

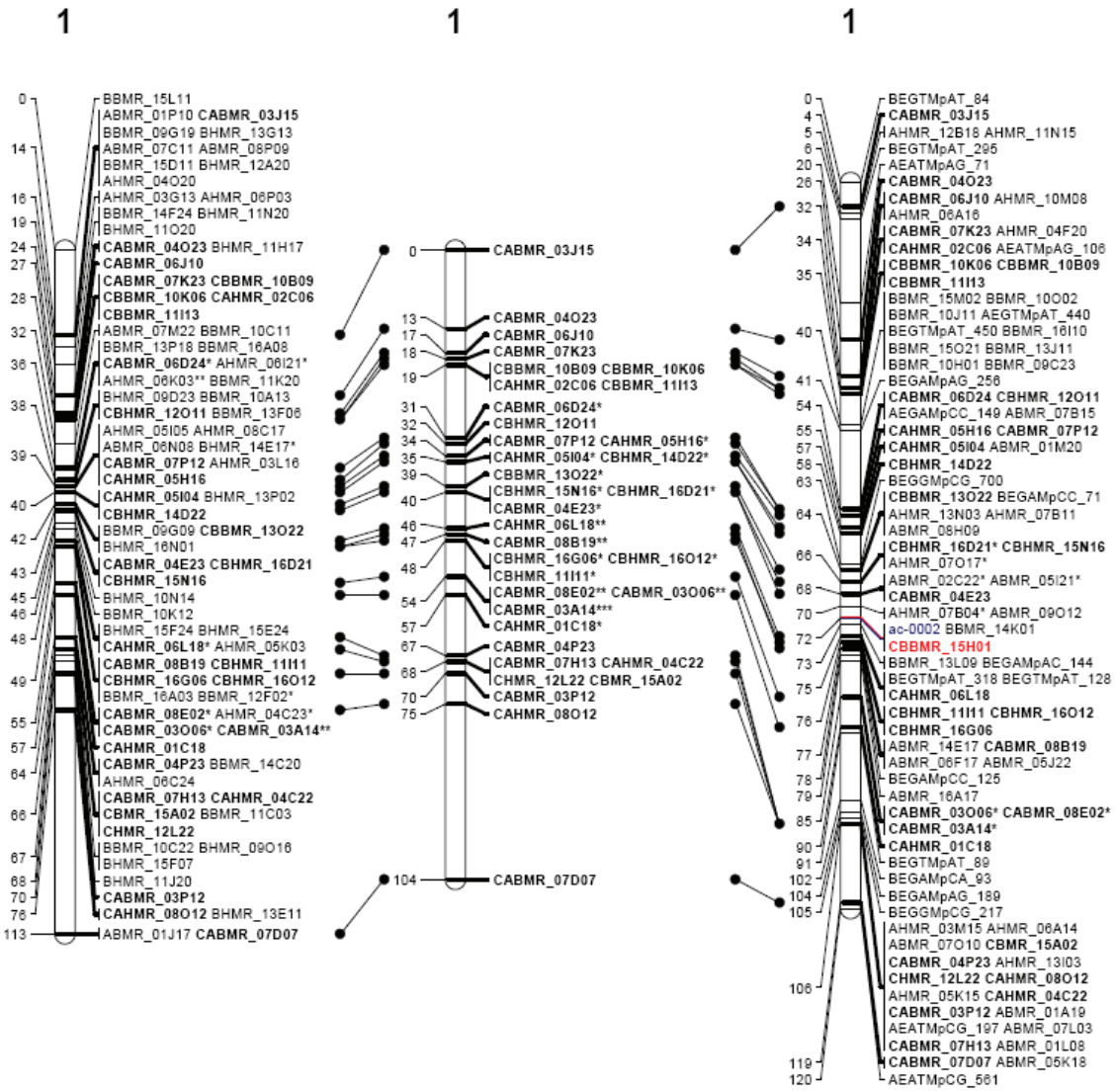
Genetic linkage maps generated primarily with

Diversity Arrays Technology

Left: linkage groups from segregations of markers in progeny of the bread wheat-derived isolate IPO323 and the durum wheat-derived isolate IPO95052.

Right: linkage groups from segregations of markers in progeny of the bread wheat-derived isolates IPO323 and IPO94269.

Middle: linkage groups in bridge map generated with shared segregating markers from both crosses; integration made possible by the common shared parental isolate IPO323.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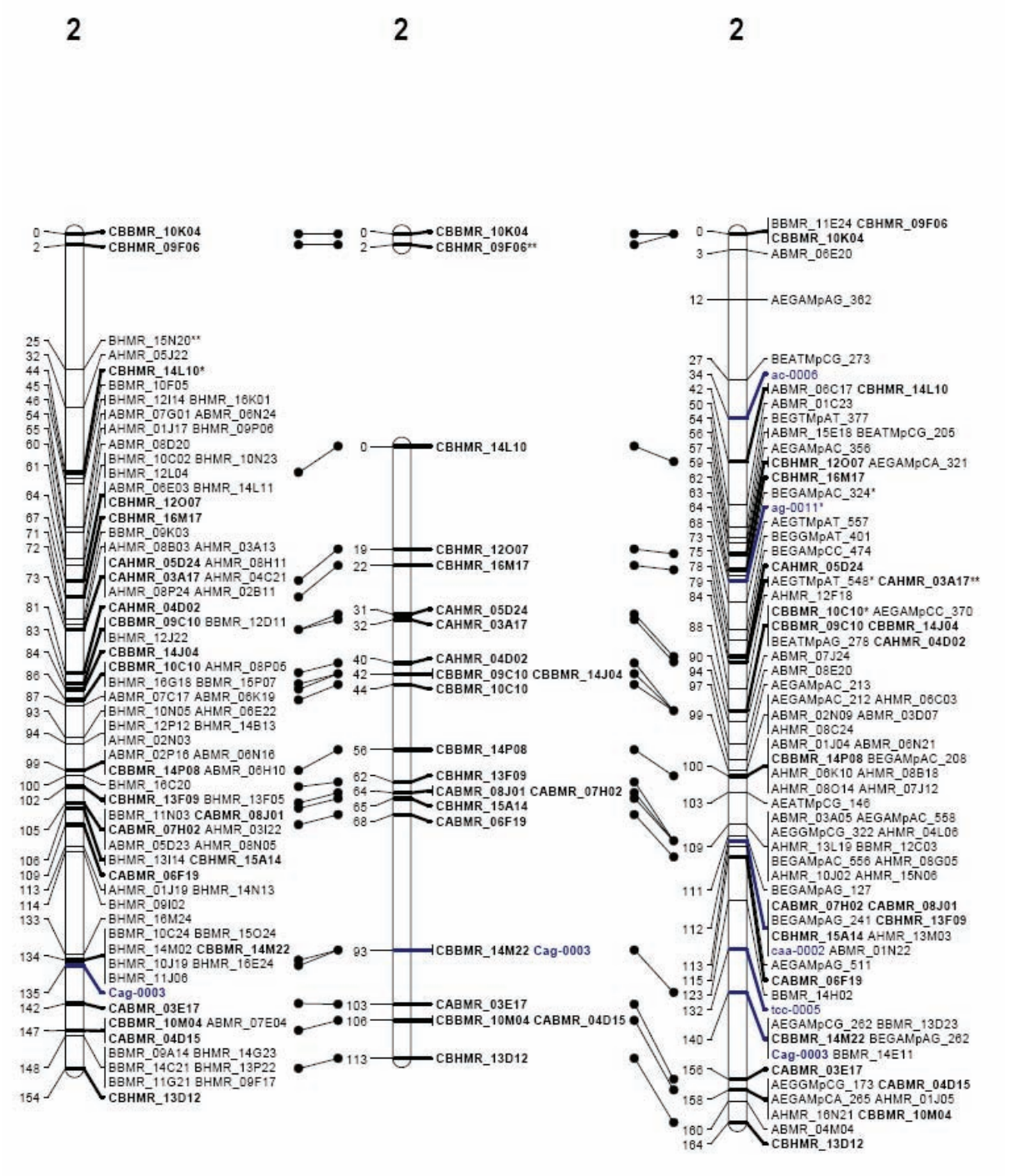
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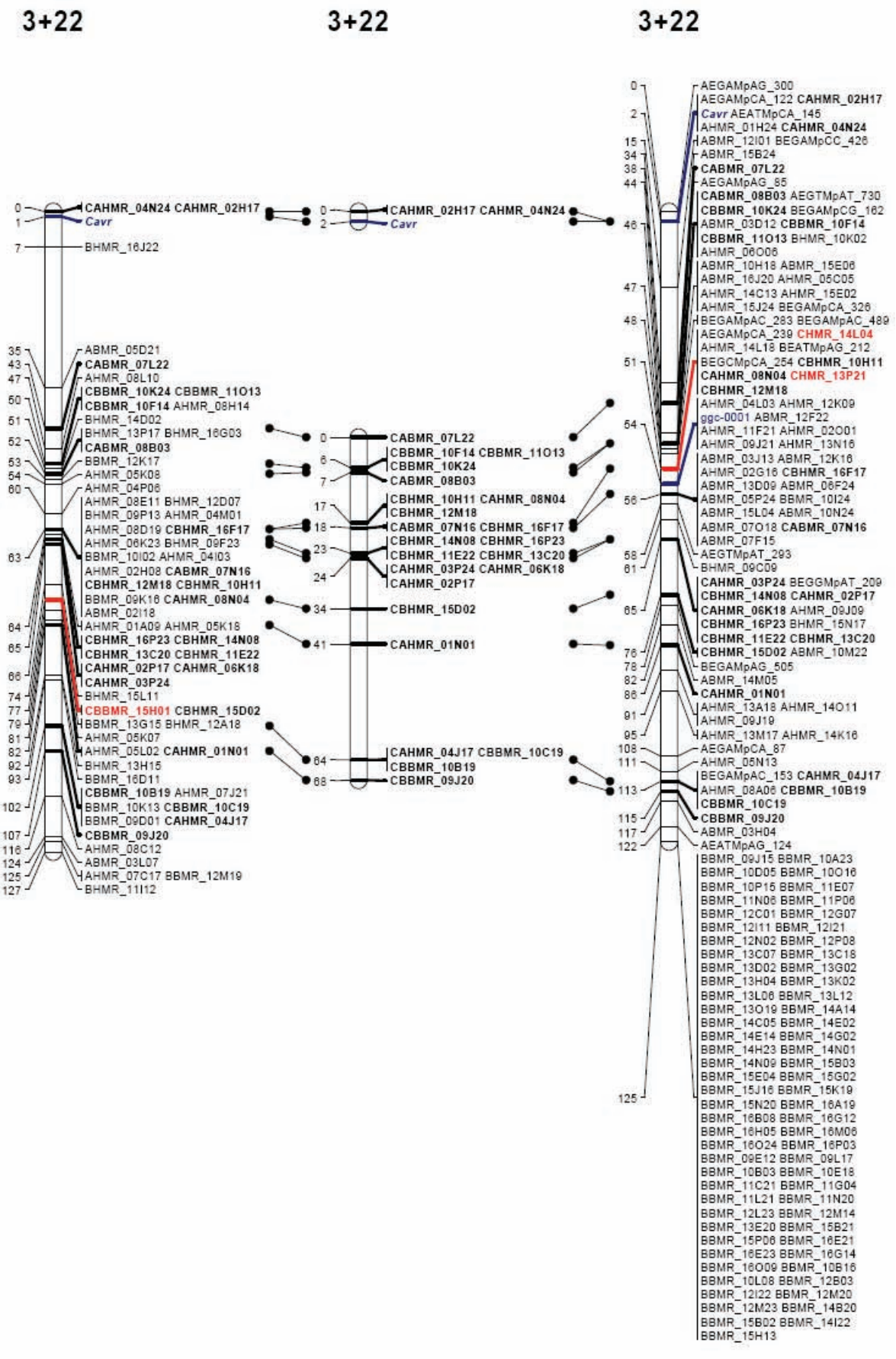
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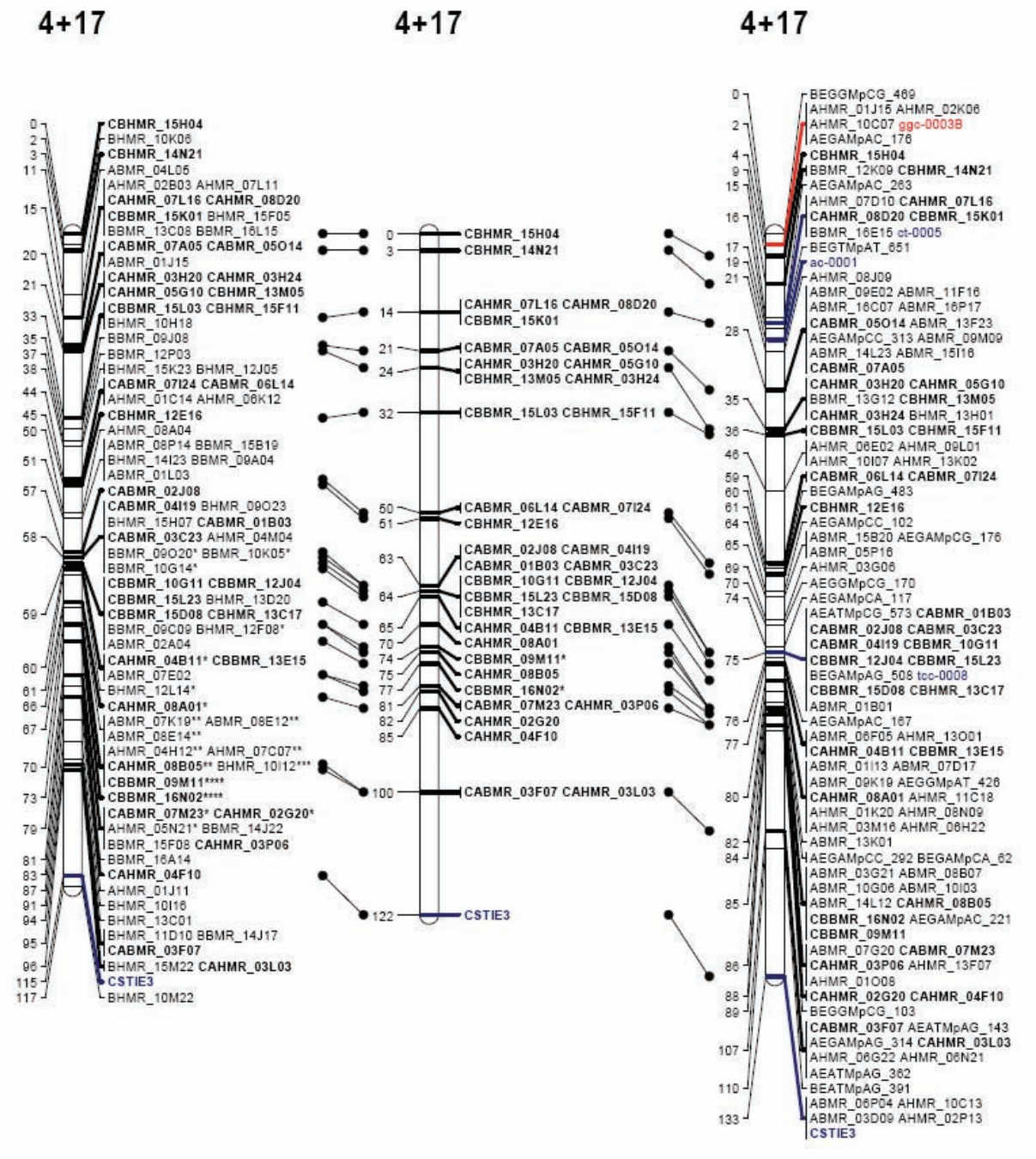
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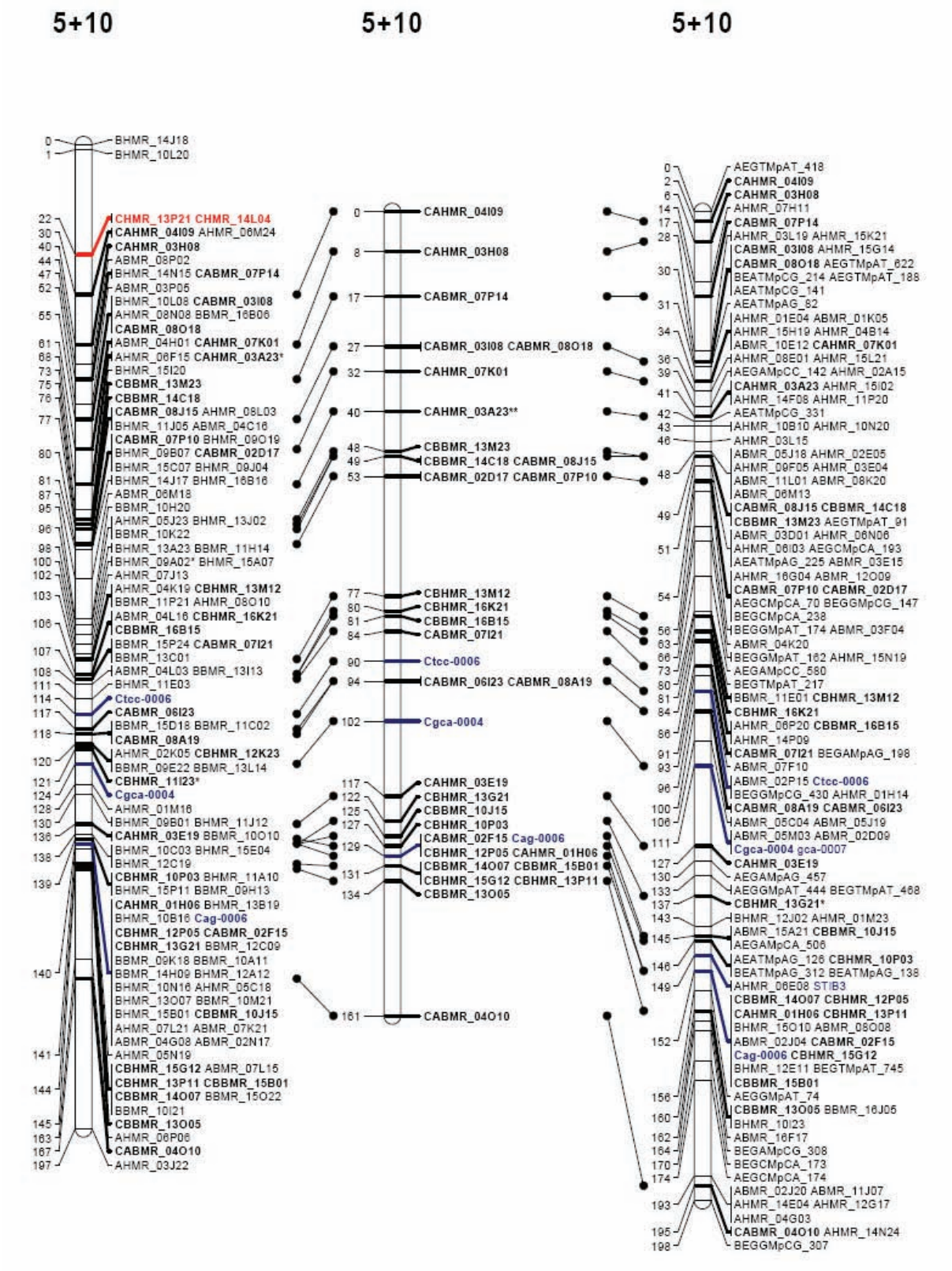
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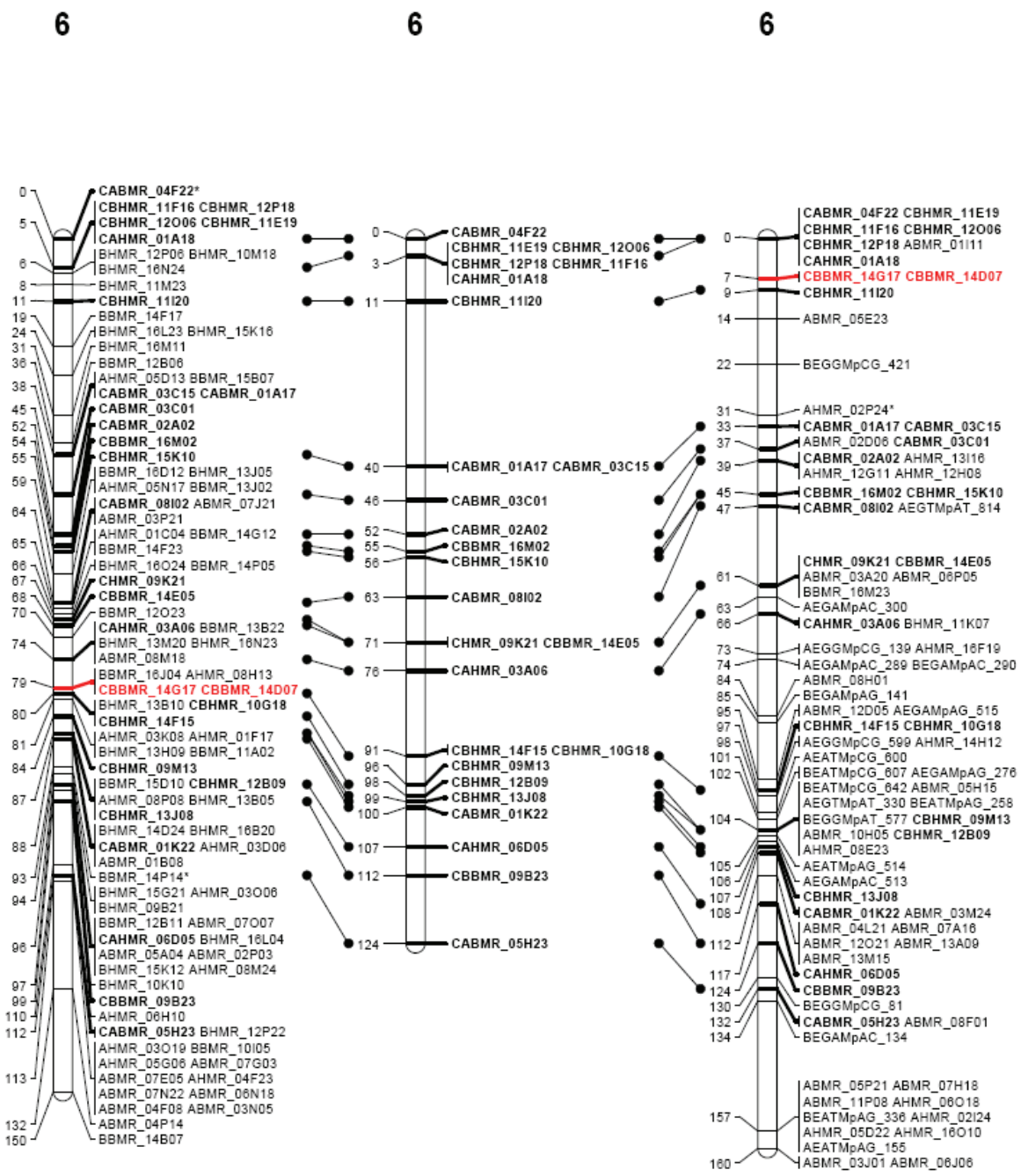
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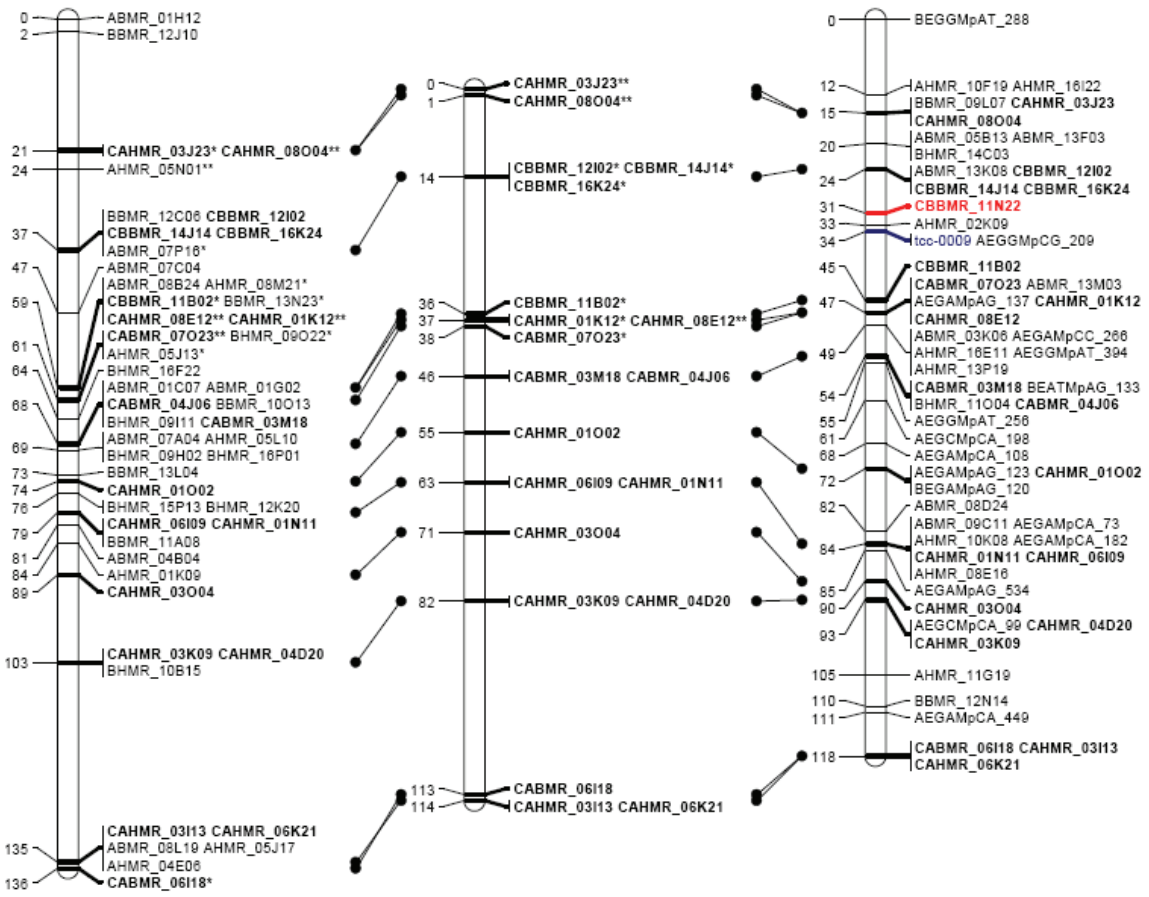
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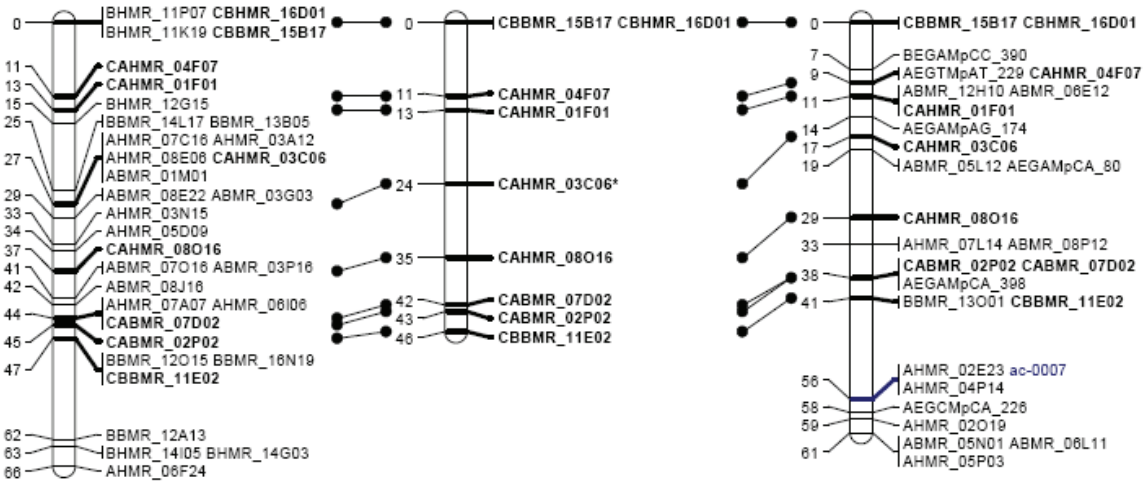
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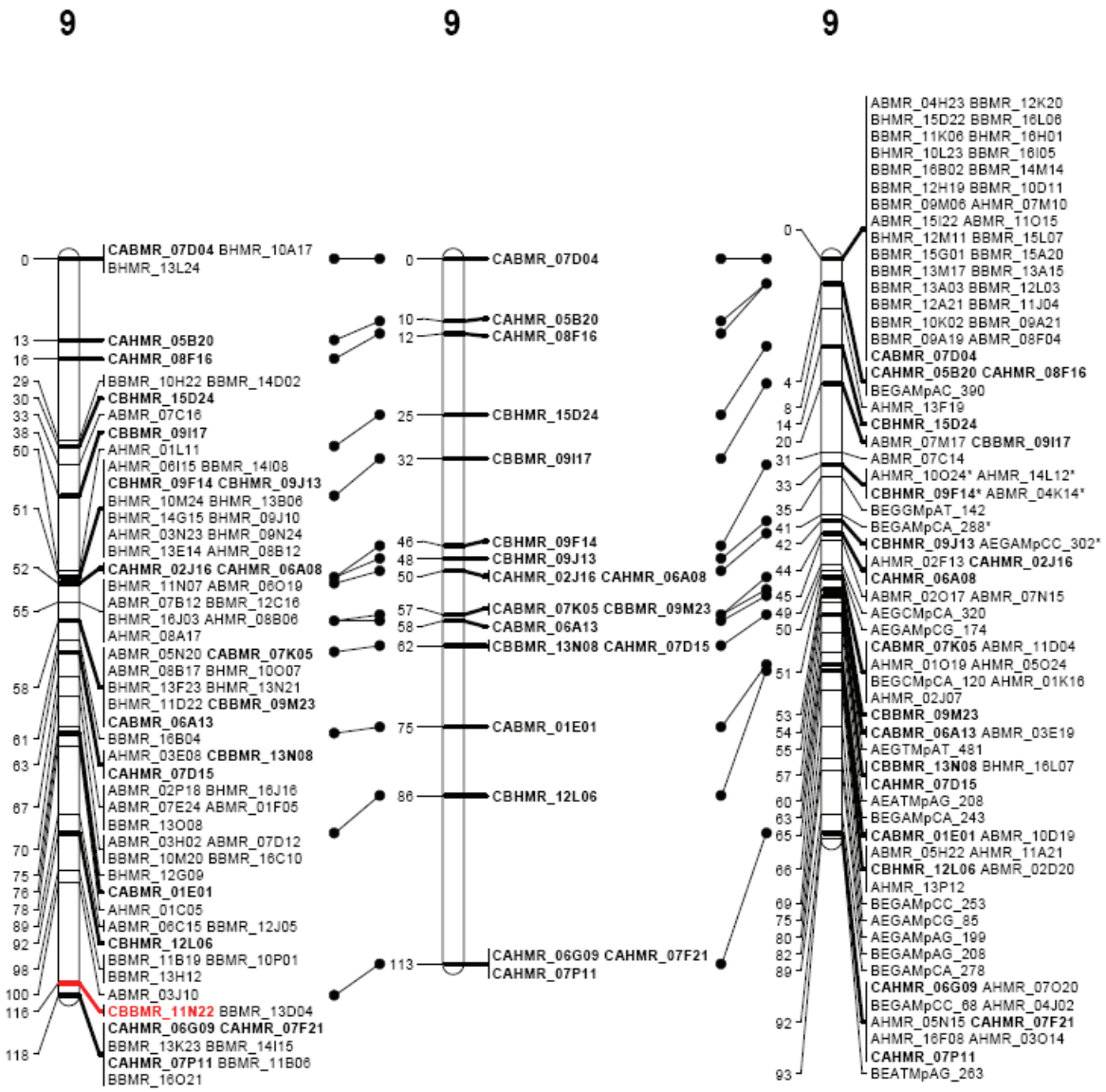
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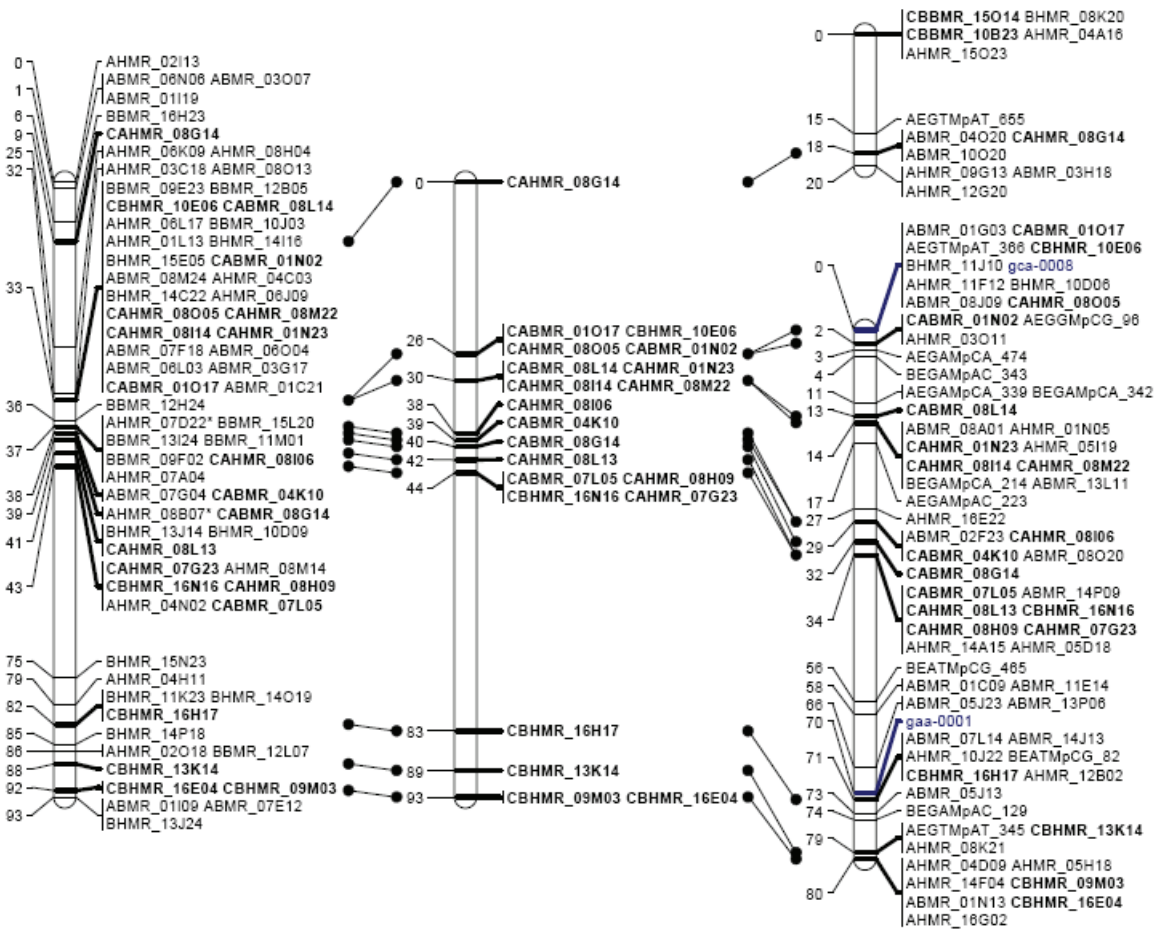
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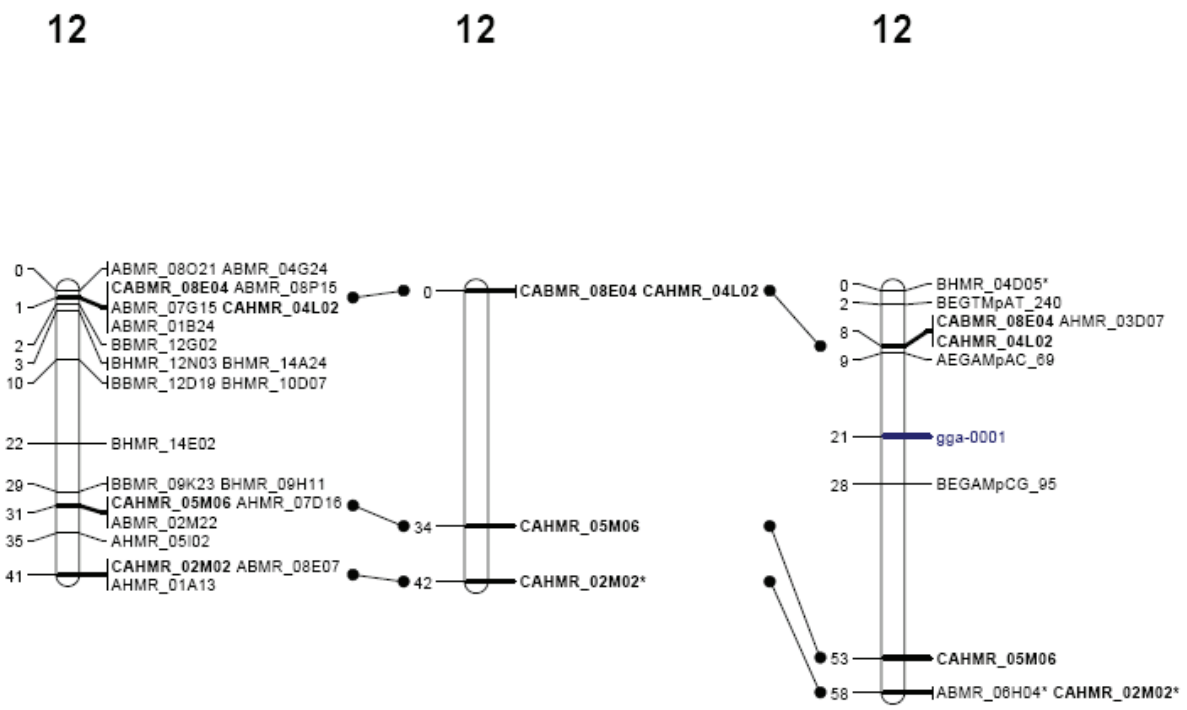
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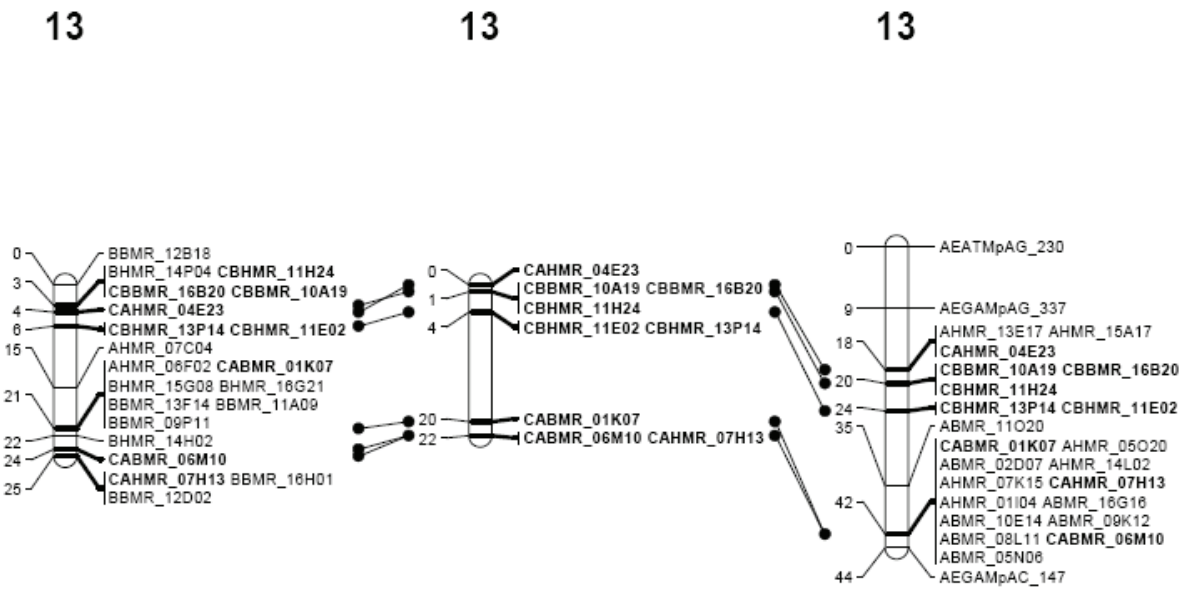
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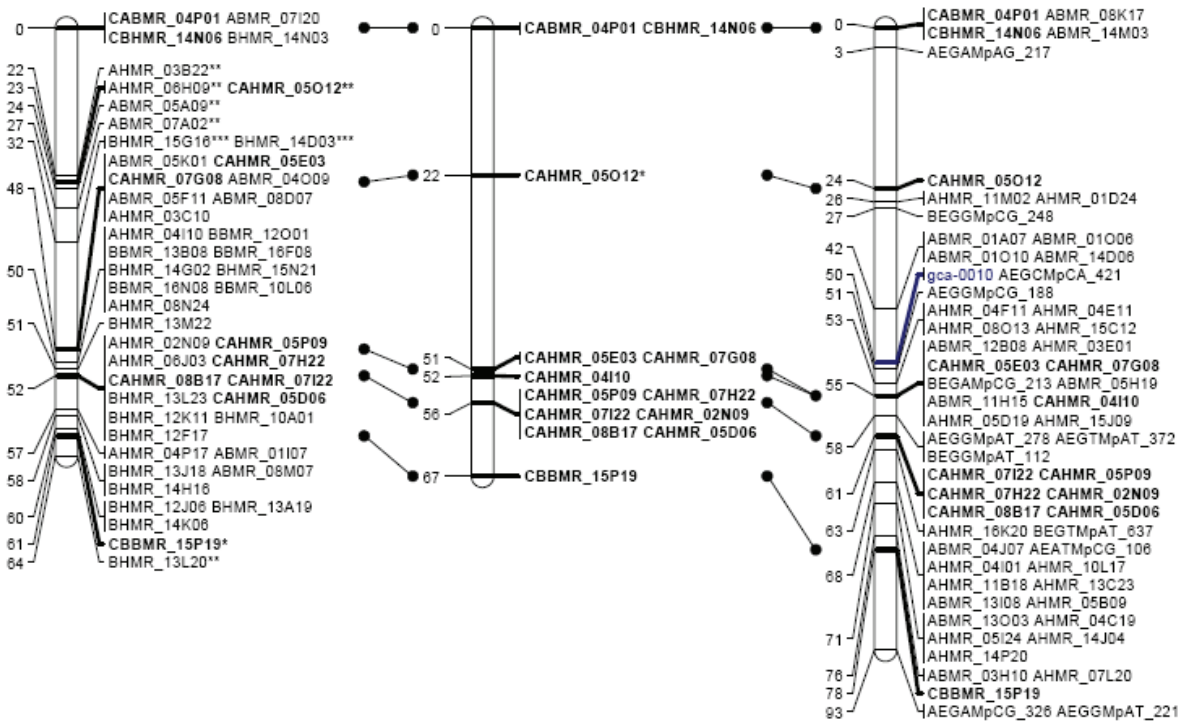
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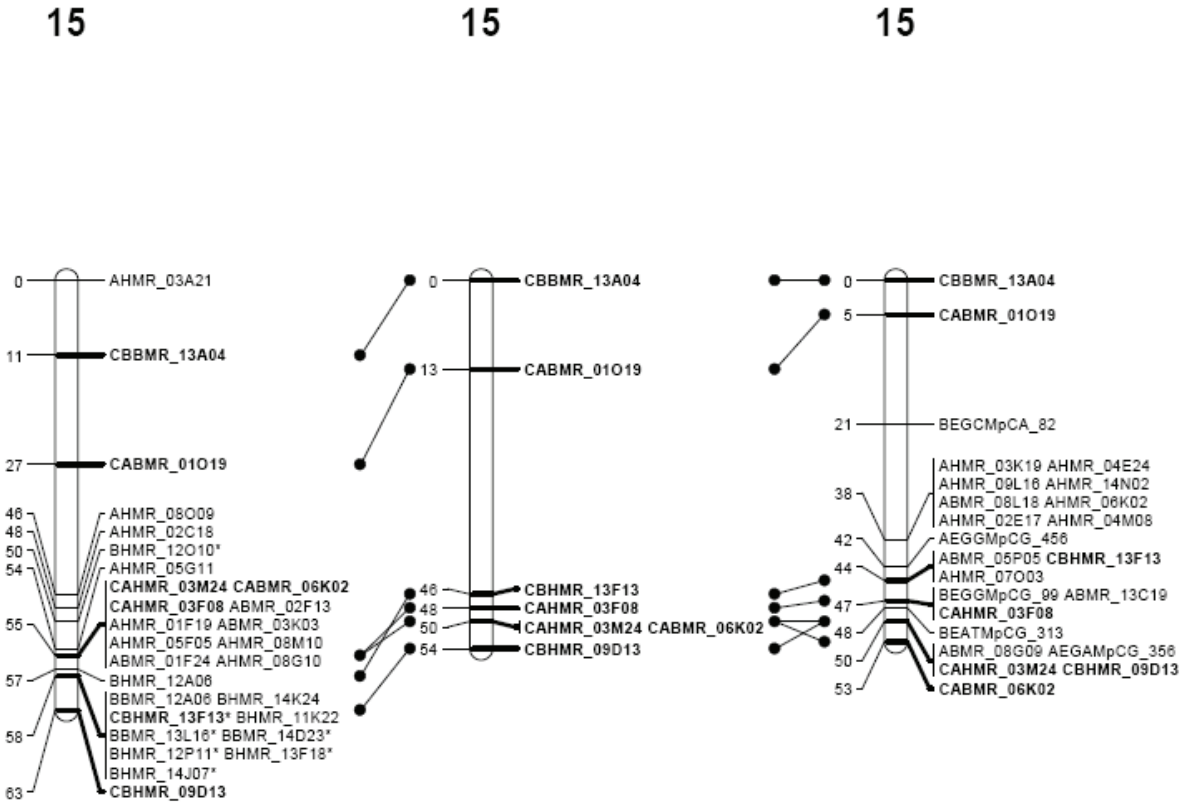
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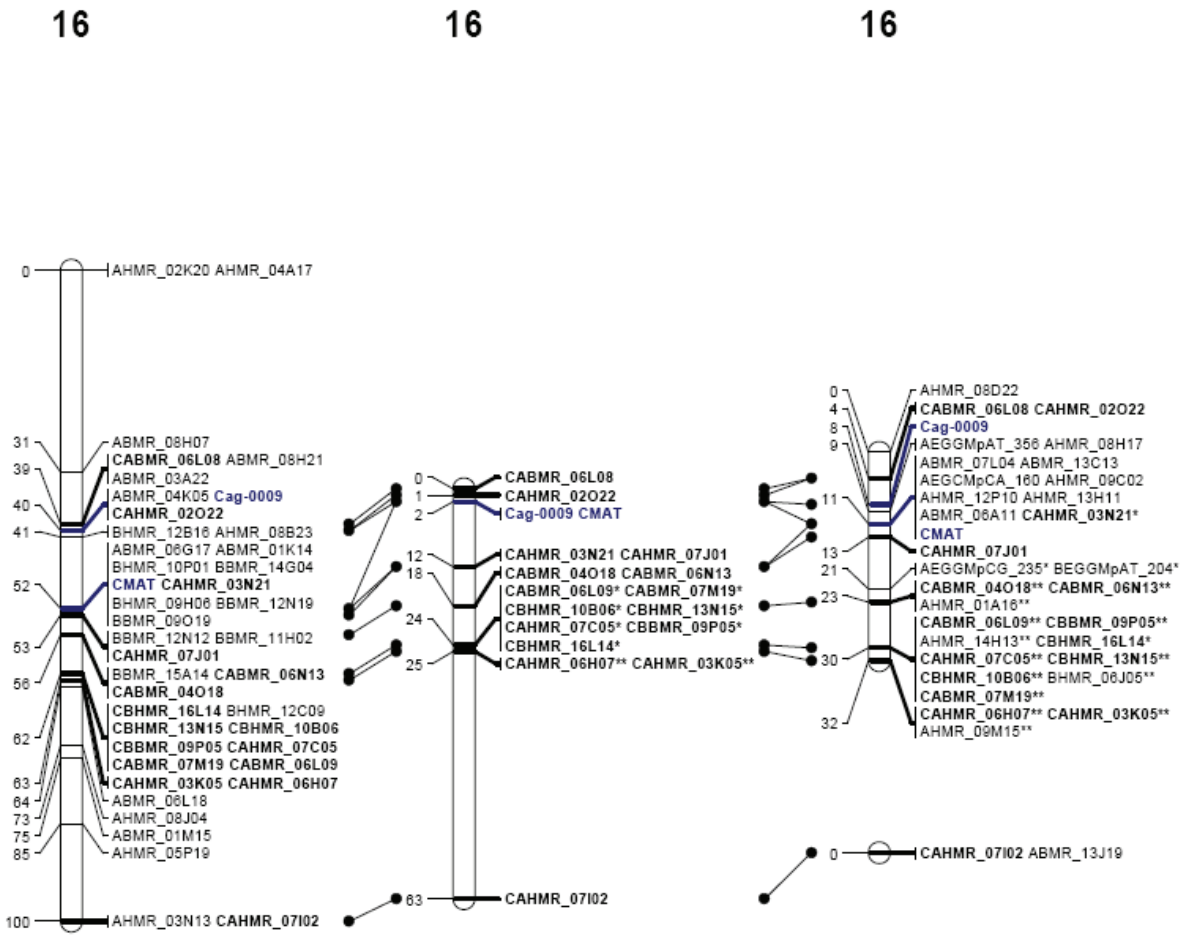
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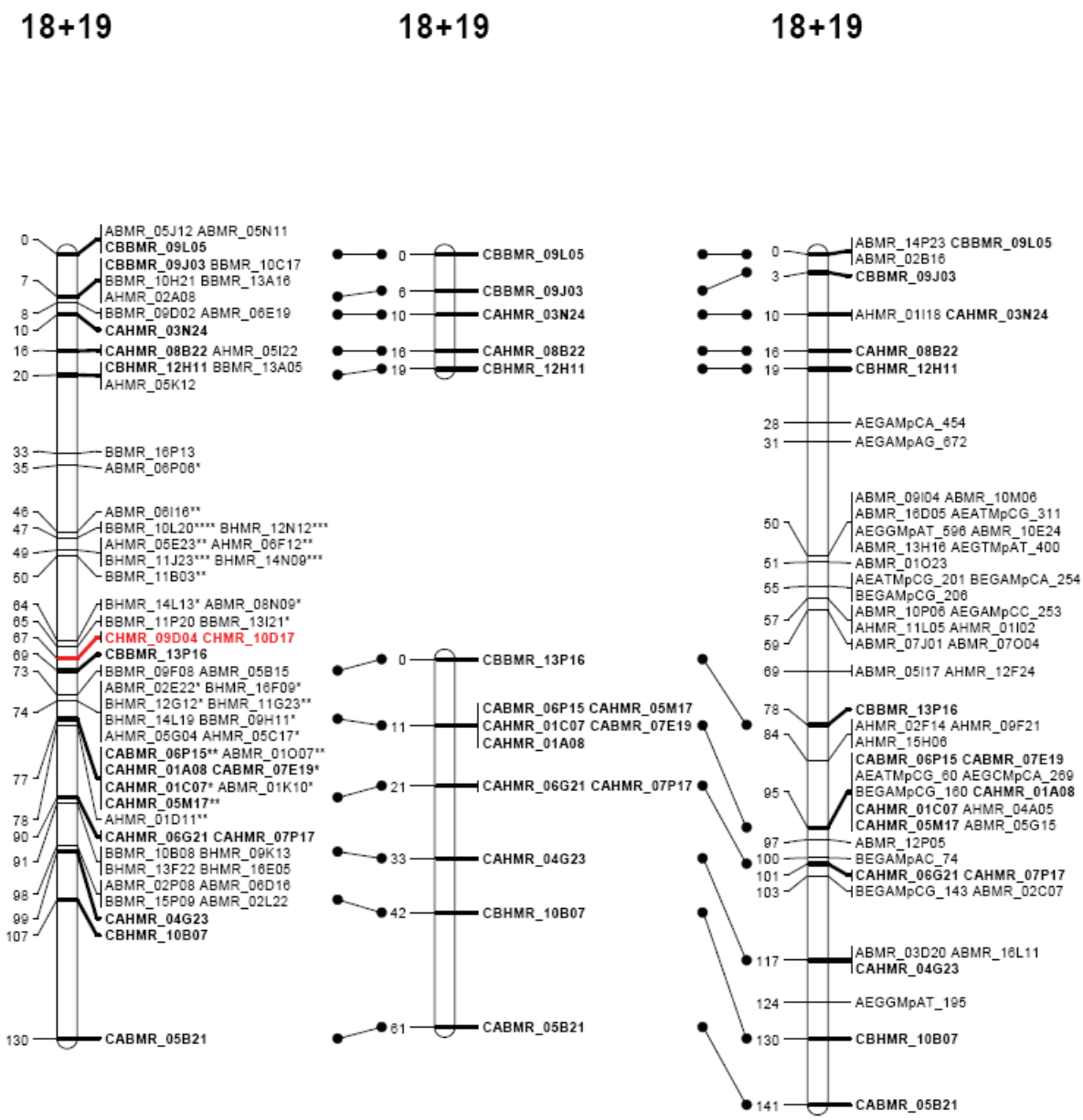
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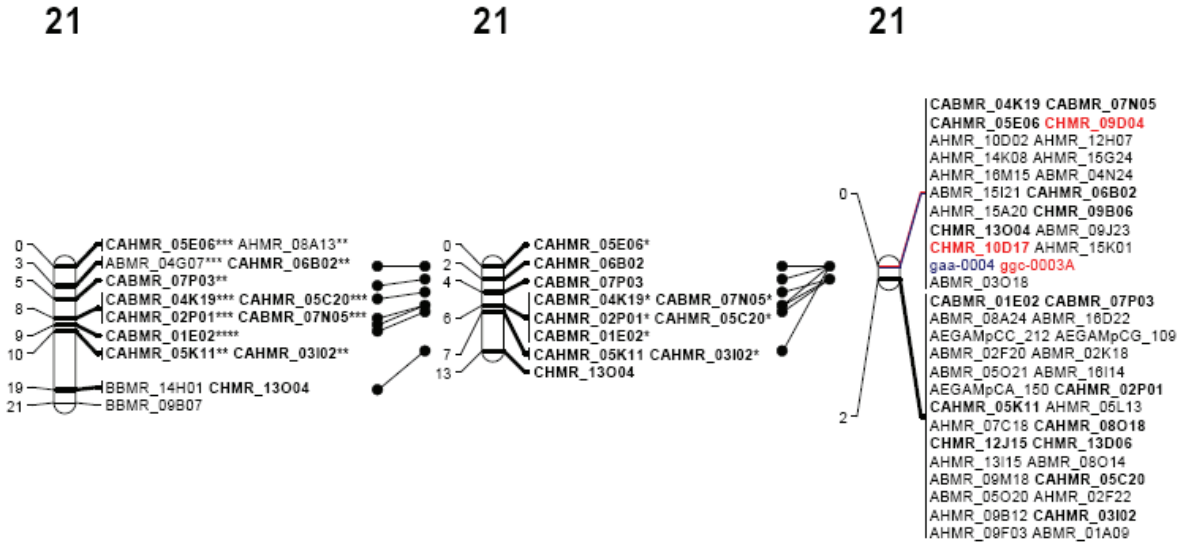
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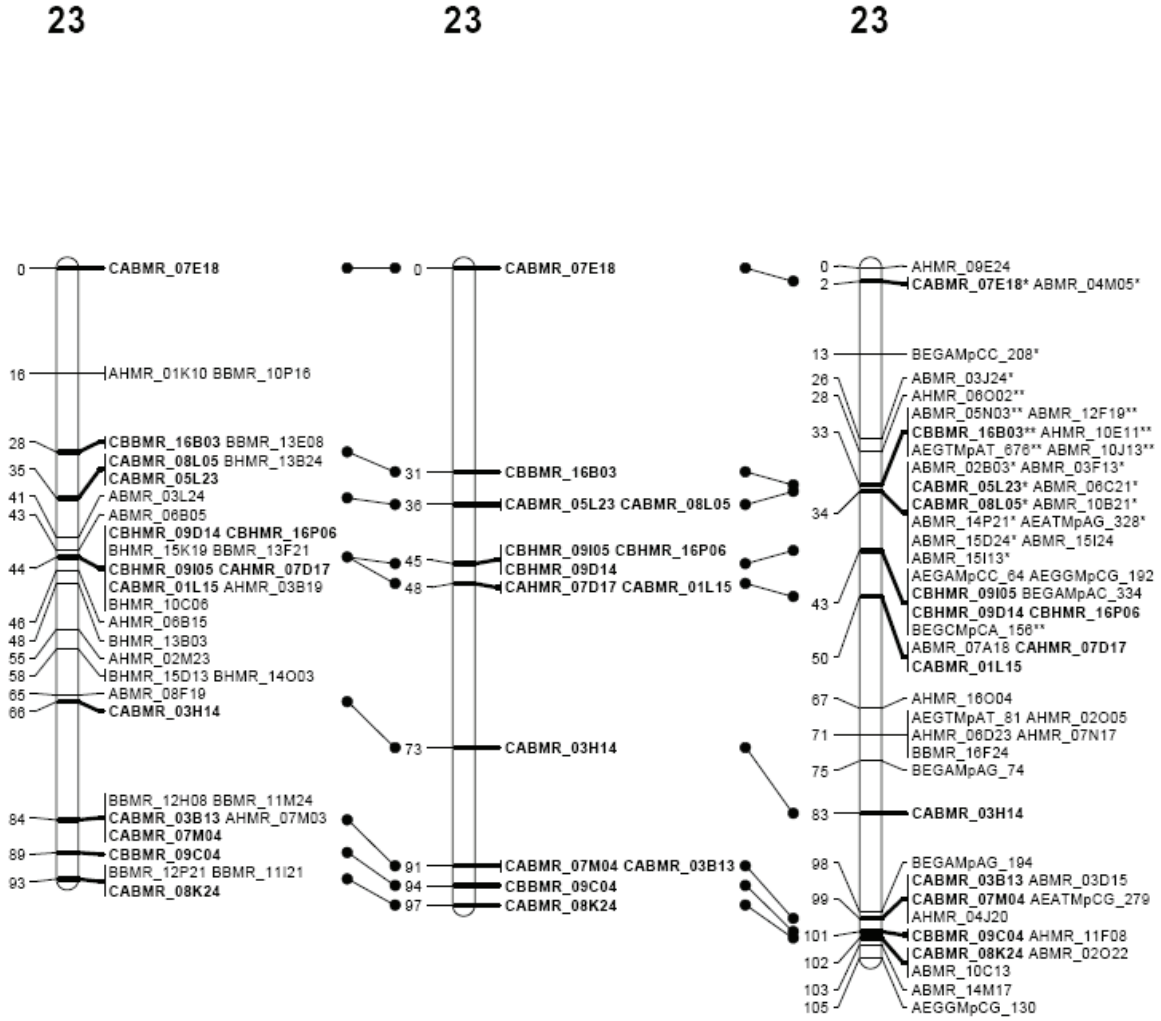
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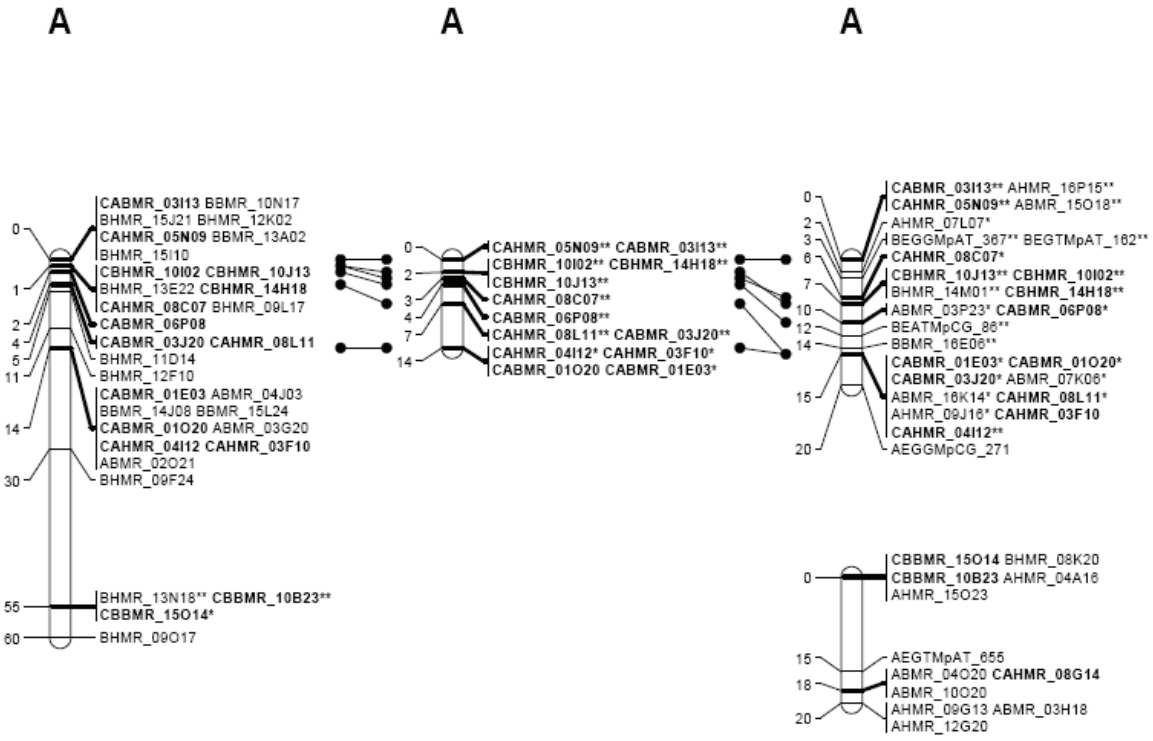
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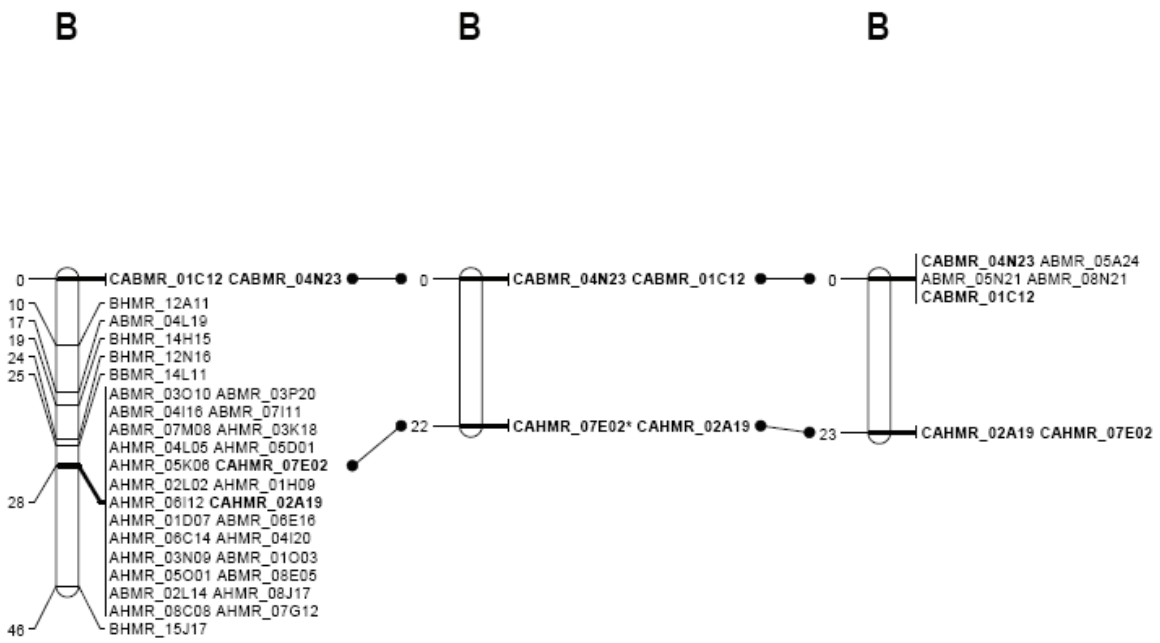
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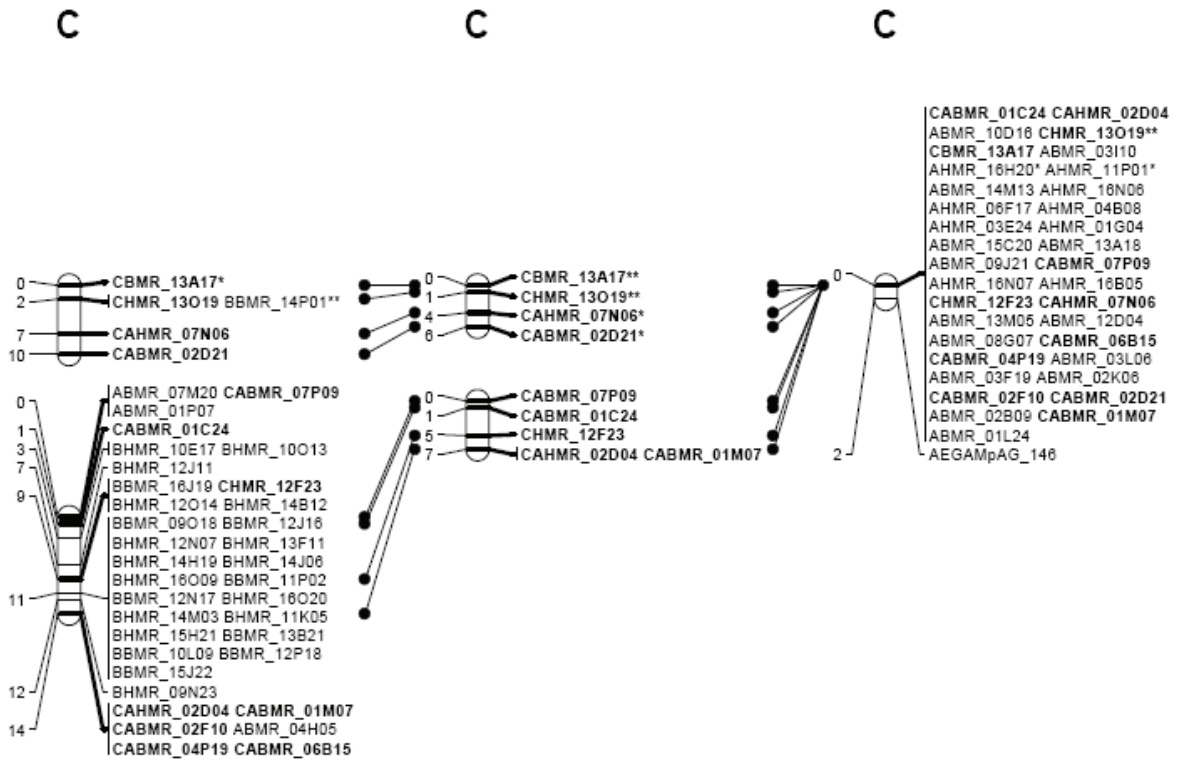
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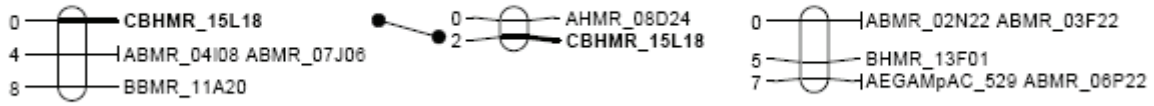
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Summary

Mycosphaerella species are haploid ascomycetes that cause major economic losses in crops that include cereals, citrus fruits, and bananas, among others. Two organisms in this genus are *Mycosphaerella graminicola* (Fuckel) J. Schröt (anamorph *Septoria tritici*) and *Septoria passerinii*. *M. graminicola* is the causal agent of septoria tritici blotch of both bread wheat and durum wheat species, and *S. passerinii* causes septoria speckled leaf blotch of barley. *M. graminicola* is a heterothallic fungus with a very active sexual cycle, while no sexual cycle has been reported for *S. passerinii*.

This thesis includes studies on mating and genetics of both *M. graminicola* and *S. passerinii*. **Chapter 1** gives an introduction to these pathogens and an overview of the research topics. In **Chapter 2**, we studied the possibility of *in planta* generation of sexual progeny of the fungal wheat pathogen *M. graminicola* when one of the parents was avirulent on a resistant host. We found that avirulent isolates are able to survive and even increase in biomass after inoculation onto resistant wheat cultivars and can complete sexual cycles on resistant cultivars to yield viable ascospores as long as the other parent is virulent. To our knowledge, this is the first time such a phenomenon has been described, and the possibility to generate such crosses opened the door for studies in **Chapters 3 and 4**.

Chapter 3 describes the construction of two high-density genetic linkage maps of *M. graminicola* using Diversity Arrays Technology (DArT) and the integration of these into a core map with common markers due to a common parental isolate. One of the maps was constructed based on segregations of progeny of two bread wheat-derived isolates, IPO323 and IPO94269, and the other was constructed from segregations of progeny of IPO323 and the durum wheat-derived isolate IPO95052. In total, 1,144 markers made up the integrated core map. Analyses from this study revealed that progeny had translocations, diploid and partial diploid linkage groups, and loss of entire linkage groups.

Although *M. graminicola* causes disease on both bread wheat and durum wheat, isolates within the population show clear distinctions in either virulence on bread wheat or on durum wheat (host specificity). In **Chapter 4**, we studied the genetic basis of host specificity in *M. graminicola* using 163 progeny from crosses between the Dutch bread wheat-derived IPO323 and the Algerian durum wheat-derived IPO95052. Phenotyping of progeny was performed on a set of seven differential cultivars, and progeny crossed on either bread wheat or durum wheat could infect cultivars of bread wheat, durum wheat, both, or neither. These results were used to map nine quantitative trait loci (QTLs) on seven linkage groups in the high-density genetic linkage map from **Chapter 3**. One of these loci was previously mapped

for cultivar specificity of IPO323in bread wheat, and the same locus was now mapped for host specificity of IPO323 to durum wheat. Our results show that the reported host specificity is probably the result of combinations of a number of independently inherited avirulence factors.

In addition to avirulence genes, fungi can inherit other traits for survival. One such heritable trait is a point mutation in the mitochondrial genome that conveys resistance to strobilurin fungicides. **Chapter 5** describes a study on the inheritance of strobilurin resistance. Resistant and sensitive isolates of *M. graminicola* were crossed on wheat seedlings that were both untreated and preventively treated with various concentrations of azoxystrobin (Amistar™), and progeny were analyzed to determine the rate of inheritance of the aforementioned mutation. Preventive rates from 3.125-200% Amistar™ resulted in completely resistant progeny populations despite the fact that the segregation of nuclear genes confirmed regular meiotic behavior. We conclude that sensitive isolates overcome the disruption of mitochondrial respiration and participate in sexual reproduction even under high fungicide pressure and that fungicide stress induces or results in preferential mating in *M. graminicola*.

The barley pathogen *S. passerinii* clusters closely to *M. graminicola* in phylogenetic studies based on ITS sequences, and a high degree of genetic variation among isolates is found in nature. However, no teleomorph has been reported for this *S. passerinii*, and hence, it was considered to be asexual. Nevertheless, mating type idiomorphs were recently detected and isolated. In **Chapter 6**, we studied the possibility of a *Mycosphaerella* teleomorph associated with *S. passerinii*. Isolates with opposite mating types were co-inoculated onto barley cultivars, and leaves were monitored for the discharge of ascospores. Characterization of a segregating population by both molecular and phenotypic analyses confirmed that we successfully generated the hitherto unknown *Mycosphaerella* teleomorph of *S. passerinii*.

Finally, the results of this thesis are discussed in a broader perspective in **Chapter 7** in relation to epidemiology, co-evolution, and durability of resistance in the wheat-*M. graminicola* pathosystem. The proven ability of avirulent isolates of *M. graminicola* to generate sexual progeny on resistant cultivars represents a new dynamic in population genetics that has not previously been considered in epidemiology. Results from this thesis emphasize the complex ways in which the sexual cycle contributes to the overall success of *M. graminicola* on wheat.

Samenvatting

Mycosphaerella soorten zijn haploïde ascomyceten die zeer grote opbrengstverliezen veroorzaken in onder andere granen, citrusvruchten en bananenplanten. Twee soorten binnen dit geslacht zijn *Mycosphaerella graminicola* (Fuckel) J. Schröt (anamorph *Septoria tritici*) en *Septoria passerinii*. *M. graminicola* is de veroorzaker van septoria bladvlekkenziekte van brood- en durumtarwe, en *S. passerinii* veroorzaakt bladvlekkenziekte in gerst. *M. graminicola* is een heterothallische schimmel met een zeer actieve seksuele cyclus, terwijl voor *S. passerinii* tot nu toe geen geslachtelijke cyclus is beschreven.

Dit proefschrift beschrijft onderzoek naar de geslachtelijke voortplanting en de genetica van *M. graminicola* and *S. passerinii*. **Hoofdstuk 1** is een introductie tot deze pathogenen en geeft een overzicht van de onderzoeksonderwerpen. In **Hoofdstuk 2** wordt de *in planta* ontwikkeling van geslachtelijke nakomelingen van de pathogene tarweschimmel *M. graminicola* beschreven terwijl één van de ouders avirulent is. Wij hebben vastgesteld dat avirulente isolaten kunnen overleven en zelfs in biomassa toenemen na inoculatie van resistente tarweplanten en bovendien aan een geslachtelijke cyclus kunnen deelnemen en die voltooien tot en met de productie van kiemkrachtige ascosporen zolang de andere ouder in de geslachtelijke cyclus een virulent isolaat betreft. Naar ons beste weten is dit de eerste keer dat dit fenomeen is beschreven en dit resultaat bood de mogelijkheid tot het in **Hoofdstuk 3** en **4** beschreven onderzoek.

Hoofdstuk 3 beschrijft de constructie van twee hoge-dichtheids genetische koppelingskaarten waarbij gebruik is gemaakt van Diversity Arrays Technology (DArT). De integratie van deze kaarten tot een brugkaart was mogelijk door gebruik te maken van een gezamenlijke ouder in beide kruisingen. Eén kaart werd gemaakt door de uitsplitsing van merkers in de nakomelingschap van een kruising tussen twee broodtarwe-isolaten, IPO323 en IPO94269, te bestuderen. De andere kaart is gebaseerd op de uitsplitsing van merkers in een populatie die werd verkregen uit een kruising tussen IPO323 en het durumtarwe-isolaat IPO95052. In totaal werden 1.144 merkers op de geïntegreerde kaart geplaatst. Analyse van deze kaarten liet zien dat translocaties en aneuploïdie regelmatig voorkwamen in de nakomelingschappen.

Hoewel *M. graminicola* in zowel brood- als durumtarwe septoria bladvlekkenziekte veroorzaakt, vertonen isolaten in natuurlijke populaties een duidelijke specificiteit voor deze soorten (waardplantspecificiteit). In **Hoofdstuk 4** is de genetische basis voor deze waardplantspecificiteit in *M. graminicola* onderzocht in de nakomelingschappen (N=163) van de kruisingen tussen het Nederlandse broodtarwe-isolaat IPO323 en het Algerijnse

durumtarwe-isolaat IPO95052. De fenotypering, van zowel op broodtarwe als op durumtarwe verkregen nakomelingen, werd uitgevoerd op zeven differentiërende tarwerassen waaruit bleek dat deze isolaten pathogeen waren op individuele brood- en/of durumtarwe rassen, maar dat er ook isolaten zijn die geen van de getoetste rassen kunnen aantasten. Deze resultaten zijn gebruikt om negen quantitative trait loci (QTLs) op zeven koppelingsgroepen van de in **Hoofdstuk 3** beschreven hoge-dichtheids genetische koppelingskaart te plaatsen. Eén van deze loci werd eerder geplaatst als een broodtarwe cultivar-specifiek locus in IPO323 en blijkt nu ook verantwoordelijk te zijn voor de waardplantspecificiteit van dit isolaat voor de getoetste durumtarwe rassen. Onze gegevens laten zien dat waardplantspecificiteit mogelijk het resultaat is van de combinatie van een aantal onafhankelijk overerfbare avirulentiefactoren.

Naast avirulentiefactoren kunnen schimmels ook andere eigenschappen overdragen naar nakomelingen. Een dergelijke eigenschap is een door een puntmutatie in het mitochondrium veroorzaakte resistentie tegen strobilurine fungiciden. **Hoofstuk 5** beschrijft een onderzoek naar de overerving van strobilurine resistentie. Resistente en gevoelige *M. graminicola* isolaten werden gekruist op onbehandelde en met diverse concentraties azoxystrobine (Amistar™) behandelde tarwe zaailingen. De nakomelingschappen werden geanalyseerd op de overerving van de hiervoor genoemde mutatie. Preventieve behandelingen met 3.125-200% Amistar™ resulteerde in volledig resistente nakomelingschappen ondanks het feit dat door nucleair gecodeerde eigenschappen normaal uitsplitsen en daarmee regelmatig meiotisch gedrag bevestigden. Wij concluderen dat gevoelige isolaten de uitschakeling van de mitochondriale ademhaling omzeilen door deel te nemen aan de geslachtelijke reproductie; zélfs onder hoge selectiedruk die dit gedrag in *M. graminicola* induceert of waaruit dit gedrag resulteert.

Uit fylogenetisch onderzoek, waarin gebruik is gemaakt van ITS sequenties, blijkt dat het gerstepathogeen *S. passerinii* nauw verwant is aan *M. graminicola* en dat natuurlijke populaties een grote genetische diversiteit vertonen. Echter, tot nu toe is er geen teleomorf voor deze schimmel gevonden en wordt zij daarom als ongeslachtelijk beschouwd. Desalniettemin zijn recent wel de paringsgenen geïdentificeerd en gekloneerd. In **Hoofdstuk 6** is nagegaan of *S. passerinii* een *Mycosphaerella* teleomorf zou kunnen hebben. Isolaten met een tegengesteld paringstype werden gezamenlijk geïnoculeerd op gerstrassen en de bladeren werden onderzocht op het uitschieten van ascosporen. De karakterisering van een moeizaam verkregen uitsplitsende populatie met behulp van moleculaire en fenotypische

kenmerken bevestigde uiteindelijk de productie van een tot nu toe onbekende *Mycosphaerella* teleomorf van *S. passerinii*.

Tenslotte worden de in dit proefschrift beschreven resultaten in **Hoofdstuk 7** in een breder kader geplaatst en worden de implicaties voor de epidemiologie, co-evolutie en duurzaamheid van resistentie in het tarwe-*M. graminicola* pathosysteem besproken. Het feit dat avirulente *M. graminicola* isolaten een geslachtelijke cyclus kunnen aangaan op resistente rassen geeft een nieuwe invalshoek op de populatiegenetica van dit pathogeen die tot nu toe ook niet in overweging is genomen in de epidemiologie. De resultaten van dit proefschrift benadrukken de belangrijke rol van de geslachtelijke voortplanting voor het succes van *M. graminicola* als pathogeen van tarwe.

First and foremost, I praise God for providing physical and emotional strength that was needed for the completion of this thesis.

The work presented herein has truly been a collaborative effort involving many research groups both in the Netherlands and abroad. The majority of the research was carried out at PRI under the supervision of my co-promoter, Dr. Gert Kema. Gert, I am extremely thankful to you for giving me this opportunity to learn from your experience and expertise in the field of plant pathology and especially dealing with *Mycosphaerella graminicola* on wheat. The scientific content of our work discussions was always constructive and insightful, and I genuinely appreciate how you always respectfully listened to and encouraged my reports, opinions, and even intuitions concerning my research. I am also grateful for the freedom I was given to plan my own experiments and test my own hypotheses. In this environment, I was able to discover and shape my own personal research interests. Thanks also to Hanna and the rest of your family for your warm hospitality.

To my co-promoter, Prof. Maarten de Waard, you have been very kind, supportive, and encouraging throughout my studies. Thank you for all of the time spent on proof-reading and commenting on my thesis chapters. Your experience in fungicide resistance was particularly valuable for Chapter 5 dealing with strobilurins. I am honored to be one of your last PhD students, and I wish you all success in your retirement. To my promoter, Prof. Pierre de Wit, although you are the head of the Laboratory of Phytopathology, you were always surprisingly accessible, caring, and ready to listen. Thank you also for your quick turn-around and helpful comments on my thesis chapters and for your belief in the novelty and value of my work.

I have had a very unique opportunity working with the members of the Pathogen Genetics Cluster. Theo, you are very knowledgeable about so many different topics. I appreciate the way that you can be serious about research without being too serious about life in general. Cees, we share many of the same research opinions and interests concerning mating of fungi, and our discussions have been good for building character. Rahim, it was decidedly beneficial, both scientifically and socially, that I was able to do a parallel PhD study with you during the duration of my stay, and my family has enjoyed knowing you, Akram, and Omid. You are an excellent scientist with very efficient and inventive ideas. All of us, including Gert, have traveled extensively together on excursions and to conferences in Belgium, France, Germany, Tunisia, Denmark, Austria, and the US.

Els, Ineke, and Odette, you are all excellent technicians and excellent friends. Els, you trained me in greenhouse and lab work when I first came to PRI, and you also showed me around Wageningen and helped me equip my bike with all of the Dutch accessories. Ineke, you did countless DNA extractions for me and helped in finding Hannah's school in Ede, and you and Catrien spent many hours babysitting Hannah and Hope. Odette, we weren't involved in the same work projects, but that didn't prevent you from helping out with the storage of the thousands of isolates from the strobilurin crosses, even in the evenings and on your own time, and it was great fun making Christmas cookies with you. We have all shared many memories over the past years, and I know I will always remember you fondly.

It has been my pleasure to have had the opportunity to supervise three students over the course of my studies: Master's students Sarrah Ben M'Barek and Caucasella Díaz-Trujillo and undergraduate student Casper van Schaik. Sarrah, you were the first of my students, and we worked closely for nine months on various projects. We learned many things together as we went, especially the development of a TaqMan® assay (250!). It was a wonderful coincidence that the *Septoria* and *Stagonospora* Diseases of Cereals conference was held in your home country of Tunisia while you were with us. You took us to some great spots while we were there, including the ancient city of Carthage and a traditional Tunisian coffee house. Your driving in Tunisia still impresses me. Casper, you worked for a few months on DNA extractions and microsatellite marker testing. You definitely have a colorful character, and I was enlightened on many aspects of the Punk culture. I hope you are doing what you enjoy now, whether it is lab work or selling music from your internet company. Caucasella, you performed much of the work in Chapter 2 related to TaqMan® analysis and microsatellite marker testing. I appreciate your attention to detail and your inquisitive attitude. You probably became tired of hearing about how much I like Mexican food. Thanks for bringing some on my birthday –that was great! Sarrah and Cauca, you have both decided to continue your PhD studies in our group, and I wish you both all success.

Other collaborators for this thesis include Alexander Wittenberg and Dr. Henk Schouten of PRI and the Laboratory of Plant Breeding at Wageningen University; Dr. Steve Goodwin and Jessica Cavaletto of the USDA and Department of Botany and Plant Pathology at Purdue University in West Lafayette, Indiana, USA; and Prof. Pedro Crous, director of the Fungal Biodiversity Center (Centraalbureau voor Schimmelcultures, CBS) in Utrecht, The Netherlands. Alexander, we have discussed many times how we are happy with our collaborative efforts on two chapters for our PhD theses, which in this thesis are Chapters 3 and 4. You have done a great job with DArT and mapping. I honestly believe that you,

Henk, and Theo were nearly as committed as I to help me finish on time. Henk, thank you for always being kind and positive and for bringing such statistically marvelous ideas to the table that have greatly enhanced these chapters. Steve and Jessica, I met both of you at Purdue University at the same time that I met Gert and Theo before I began my studies in Wageningen, and we have since collaborated on Chapter 6 dealing with *Septoria passerinii*. You have both been friendly and supportive since day one, and I have enjoyed our personal and professional interactions immensely. Pedro, I always think of you as the Steve Irwin of fungal researchers. Your presentations are always dynamic (just look at how *beautiful* this *Mycosphaerella* species is!), and I think no one who attends one will ever forget you. We also collaborated on *S. passerinii*, and I appreciate your enthusiastic efforts to find this elusive sexual structure.

In addition to those mentioned above, I appreciate research discussions with Ramin Roohparvar, Lute-Harm Zwiers, and Ioannis Stergiopolous from the laboratory of Prof. Maarten de Waard and Ewald Groenewald, Marizeth Groenewald, Mahdi Arzanloo, and Edwin Abeln from the laboratory of Prof. Pedro Crous; of which these groups plus our group formed the Dutch Mycosphaerella Group. Working at PRI was a very positive experience overall. With hundreds of bright and imaginative researchers, there was always an expert on hand for discussions on every topic. I really enjoyed the productive atmosphere in which competition among groups was shunned in favor of collaborative projects. I thank Richard van Hoof, Michel Klerks, Cor Schoen, and Ineke de Vries for technical advice on TaqMan® assays, and I appreciate interactions with all of the members of the new Molecular Phytopathology Cluster, as well as the Phytophthora Cluster. Thanks also for administrative help, especially from Peter, Ali, Jan-Maarten, and Ecevit.

Before I began my PhD studies, there were several people who were very supportive and encouraging, including Profs. D. Mack Ivey, David TeBeest, and Jim Correll from the University of Arkansas. Dr. Ivey, you gave me my first opportunity to do original scientific research when I was granted a Howard Hughes Undergraduate Research Fellowship in the Department of Biological Sciences. I remember thinking it was the coolest thing ever to get paid to do research –I still think so! Dr. TeBeest, you allowed me to work in your lab for another summer internship with the C. Roy Adair Fellowship in the Department of Plant Pathology, and I later continued with you for my Master's studies. Thank you both for these opportunities. Claudia, thanks for your patient training in Dr. TeBeest's lab and for the continued friendship of you and John. Dr. Correll, you served on my Master's thesis

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Personal support is so much needed in addition to professional support, and I would like to thank the Church in Wageningen, including the Vineyard Gemeente, the International Christian Fellowship (ICF), and the Wagenburcht Gereformeerde Kerk Vrijgemaakt. While we were so far from home, the Church many times served as our family, and I am so thankful for that. Pastors Johan and Frannie Vink and George and Paulien Zeeman, you head a large congregation, but you always fulfilled your pastoral roles on a personal level. A big thanks also goes out to the church translators, especially Evert-Jan, Wim, Suheyla, Remco, Gineke, Gera, and Paulien, and Doortje. Marco and Virny (and baby on the way), thanks so much for showing kindness by giving us many start-up items, including a refrigerator and washing machine, even before we met you. We have become great friends and have had some great times! Evert-Jan and Esther, we have enjoyed getting to know you and your family. Thanks for including us in cultural traditions such as Queen's Day and Sinter Klaas. Ineke, you have been a wonderful colleague but also a great support in the Church and a great help in administrative things such as translation of all kinds of Dutch documents. Thank you for all of the furniture that you gave to us and for sponsoring a Thanksgiving turkey the first year we were here. Thanks to Tonia and Luis Ledelay and family for hospitality and assistances dealing with Hannah's school. Samson and Lia and family, we always enjoyed your down-to-earth lifestyle and excellent fufu and soups. Robert and Finda and family; Alex and Ola and Anthony; Doortje, Faith, and Beatrice; Wally and John; Esnati and Jeoffrey; Harrison; Stephen; and so many others; we appreciate your friendships and your dedication to prayer groups and church-planting. We did manage to make some American friends while in the Netherlands, as well. Mitch and Michelle and family, we enjoyed getting together, letting the kids play, and exchanging our experiences as ex-pats. Dan and Beth (and baby on the way), it was fun to get to know you guys in the last months of our stay. Thanks for your prayers and for your friendship. We also appreciate so many others from the Church!

Because of the multi-cultural population in Wageningen, I have been blessed to have friends from diverse parts of the world. In addition to those already mentioned, my family

and I are thankful for our French friends, Franck and Veronique and family, for entertaining evenings including French food (always at their house –sorry, Guys!). As an American, I have had the unique opportunity to interact with many Iranian families. My family and I want to thank the Iranian community in Wageningen for friendships, hospitality, and so much practical assistance (such as with moving furniture and offering a place to stay on our last night). We have especially enjoyed interactions with Rahim, Akram, and Omid; Ramin and Mahnaz; Mostafa, Mariam, and Alireza; Hossein and family; Reza and family; Farhad and family; and Mahdi.

Finally, I come to the thanks due to my family, without whose support I could not have completed this thesis. To Mom and Dad, thank you for valuing education and for encouraging me and assisting me so that I could finish my Bachelor's degree during some rough circumstances. For you both, and for Barbara and Terry, I know it was hard to have your children and grandchildren so far away, but thank you for not protesting too strongly about it and for flying us home numerous times during our stay. To my husband, Jonathan, and my daughters, Hannah and Hope, my long hours at work have been difficult for all of us. The strength of our family has really been tested, and I praise God that we made it through. Hannah and Hope, it is my desire that you both will come out with the positive aspects of living in the Netherlands while I studied. Hannah, you embraced the Dutch culture from the beginning. You jumped right into the Dutch school system and learned the language fluently. You have made friends from all over the world, and I hope that you will remember to appreciate diversity of cultures throughout your life. Hope, you were born in the Netherlands, and because of that your father was able to stay home and bond with you and take care of you (and also Hannah). I hope that someday when you're older, you will be able to visit the country of your birth and that you will have a desire to know about other countries and cultures. Jonathan, you have been my best friend since I was 14 years old. Of course, at 14 I may have thought about marrying you someday, but I never could have imagined what you would sacrifice for me and my education. You left your career and put your education on hold, sold our first home together that we both loved, and moved with Hannah to the Netherlands to be in a supporting role for our family. If you hadn't have stuck it out, there is absolutely no way that I would have finished this thesis. I am forever grateful and forever in love with you.

Even after five pages, I still have not thanked everyone by name. I appreciate having known all of you, and I hope to see you again. Tot ziens! Hup Holland! Ik ben klaar!

Sarah Blossom Ware was born in 1974 in Cleveland, Ohio, U.S.A. She was granted an academic scholarship and began undergraduate studies at the University of Arkansas in 1995. Sarah received a BSc degree with honors in Microbiology with a medical emphasis in Spring 2000, during which she conducted research on the human bacterial pathogen *Clostridium difficile*. Sarah was then granted a graduate assistantship in the Department of Plant Pathology at the University of Arkansas. During this time, her research focused on the genetic characterization of *Colletotrichum sublineolum*, a fungal pathogen of sorghum, and she received an MSc degree in Plant Pathology in December 2001. Following Master's studies, Sarah was granted an AIO PhD Assistantship from the graduate school Experimental Plant Sciences in Wageningen, The Netherlands. She conducted her thesis research at Plant Research International and Wageningen University's Laboratory of Phytopathology on aspects of sexual reproduction in the cereal pathogens *Mycosphaerella graminicola* and *Septoria passerinii*.

LIST OF PUBLICATIONS

Full papers:

Ware SB, Verstappen ECP, Breeden J, Cavaletto JR, Goodwin SB, Waalwijk C, Crous PW, Kema GHJ. 2006. Discovery of a functional *Mycosphaerella* teleomorph in the presumed asexual barley pathogen *Septoria passerinii*. Submitted.

Ware SB, van der Lee TAJ, Díaz-Trujillo C, de Wit PJGM, de Waard MA, Kema GHJ. 2006. Host resistance and fungicides do not prevent sex in a plant pathogen. In preparation for submission.

Wittenberg AHJ, van der Lee TAJ, **Ware SB**, Kilian A, Visser RGF, Kema GHJ, Schouten HJ. 2006. High-density maps of the fungus *Mycosphaerella graminicola* using Diversity Arrays Technology (DArT) reveal frequent loss of chromosomes. In preparation for submission.

Ware SB, Wittenberg AHJ, Verstappen ECP, van der Lee TAJ, Schouten HJ, Kema GHJ. 2006. Quantitative trait loci determine specificity in the fungal wheat pathogen *Mycosphaerella graminicola* to bread and durum wheat cultivars. In preparation for submission.

Abstracts and short papers:

Ware SB, van der Lee TAJ, Kema GHJ. 2006. Strobilurins and sex in *Mycosphaerella graminicola.* Page 108 in: Proceedings of the 8th European Conference on Fungal Genetics, Vienna, Austria.

Ware SB, Kema GHJ. 2005. Genetics of avirulence in *Mycosphaerella graminicola*, the wheat septoria leaf blotch fungus. Page 146 in: Proceedings of the 23rd Fungal Genetics Conference, California, U.S.A.

Kema GHJ, de Vries PM, van der Lee TAJ, Mendes O, Verstappen ECP, Mehrabi R, **Ware SB**, Jalink H, van der Schoor R, Waalwijk C. 2004. Monitoring and Molecular Diagnostics of Pathogens. 14th International Reinhardsbrunn Symposium; Modern Fungicides and Antifungal Compounds. Friedrichroda, Germany.

S.B. Ware, Verstappen ECP, Cavaletto JR, Goodwin SB, Waalwijk C, Kema GHJ. 2004. Identification of the teleomorph of *Septoria passerinii*, the barley speckled leaf pathogen. Page 94 in: Proceedings of the 7th European Conference on Fungal Genetics, Copenhagen, Denmark.

Ben M'Barak S, **Ware SB**, Waalwijk C, van der Lee TAJ, Mehrabi R, Kema GHJ. 2003. A TaqMan® approach to monitor and quantify *Mycosphaerella graminicola*. Page 161 in: Global insights into the *Septoria* and *Stagonospora* diseases of cereals. Procedings of the 6th International Symposium on *Septoria* and *Stagonospora* Diseases of Cereals. Edited by Kema GHJ, van Ginkel M, Harrabi M. Tunis, Tunisia.

Ware SB, Verstappen ECP, Goodwin SB, Waalwijk C, Kema GHJ. 2003. Experimental identification of the teleomorph of the barley pathogen *Septoria passerinii*. Pages 107-110 in: Global insights into the *Septoria* and *Stagonospora* diseases of cereals. Procedings of the 6th International Symposium on *Septoria* and *Stagonospora* Diseases of Cereals. Edited by Kema GHJ, van Ginkel M, Harrabi M. Tunis, Tunisia.

Ware SB, Verstappen ECP, Groenewald E, Waalwijk C, Kema GHJ. 2003. Host specificity of *Mycosphaerella graminicola*, the septoria leaf blotch pathogen of wheat. Page 163 in: Global insights into the *Septoria* and *Stagonospora* diseases of cereals. Procedings of the 6th International Symposium on *Septoria* and *Stagonospora* Diseases of Cereals. Edited by Kema GHJ, van Ginkel M, Harrabi M. Tunis, Tunisia.

Ware SB, Guerber CA, TeBeest DO. 2001. Genetic diversity of *Colletotrichum sublineolum* in a worldwide sampling of isolates from sorghum. *Phytopahology* 91:S93. Publication no. P-2001-0679-AMA.

Ware SB, Ivey DM. 2001. The *Clostridium difficile* EIIA protein of the mannitol phosphotransferase system is a repressor of the oligopeptide permease. Page A205 in: Proceedings of the Experimental Biology Annual Meeting (American Society for Biochemistry and Molecular Biology), Orlando, Florida.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Sarah Blossom Ware
Date: 30 October 2006
Group: Laboratory of Phytopathology (Wageningen University) & Biointeractions and Plant Health (Plant Research International)

1) Start-up phase	date	cp
► **First presentation of your project**		
Genes in *Mycosphaerella graminicola* controlling host plant specificity	Feb 21, 2003	1.5
► **Laboratory use of isotopes**		
Radiation hygiene, level 5B	Jun 4-7, 2002	1.5
Subtotal Start-up Phase		3.0

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
PhD student day 2003, Vrije Universiteit Amsterdam	Jun 3, 2004	0.3
PhD student day 2004, Radboud University Nijmegen	Jun 2, 2005	0.3
► **EPS theme symposia**		
Theme II symposium 2003 'Interactions between Plants and Biotic Agents', Wageningen University	Dec 12, 2003	0.3
Theme II symposium 2004, 'Interactions between Plants and Biotic Agents', Utrecht University	Sep 17, 2004	0.3
► **NWO Lunteren days and other National Platforms**		
NWO-ALW, Platform molecular genetics annual meeting, Lunteren	Apr 15-16, 2002	0.6
Meeting: Mycological section of the Dutch microbiological society, Utrecht University	Nov 7, 2003	0.3
WCS day	Jan 22, 2004	0.3
► **Seminars (series), workshops and symposia**		
CBS/ Wageningen Phytopathology Symposium, Utrecht	Jun 27, 2003	0.3
GeNeYous symposium 2004, Utrecht	Jan 20, 2004	0.3
GeNeYous symposium 2005, Oss	April 27, 2005	0.3
► **Seminar plus**		
► **International symposia and congresses**		
6th Int. Symp. on Septoria & Stagonospora Diseases of Cereals, Tunis (Tunisia)	Dec 8-12, 2003	1.5
7th Eur. Conf. on Fungal Genetics, Copenhagen (Denmark)	Apr 17-20, 2004	1.2
23rd Fungal Genetics Conference, Asilomar, California (U.S.A.)	Mar 15-20 2005	1.8
8th European Conference on Fungal Genetics, Vienna, (Austria)	Apl 8-11, 2006	1.2
► **Presentations**		
Autumn School 'Disease resistance in plants', poster and oral presentation	Oct 14-16, 2002	1.4
Meeting: Mycological section of the Dutch microbiological society, Utrecht, oral	Nov 7, 2003	0.7
6th Int. Symp. On *Septoria & Stagonospora* Diseases of Cereals, Tunis (Tunisia), oral (1) and posters (3)	Dec 8-12, 2003	2.8
7th Eur. Conf. on Fungal Genetics, Copenhagen (Denmark), poster	Apr 17-20, 2004	0.7
Dept. of Plant Pathology, University of Arkansas (USA), oral	Oct 29, 2004	0.7
23rd Fungal Genetics Conference, Asilomar, California (U.S.A.), poster	Mar 15-20 2005	0.7
8th European Conference on Fungal Genetics, Vienna, (Austria), poster	Apr 8-11, 2006	0.7
► **IAB interview**	Jun 3, 2005	0.7
► **Excursions**		
Field visit, breeding for resistance to *Septoria* and *Fusarium*, Lille (France)	Jun 25, 2003	0.3
Control of cereal diseases by new fungicides, Syngenta (Germany)	Jul 4, 2003	0.3
Summer excursion for cereal diseases in Belgium and France	Jun 28, 2004	0.3
Subtotal Scientific Exposure		18.3

3) In-Depth Studies	date	cp
► **EPS courses or other PhD courses**		
Autumn School 'Disease resistance in plants', Wageningen University	Oct 14-16, 2002	0.9
Course 'Confocal light microscopy', University of Amsterdam	May 12-16, 2003	1.5
Summer School 'Molecular basis of microbe-plant interactions', Leiden University	Jun 5-7, 2003	0.9
Course 'Functional genomics: theory and hands-on analysis', Utrecht University	Aug 25-28, 2003	1.2
► **Journal Club**		
Weekly phytopathology lab meeting	2002-2006	1.0
Biweekly pathogen genetics cluster discussions	2002-2006	1.0
Monthly Dutch *Mycosphaerella* group discussions	2002-2006	1.0
► **Individual research training**		
Training for detached leaf assay, Centre de Recherche sur les Plantes, Orsay (France)	May 2-3, 2002	0.6
Subtotal In-Depth Studies		8.1

4) Personal development	date	cp
► **Skill training courses**		
Techniques for writing and presenting a scientific paper, Wageningen	Mar 26-28, 2003	1.2
Dutch for self-study I, Wageningen	Fall 2003	1.5
Scientific Publishing, Wageningen	Oct 13, 2005	0.3
► **Membership of Board, Committee or PhD council**		
Visiting Scientists' Task Force	2003-2004	0.7
Subtotal Personal Development		3.7

TOTAL NUMBER OF CREDIT POINTS*		**33.1**

** A credit represents a normative study load of 28 hours of study*

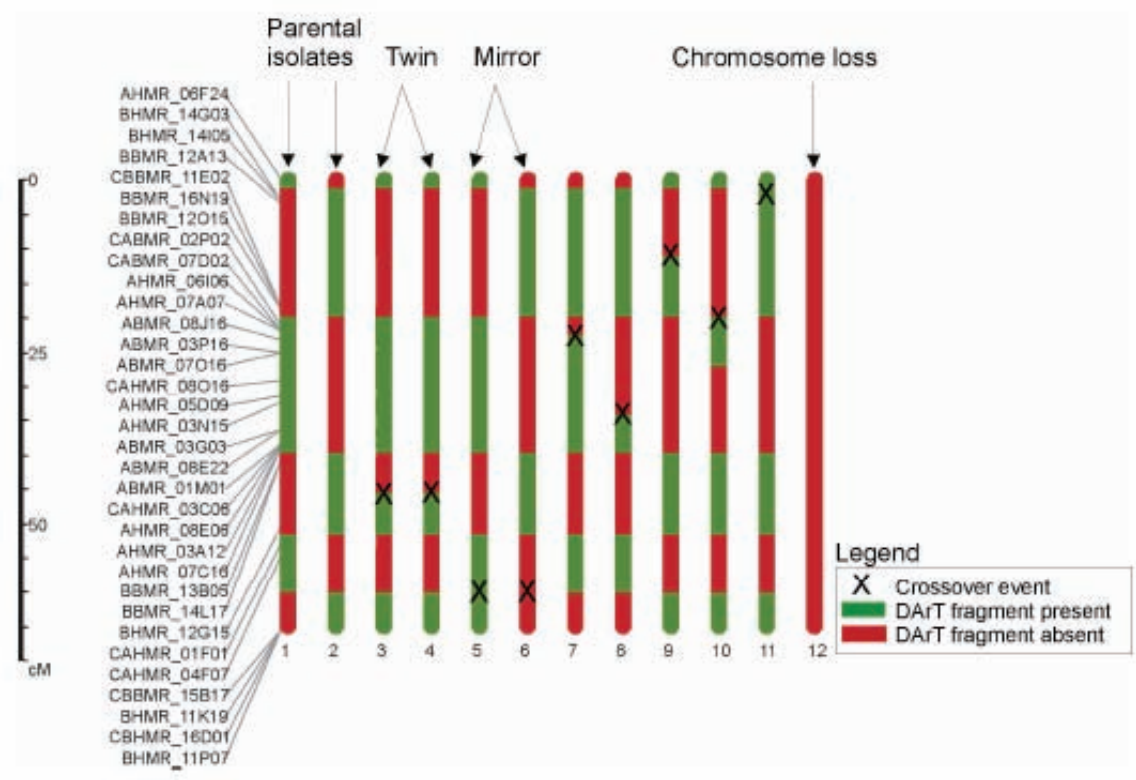
This research was financially supported by the graduate school Experimental Plant Sciences at Wageningen University in Wageningen, The Netherlands

Layout and design: by the author.

Front cover: Top left: ascospore of *Mycosphaerella graminicola* (photo: Pedro Crous). Bottom right: genetic linkage group (LG 3+22) of *M. graminicola* with positions and LOD-values for avirulence loci corresponding to five wheat cultivars. Center: uninfected wheat leaf fragment.

Back cover: Virulence phenotypes of seven wheat cultivars after inoculation with isolates of *M. graminicola.*

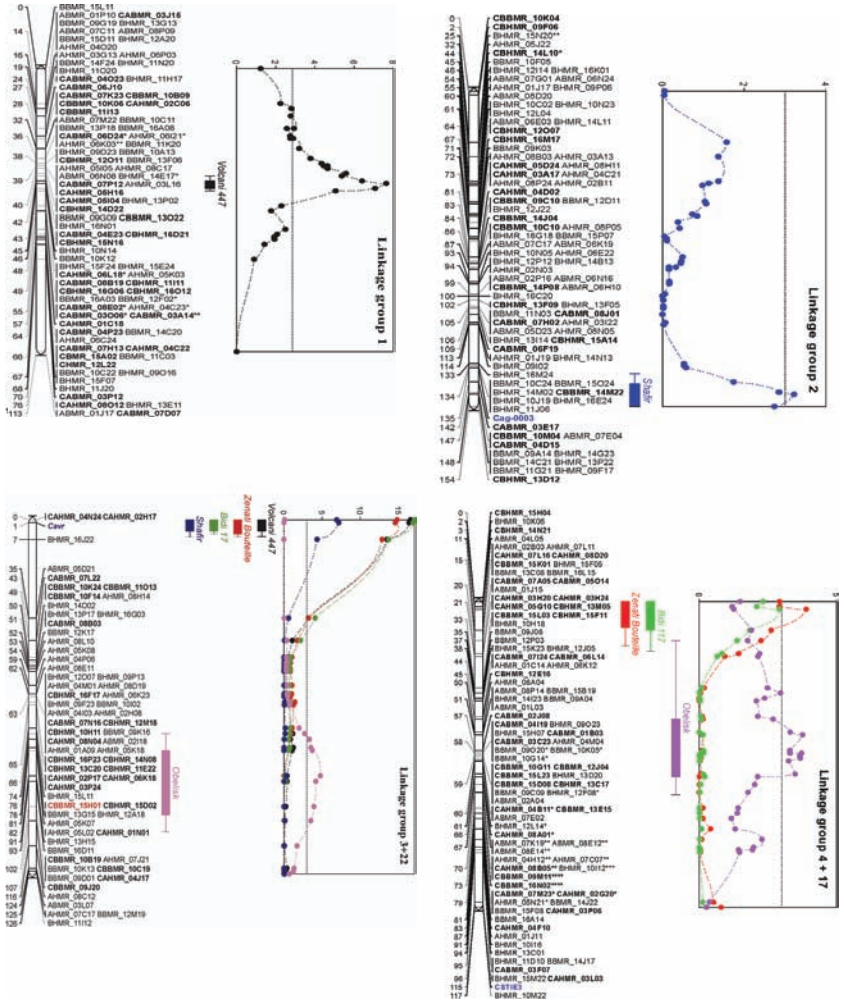
Color Figure, Chapter 3



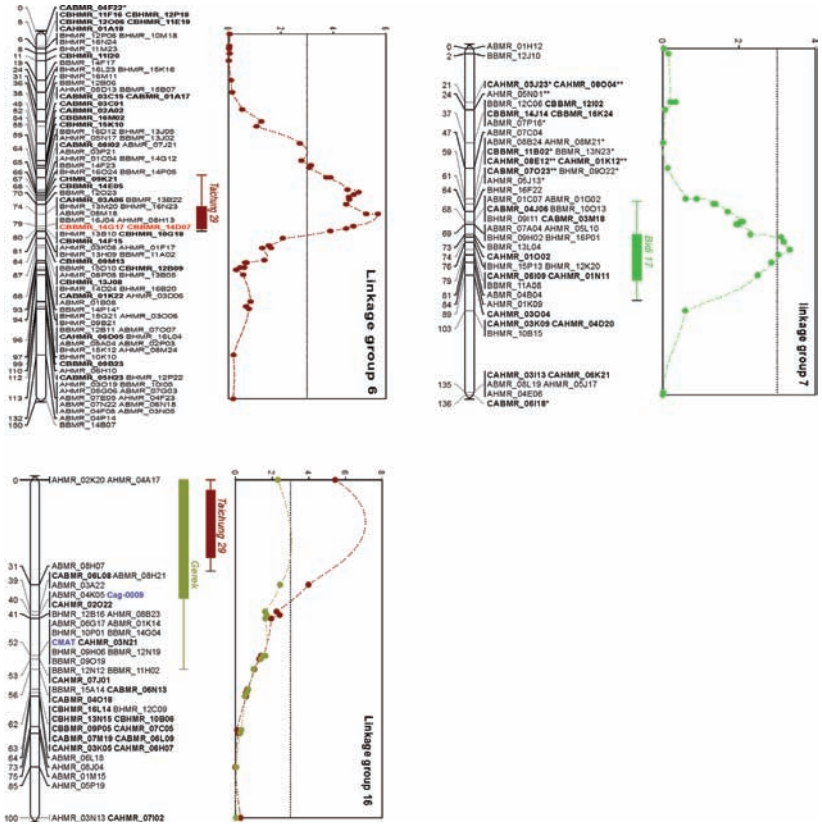
Chapter 3, Page 65, Figure 4

Color Figures, Chapter 4

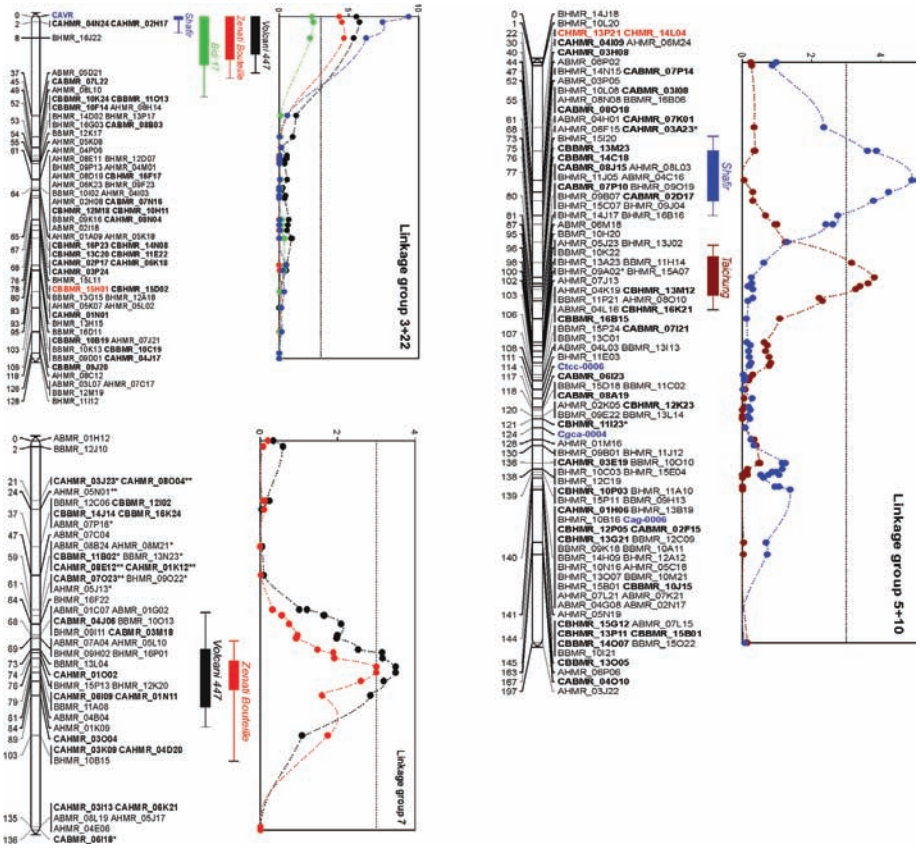
Chapter 4, Page 80, Figure 1A



Chapter 4, Page 86, Figure 3A

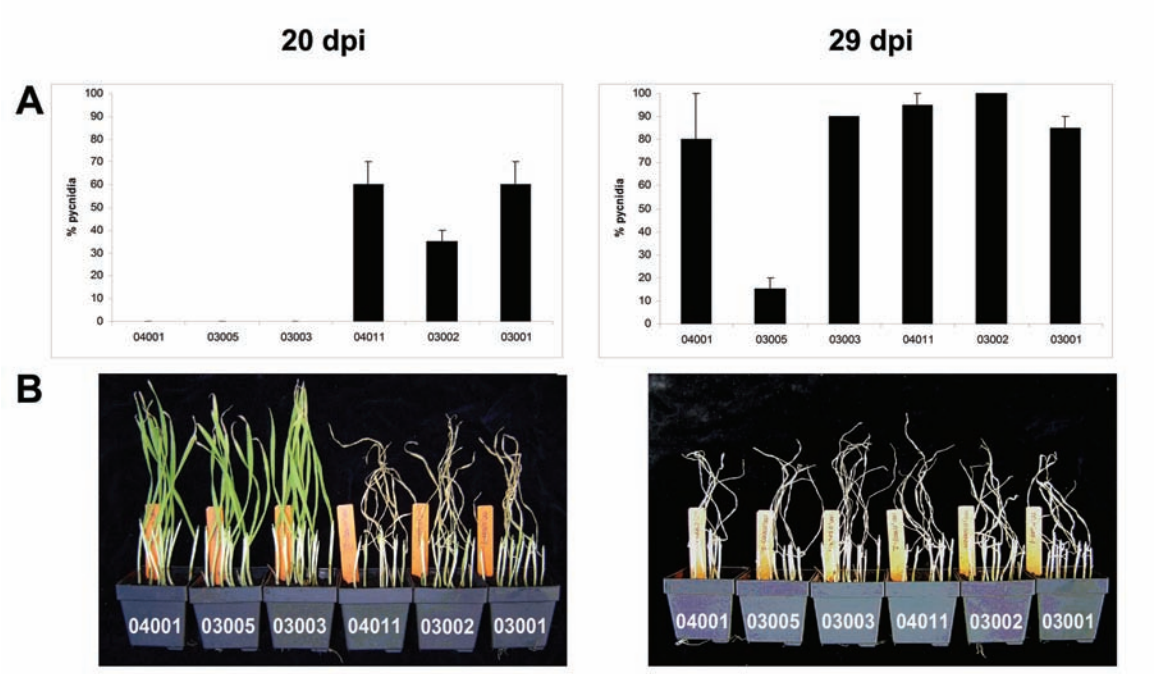


Chapter 4, Page 87, Figure 3B

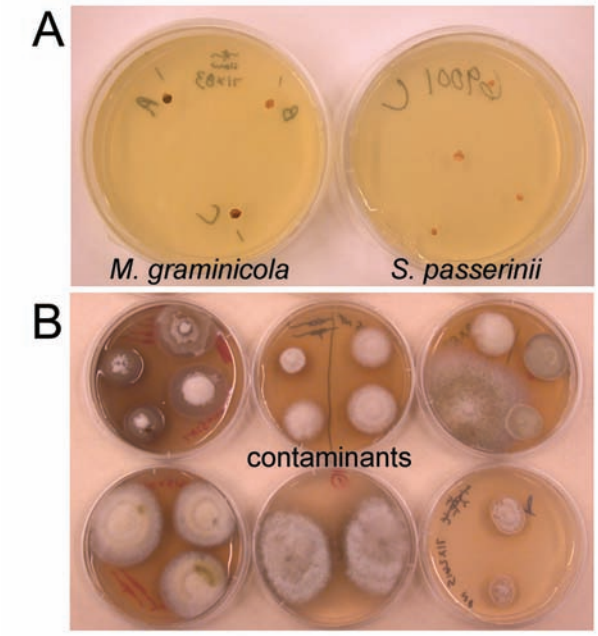


Chapter 4, Page 89, Figure 4

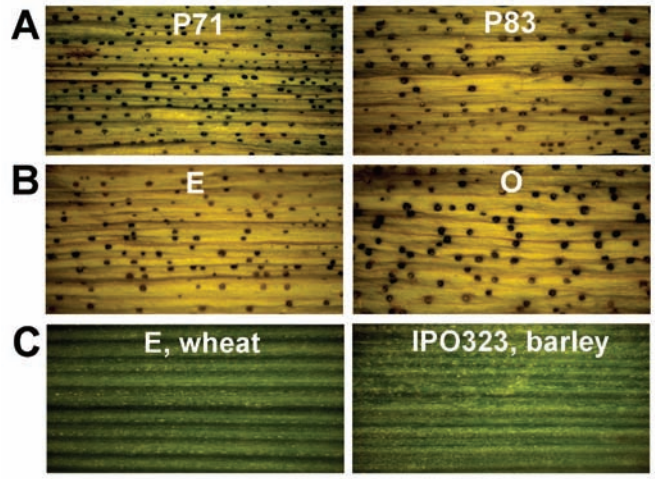
Color Figures, Chapters 5 and 6



Chapter 5, Page 111, Figure 2



Chapter 6, Page 132, Figure 2



Chapter 6, Page 135, Figure 4

Pathogen Genetics Cluster, 2004

Molecular Phytopathology Cluster, 2006