A Combined Amplified Fragment Length Polymorphism and Randomly Amplified Polymorphism DNA Genetic Linkage Map of *Mycosphaerella graminicola*, the Septoria Tritici Leaf Blotch Pathogen of Wheat

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ABSTRACT

An F_1 mapping population of the septoria tritici blotch pathogen of wheat, *Mycosphaerella graminicola*, was generated by crossing the two Dutch field isolates IPO323 and IPO94269. AFLP and RAPD marker data sets were combined to produce a high-density genetic linkage map. The final map contained 223 AFLP and 57 RAPD markers, plus the biological traits mating type and avirulence, in 23 linkage groups spanning 1216 cM. Many AFLPs and some RAPD markers were clustered. When markers were reduced to 1 per cluster, 229 unique positions were mapped, with an average distance of 5.3 cM between markers. Because *M. graminicola* probably has 17 or 18 chromosomes, at least 5 of the 23 linkage groups probably will need to be combined with others once additional markers are added to the map. This was confirmed by pulsed-field gel analysis; probes derived from 2 of the smallest linkage groups hybridized to two of the largest chromosome-sized bands, revealing a discrepancy between physical and genetic distance. The utility of the map was demonstrated by identifying molecular markers tightly linked to two genes of biological interest, mating type and avirulence. Bulked segregant analysis was used to identify additional molecular markers closely linked to these traits. This is the first genetic linkage map for any species in the genus Mycosphaerella or the family Mycosphaerellaceae.

MAPPING fungal genomes with the aid of molecular markers is a rapidly developing area in biological science. Particularly, the application of PCR-based strategies such as randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLPs) allows a highly efficient generation of markers. Integration of biological traits and molecular markers into one map allows a quick focus on particular areas of the genome to develop strategies for cloning genes of interest. In plant pathogenic fungi, typical genes of interest are those that control mating, avirulence, and fungicide resistance (Whisson *et al.* 1995; Xu and Leslie 1996; Van der Lee *et al.* 1997; Turgeon 1998).

More than 1800 species names are in the genus Mycosphaerella, including synonyms. At least 500 are recognized as true species (Corlett 1991), some of which cause huge economic losses in important cash crops. For example, sigatoka disease in banana is caused by *M. fijiensis* and *M. musicola*; ringspot disease of *Brassica* spp. is caused by *M. brassicicola*; strawberry leaf spot is caused by *M. fragariae*; and several Mycosphaerella species cause leaf spot diseases on Eucalyptus, Populus, and other tree species (Crous 1998). Septoria tritici leaf blotch is the major disease of wheat in Western Europe and is of considerable importance worldwide in areas with a temperate, high-rainfall environment during the wheat-growing season, such as the Southern Cone of South America (Van Ginkel and Rajaram 1993; Cowger *et al.* 2000). The causal agent, *Septoria tritici*, was first described by Desmazières (1842). One hundred thirty years later Sanderson identified the ascomycete *Mycosphaerella graminicola* as the sexual stage (teleomorph) of *S. tritici* (Sanderson 1972, 1976). The disease is characterized by necrotic blotches on the foliage, which contain asexual (pycnidia) and sexual (pseudothecia) fruiting structures. Recent studies revealed that *M. graminicola* populations are highly diverse and suggested that sexual reproduction has a major influence on the genetic structure of *M. graminicola* populations (McDonald *et al.* 1999).

Genetic analyses were not possible until we developed a crossing protocol for this haploid ascomycete, which showed that *M. graminicola* has a heterothallic, bipolar

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mating system (Kema *et al.* 1996b). Furthermore, AFLP and RAPD markers segregated according to Mendelian expectation so it is now possible to construct a genetic linkage map (Kema *et al.* 1996b, 2000). Genetic maps could help to answer important questions about the biology of *M. graminicola.* For example, the nature of the interaction between *M. graminicola* and its wheat host has been controversial. Although some researchers have concluded that resistance to this pathogen in wheat and, conversely, avirulence in the pathogen are quantitative traits without specific interactions, others revealed specific interactions between wheat cultivars and particular isolates of the pathogen (Kema *et al.* 1999). The basis for such specific differences between pathogen isolates could now be addressed in genetic experiments. Recently, we have identified a single avirulence locus in *M. graminicola* (Kema *et al.* 2000).

The objective of this study was to generate a high-density genetic linkage map of the *M. graminicola* genome by integrating AFLP and RAPD data and to map and physically locate loci involved in mating and host-cultivar specificity to assist positional cloning of these and other genes of interest.

MATERIALS AND METHODS

Fungal isolates and growth conditions: Two Dutch isolates of *M. graminicola* with opposite mating types were crossed according to the protocol described by Kema *et al.* (1996b). IPO323 was isolated in 1981 from the soft white winter wheat cv. Arminda, has mating type *MAT1-1*, and is avirulent on the differential cultivars Veranopolis, Kavkaz, and Shafir. IPO94269 was isolated as a single ascospore discharged from wheat stubble in 1994. It has mating type *MAT1-2* and is virulent on the aforementioned differential cultivars. The pathogenicity of both isolates has been tested intensively under growth room and field conditions (Kema and Van Silfhout 1997; Kema *et al.* 2000; Brown *et al.* 2001). Following co-inoculation of the susceptible cv. Obelisk with both isolates, infected leaves were stimulated to discharge ascospores into sterile water agar plates (Kema *et al.* 1996b). Germinated ascospores were isolated individually with a sterile needle, transferred to 2% V8-juice agar plates, and incubated at 20° under continuous light. Individual colonies were transferred to liquid YG medium (3% yeast and 1% glucose) and incubated in a reciprocal shaker at 18° for ~5 days. The resulting spores were collected, concentrated by centrifugation, and maintained at −80° and on silica gel at −20° in freezers. The total progeny population consists of 202 isolates derived from a single cross. Among these, a subset of 68 isolates was chosen for further analyses.

Bioassays and mating type identification: Bioassays and segregation analysis for avirulence to a number of wheat cultivars in this population were reported elsewhere (Kema *et al.* 1996a, 2000). The mating types of 68 progeny were determined by PCR with mating type-specific primers (Waalwijk *et al.* 2002) and by backcrossing the progeny to both parental strains. Progeny that produced ascospores with the *MAT1-2* but not the *MAT1-1* parent were scored *MAT1-1*; those that produced ascospores with the *MAT1-1* but not the *MAT1-2* parent were scored *MAT1-2.*

DNA isolation: For AFLP analysis, isolates were cultured in liquid YG medium for 5 days at 18°. Spores were allowed to sediment overnight and were subsequently collected and lyophilized in cryotubes for 48 hr. DNA from all isolates was extracted using the Puregene DNA isolation kit D6000 (Gentra Systems, Minneapolis) according to the manufacturer's instructions. Tissue for RAPD analysis was grown in 250-ml Erlenmeyer flasks containing 100 ml yeast maltose broth (4 g yeast extract, 4 g malt extract, 4 g sucrose/liter distilled water) at room temperature (22°–24°) on a shaker rotating at 180 rpm. Each flask was inoculated with a lyophilized filter paper disc containing mycelia and spores taken from an actively growing culture. Tissue was harvested after 12 days (occasionally longer for slow-growing isolates) by vacuum filtration, frozen at −80° for at least 30 min, and lyophilized. DNA was extracted essentially as described by Biel and Parrish (1986), except three additional phenol:chloroform extractions were performed instead of the Sephadex-column purification step. Extracted DNA was quantified with a Hoefer DyNAQuant 2000 fluorometer (Hoefer Pharmacia Biotech).

AFLP and RAPD genotyping: The initial AFLP protocol was as described by Vos *et al.* (1995). DNA samples were digested with the restriction enzymes *Eco*RI and *Msp*I. The latter enzyme was chosen because digests with various restriction enzymes suggest a relatively high CG content of *M. graminicola,* so *Msp*I should cleave more sites than commonly used alternatives such as *Mse*I, which is specific for AT-rich regions. After screening the parental isolates for optimal polymorphisms with 64 primer combinations, 11 were chosen for the mapping analysis (Table 1). The combinations were screened on the subset of the mapping population following another AFLP protocol in which primers labeled with fluorescent dyes were processed in a thermocycler and analyzed on automated sequencing equipment (Bonants *et al.* 2000).

RAPD analysis was done essentially as described by Williams *et al.* (1990). PCR reactions (25 μl) contained 7.9 μl deionized H_2O, 2.5 μl 0.01% gelatin, 2.5 μl 10× buffer, 2.5 μl 2.0 mm dNTPs, 3.5 μl 25 mm $MgCl_2$, 0.1 μl AmpliTaq DNA polymerase, 5 μl 2.0 μm primer, and 1.0 μl template DNA (12.5 ng/μl). PCR amplifications were for 1 min at 94° and then 45 cycles of 94° for 1 min, 37° for 1 min, and 72° for 2 min, followed by 7 min at 72°. In total, 240 primers were screened from Operon primer kits I, J, K, L, M, N, Q, R, S, T, U, and W (Operon Technologies, Alameda, CA).

Amplification products were loaded onto 1.2% agarose gels, run at 150 V for 2 hr and 20 min in 0.5× TBE or TAE buffer, and stained with ethidium bromide. Gel pictures were taken with an electronic photo imaging system (Fotodyne, New Berlin, WI). Fragment sizes were estimated by comparison with a 1-kb DNA ladder (Life Technologies, Grand Island, NY) using Collage image analysis software from Fotodyne. Amplifications were performed in a Perkin-Elmer (Norwalk, CT) GeneAmp PCR system 9600.

Data analysis and mapping: AFLP and RAPD markers are designated by the primer combination used for the amplification and the approximate length of the generated fragment. The length of each AFLP marker was estimated by a fluorescently labeled 50-bp ladder (Pharmacia, Piscataway, NJ) and data analysis was performed using ImageMaster 1D Elite (Pharmacia) software. The prefix A or B indicates the origin of the marker; those originating from parent IPO323 have the prefix A while markers from parent IPO94269 are indicated by the prefix B.

Segregation ratios of all markers were analyzed with JoinMap (Stam 1993), version 2.0. Markers with segregation ratios significantly different from 1:1 ($P < 0.05$) were set aside because initial mapping analysis showed that they may cause erroneous grouping of unlinked markers.

Linkage analysis was performed on the remaining markers. Initial assignment to linkage groups was based on the loga-

rithm of the odds (LOD) ratio for each possible marker pair. The LOD value indicates the likelihood of linkage by comparing the probabilities of random association of markers in the progeny to association caused by linkage. We used LOD values in the range of 3–8, whereas the final assembly of linkage groups was completed using a LOD value of 4.5. In the second step, the calculation of a linear order of markers within a linkage group, we used a map LOD value of 0.01 and the Kosambi mapping function. JoinMap 2.0 also offers the possibility to set aside markers, which are difficult to position within a linkage group on the basis of a chi-square test comparing the direct distance of markers with their mapping distance. We used a chi-square threshold of 5, beyond which markers are not positioned on the map. Finally, we tested if the markers with a distorted segregation ratio could contribute to the map. Because most of these markers either were not linked at a LOD threshold of 4.5 or were mapped to previously identified linkage groups only with difficulty, these aberrantly segregating markers were not included in the final map.

Bulked segregant analysis: Bulked segregant analysis (Michelmore *et al.* 1994) was used to identify additional markers closely linked to the avirulence locus. The two bulks were composed of equal amounts of DNA from 34 virulent isolates and 33 avirulent isolates, respectively. The parents and both bulks were screened with the 64 AFLP primer combinations mentioned above. Primer combinations that identified differences between the bulks were then used to analyze the progeny individually and linkage analysis was performed as described above.

Pulsed-field gel electrophoresis: Contour-clamped homogeneous field electrophoresis (Bio-Rad DRII, Richmond, CA) was used to analyze the electrophoretic karyotypes of both parents. Chromosomes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were used as chromosomal size markers (Bio-Rad). The chromosomes in the small size ranges were separated on 1% SeaKem Gold agarose at 200 V and 14° with pulses increasing from 50 to 90 sec for 24 hr, whereas medium-sized chromosomes were separated at 100 V and 9° with pulses of 3000 sec for 8 hr followed by pulses of 500 sec for 64 hr. Large chromosomes were separated at 45 V and 9° on 0.8% SeaKem Gold agarose using the following consecutive conditions: electrophoresis started with pulses increasing from 600 to 900 sec for 24 hr and continued with pulses increasing from 1500 to 2700 sec for 48 hr, pulses increasing from 2700 to 3600 sec for 96 hr, and finally pulses increasing from 60 to 90 sec for 15 hr at 100 V.

Southern hybridization: Pulsed-field gels were hybridized to physically map the *MAT* and *AVR* loci of *M. graminicola.* An 850-bp PCR fragment from the *MAT1-2* gene of *Tapesia yallundae*, provided by Dr. P. Dyer (University of Nottingham, United Kingdom), was random-prime labeled (Sambrook *et al.* 1989) and used as a probe to map the *MAT* locus (Waalwijk *et al.* 2002). A 145-bp AFLP marker identified by the bulked segregant analysis, designated AVIR2, was isolated, cloned in *Escherichia coli* strain XL-1blue using pGEM-T as cloning vector, radiolabeled as mentioned above, and hybridized to pulsed-field gel blots to physically map the *AVR* locus on one of the *M. graminicola* chromosomes.

RESULTS

AFLP and RAPD analysis of the mapping population: Comparison of the DNA fingerprints identified seven pairs of "twin" genotypes that probably represent genetically identical individuals generated during the mitosis that occurs just before ascospore formation. The presence of these twins enabled us to estimate the reliability of the marker scoring, which was 99.89 and 95.63% for AFLP markers and RAPD markers, respectively. One member of each twin pair was deleted from the final mapping data set, which was thus composed of 61 remaining progeny with different recombination patterns informative for linkage analysis. Application of the 11 selected AFLP primer combinations resulted in a total of 1056 AFLP fragments of which 498 (47%) were polymorphic between the parental isolates (Table 1). All these markers segregated in the progeny; however, only 271 AFLP markers that could be scored clearly on nearly all 61 progeny were used for further analysis. Forty-one of the 240 RAPD primers tested gave scorable polymorphisms between the parents of the mapping population, with an average of 2.5 fragments per primer, resulting in 104 RAPD markers. In the single-marker analyses, 16 AFLP and 20 RAPD markers showed significant deviation from the expected 1:1 segregation ratio. These markers were omitted from further analysis. Hence, eventually 84 RAPD and 255 AFLP markers as well as mating type and avirulence on the wheat cultivars Veranopolis, Kavkaz, and Shafir were used on 61 progeny isolates to construct the genetic linkage map.

Linkage and mapping analysis: Using a LOD threshold value of 4.5, 302 of the 341 markers analyzed (nearly 90%) showed linkage to at least 3 other markers, allowing reliable mapping by three-point analysis. As expected, all linkages were in coupling phase if markers originated from the same parent and in repulsion phase if markers were from different parental origin. This indicates that a LOD value of 4.5 is sufficiently selective to discriminate between true linkage and experimental noise. After the markers were assembled into linkage groups they were ordered within the linkage groups using a chi-square jump restriction of five. Nine AFLP and 11 RAPD markers caused larger chi-square jumps and were, therefore, not positioned on the map.

Hence, eventually 282 markers were positioned on the map in 23 linkage groups (Figure 1). The average spacing between markers is 4.3 cM, but there is a significant clustering of markers, which is particularly evident for the AFLP markers (Figure 1).

Avirulence and mating types: The genetics of avirulence in *M. graminicola* isolate IPO323 to the wheat cvs. Shafir, Kavkaz, and Veranopolis was recently discussed (Kema *et al.* 2000) and appeared to be controlled by a single locus ($\chi^2 = 0.15$, $P = 0.95$–0.90; Table 2). This avirulence locus mapped to linkage group 22 along with four AFLP markers (Figure 1).

Backcrossing to both parents successfully identified the mating type of progeny isolates (Table 2). In all cases, ascospores were produced only from crosses with either one or the other parent. *MAT* genotypes were later successfully verified using *MAT*-specific PCR primers (Waalwijk *et al.* 2002). Both mating type idi-

TABLE 1

Distribution of AFLP fragments in the parental *M. graminicola* isolates IPO323 and IPO94269 generated by the 11 primer combinations used in the F_1 mapping population

Primer extensions		No. of AFLP fragments			
*Eco*RI	*Msp*I	IPO323 specific	IPO94269 specific	IPO323 and IPO94269	Total
AT	AG	16	20	43	79
AT	CG	32	10	44	86
GA	AC	26	20	52	98
GA	AG	27	22	63	112
GA	CC	17	17	51	85
GA	CA	30	23	47	100
GA	CG	34	31	63	128
GC	CA	22	26	55	103
GG	AT	14	25	47	86
GG	CG	18	19	52	89
GT	AT	26	23	41	90
Total		262	236	558	1056

omorphs appeared in nearly equal frequencies (χ^2 = 2.20; P = 0.5–0.3; Table 2), confirming the heterothallic, bipolar nature of the mating system in *M. graminicola* (Kema *et al.* 1996b). The *MAT* locus mapped to linkage group 16, which also contained four AFLP and two RAPD markers (Figure 1).

Bulked segregant analysis: Due to the size of the bulks only two additional AFLP markers were generated by bulked segregant analysis, one with primer combination *Eco*RI + GA/*Msp*I + CA (AVIR1, 122 bp) and one with primer combination *Eco*RI + AT/*Msp*I + CA (AVIR2, 145 bp). Both markers cosegregated perfectly with the avirulence locus (Figure 1).

Electrophoretic karyotypes: Pulsed-field gel electrophoresis (PFGE) under different conditions revealed chromosomal polymorphisms between the parental strains (Figure 2). The maximal count of chromosomal bands in IPO323 was 15, whereas in IPO94269 13 bands were visible. The largest chromosomal band of the PFGE gels coincides with the compression zone that, as a result of the applied PFGE technique, usually contains a variety of chromosomal fragments and thus most likely is not an individual chromosome. Southern analysis revealed a discrepancy between physical and genetic linkage. Linkage groups 16 and 22 appear to be among the smallest of the entire genome (Figure 1). However, Southern analyses with the *MAT1-2* probe of *T. yallundae* as well as with a probe derived from AVIR2 showed that both loci are indeed on different, large chromosomes of the *M. graminicola* genome (Figure 3).

DISCUSSION

Despite the economic importance of plant pathogenic fungi, the number of genetic maps available remains limited. Among the four major groups of true fungi, genetic linkage maps are available for only 6 species of basidiomycetes, 1 zygomycete, 1 chytridiomycete, and 13 ascomycetes (O'Brien 1993; Xu and Leslie 1996; Chibana *et al.* 1998; Cozijnsen *et al.* 2000; Forche *et al.* 2000). Among these 21, only 8 are plant pathogens. Current taxonomy divides the Ascomycetes into 46 orders (Hawksworth *et al.* 1995), among which Saccharomycetales is represented by the complete genome sequence of the yeast *S. cerevisiae* (Goffeau *et al.* 1996). However, genetic linkage maps are available only for representatives from 6 of the remaining 45 orders. Within the Dothideales, the only map available is from *Cochliobolus heterostrophus* (Tzeng *et al.* 1992), but that species is in a different family from Mycosphaerella (Hawksworth *et al.* 1995). The present results provide the first genetic map for any species within Mycosphaerella and also within the family Mycosphaerellaceae. The genus Mycosphaerella contains over 500 spe-

Figure 1.—Genetic linkage map of the *M. graminicola* genome. Markers beginning with A originated from the IPO323 parent; those beginning with B were from the IPO94269 parent. AFLP markers are indicated by their origin followed by the *Eco*RI (E) and *Msp*I (Mp) primer combination and the size of the marker, *e.g.*, (white "1" in black circle) AFLP marker (150 bp) on linkage group 21 originates from IPO323 and is therefore designated as AEGAMpCA, 150. RAPD markers are indicated by the primer set and number followed by a dash and the approximate size of the fragment in base pairs, *e.g.*, BM14-918 is a 918-bp fragment originating from IPO94269 using Operon primer M14. The (black star) *MAT* and (white star) *AVR* locus mapped to linkage groups 16 and 22, respectively.

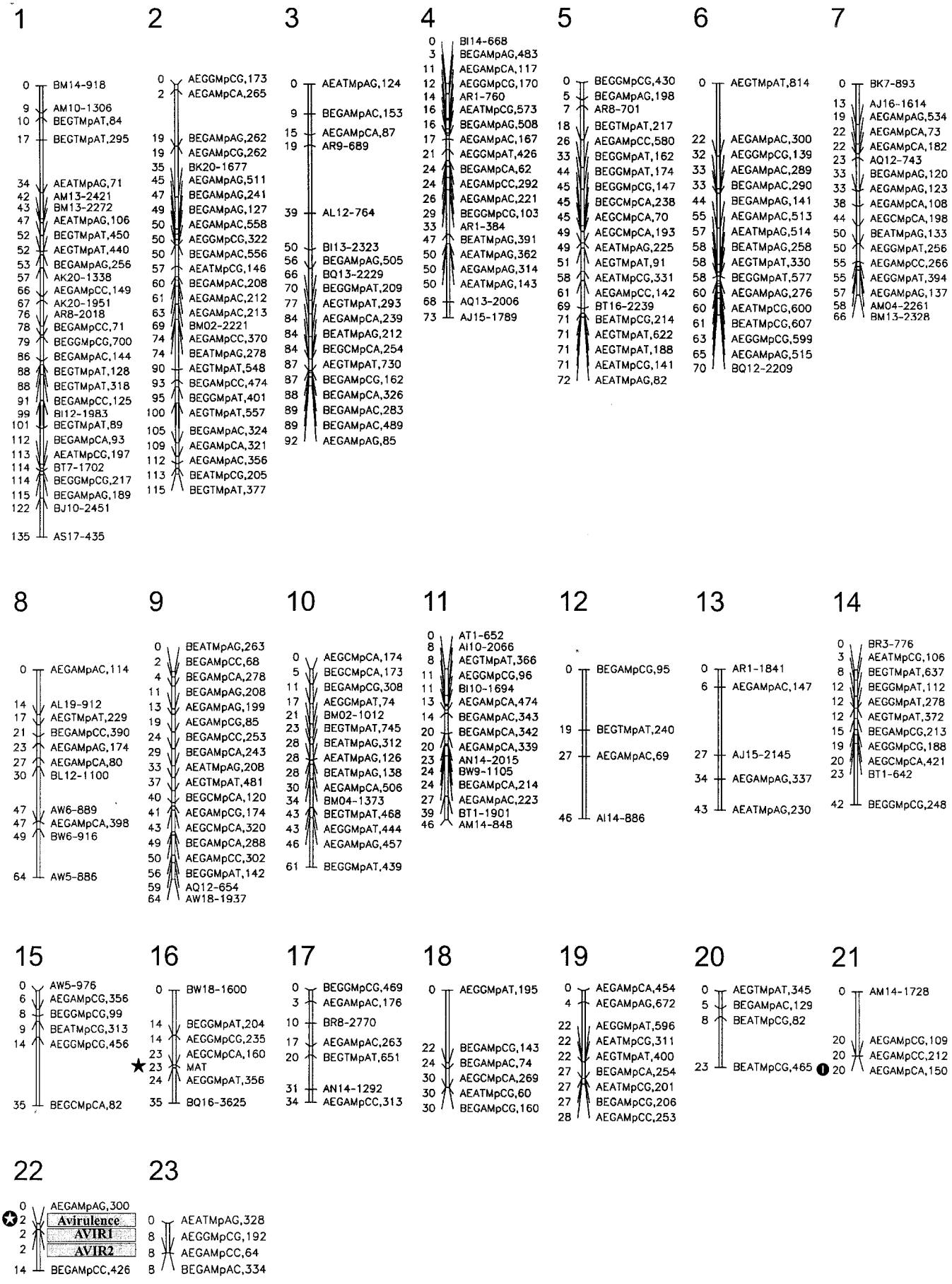
1
0 BM14-918
9 AM10-1306
10 BEGTMpAT,84
17 BEGTMpAT,295
34 AEATMpAG,71
42 AM13-2421
43 BM13-2272
47 AEATMpAG,106
52 BEGTMpAT,450
52 AEGTMpAT,440
53 BEGAMpAG,256
57 AK20-1338
66 AEGAMpCC,149
67 AK20-1951
76 AR8-2018
78 BEGAMpCC,71
79 BEGGMpCG,700
86 BEGAMpAC,144
88 BEGTMpAT,128
88 BEGTMpAT,318
91 BEGAMpCC,125
99 BI12-1983
101 BEGTMpAT,89
112 BEGAMpCA,93
113 AEATMpCG,197
114 BT7-1702
114 BEGGMpCG,217
115 BEGAMpAG,189
122 BJ10-2451
135 AS17-435
2
0 AEGGMpCG,173
2 AEGAMpCA,265
19 BEGAMpAG,262
19 AEGAMpCG,262
35 BK20-1677
45 AEGAMpAG,511
47 BEGAMpAG,241
49 BEGAMpAG,127
50 AEGAMpAC,558
50 AEGGMpCG,322
50 BEGAMpAC,556
57 AEATMpCG,146
60 BEGAMpAC,208
61 AEGAMpAC,212
63 AEGAMpAC,213
69 BM02-2221
74 AEGAMpCC,370
74 BEATMpAG,278
90 AEGTMpAT,548
93 BEGAMpCC,474
95 BEGGMpAT,401
100 AEGTMpAT,557
105 BEGAMpAC,324
109 AEGAMpCA,321
112 AEGAMpAC,356
113 BEATMpCG,205
115 BEGTMpAT,377
3
0 AEATMpAG,124
9 BEGAMpAC,153
15 AEGAMpCA,87
19 AR9-689
39 AL12-764
50 BI13-2323
56 BEGAMpAG,505
66 BQ13-2229
70 BEGGMpAT,209
77 AEGTMpAT,293
84 AEGAMpCA,239
84 BEATMpAG,212
84 BEGCMpCA,254
87 AEGTMpAT,730
87 BEGAMpCG,162
88 BEGAMpCA,326
89 BEGAMpAC,283
89 BEGAMpAC,489
92 AEGAMpAG,85
4
0 BI14-668
3 BEGAMpAG,483
11 AEGAMpCA,117
12 AEGGMpCG,170
14 AR1-760
16 AEATMpCG,573
16 BEGAMpAG,508
17 AEGAMpAC,167
21 AEGGMpAT,426
24 BEGAMpCA,62
24 AEGAMpCC,292
26 AEGAMpAC,221
29 BEGGMpCG,103
33 AR1-384
47 BEATMpAG,391
50 AEATMpAG,362
50 AEGAMpAG,314
50 AEATMpAG,143
68 AQ13-2006
73 AJ15-1789
5
0 BEGGMpCG,430
5 BEGAMpAG,198
7 AR8-701
18 BEGTMpAT,217
26 AEGAMpCC,580
33 BEGGMpAT,162
44 BEGGMpAT,174
45 BEGGMpCG,147
45 BEGCMpCA,238
45 AEGCMpCA,70
49 AEGCMpCA,193
49 AEATMpAG,225
51 AEGTMpAT,91
58 AEATMpCG,331
61 AEGAMpCC,142
69 BT16-2239
71 BEATMpCG,214
71 AEGTMpAT,622
71 AEGTMpAT,188
71 AEATMpCG,141
72 AEATMpAG,82
6
0 AEGTMpAT,814
22 AEGAMpAC,300
32 AEGGMpCG,139
33 AEGAMpAC,289
33 BEGAMpAC,290
44 BEGAMpAG,141
55 AEGAMpAC,513
57 AEATMpAG,514
58 BEATMpAG,258
58 AEGTMpAT,330
58 BEGGMpAT,577
60 AEGAMpAG,276
60 AEATMpCG,600
61 BEATMpCG,607
63 AEGGMpCG,599
65 AEGAMpAG,515
70 BQ12-2209
7
0 BK7-893
13 AJ16-1614
19 AEGAMpAG,534
22 AEGAMpCA,73
22 AEGAMpCA,182
23 AQ12-743
33 BEGAMpAG,120
33 AEGAMpAG,123
38 AEGAMpCA,108
44 AEGCMpCA,198
50 BEATMpAG,133
50 AEGGMpAT,256
55 AEGAMpCC,266
55 AEGGMpAT,394
57 AEGAMpAG,137
58 AM04-2261
66 BM13-2328
8
0 AEGAMpAC,114
14 AL19-912
17 AEGTMpAT,229
21 BEGAMpCC,390
23 AEGAMpAG,174
27 AEGAMpCA,80
30 BL12-1100
47 AW6-889
47 AEGAMpCA,398
49 BW6-916
64 AW5-886
9
0 BEATMpAG,263
2 BEGAMpCC,68
4 BEGAMpCA,278
11 BEGAMpAG,208
13 AEGAMpAG,199
19 AEGAMpCG,85
24 BEGAMpCC,253
29 BEGAMpCA,243
33 AEATMpAG,208
37 AEGTMpAT,481
40 BEGCMpCA,120
41 AEGAMpCG,174
43 AEGCMpCA,320
49 BEGAMpCA,288
50 AEGAMpCC,302
56 BEGGMpAT,142
59 AQ12-654
64 AW18-1937
10
0 AEGCMpCA,174
5 BEGCMpCA,173
11 BEGAMpCG,308
17 AEGGMpAT,74
21 BM02-1012
23 BEGTMpAT,745
28 BEATMpAG,312
28 AEATMpAG,126
28 BEATMpAG,138
30 AEGAMpCA,506
34 BM04-1373
43 BEGTMpAT,468
43 AEGGMpAT,444
46 AEGAMpAG,457
61 BEGGMpAT,439
11
0 AT1-652
8 AI10-2066
8 AEGTMpAT,366
11 AEGGMpCG,96
11 BI10-1694
13 AEGAMpCA,474
14 BEGAMpAC,343
20 BEGAMpCA,342
20 AEGAMpCA,339
23 AN14-2015
24 BW9-1105
24 BEGAMpCA,214
27 AEGAMpAC,223
39 BT1-1901
46 AM14-848
12
0 BEGAMpCG,95
19 BEGTMpAT,240
27 AEGAMpAC,69
46 AI14-886
13
0 AR1-1841
6 AEGAMpAC,147
27 AJ15-2145
34 AEGAMpAG,337
43 AEATMpAG,230
14
0 BR3-776
3 AEATMpCG,106
8 BEGTMpAT,637
12 BEGGMpAT,112
12 AEGGMpAT,278
12 AEGTMpAT,372
15 BEGAMpCG,213
19 AEGGMpCG,188
20 AEGCMpCA,421
23 BT1-642
42 BEGGMpCG,248
15
0 AW5-976
6 AEGAMpCG,356
8 BEGGMpCG,99
9 BEATMpCG,313
14 AEGGMpCG,456
35 BEGCMpCA,82
16
0 BW18-1600
14 BEGGMpAT,204
14 AEGGMpCG,235
23 AEGCMpCA,160
★ 23 MAT
24 AEGGMpAT,356
35 BQ16-3625
17
0 BEGGMpCG,469
3 AEGAMpAC,176
10 BR8-2770
17 AEGAMpAC,263
20 BEGTMpAT,651
31 AN14-1292
34 AEGAMpCC,313
18
0 AEGGMpAT,195
22 BEGAMpCG,143
24 BEGAMpAC,74
30 AEGCMpCA,269
30 AEATMpCG,60
30 BEGAMpCG,160
19
0 AEGAMpCA,454
4 AEGAMpAG,672
22 AEGGMpAT,596
22 AEATMpCG,311
22 AEGTMpAT,400
27 BEGAMpCA,254
27 AEATMpCG,201
27 BEGAMpCG,206
28 AEGAMpCC,253
20
0 AEGTMpAT,345
5 BEGAMpAC,129
8 BEATMpCG,82
23 BEATMpCG,465
21
0 AM14-1728
20 AEGAMpCG,109
20 AEGAMpCC,212
20 AEGAMpCA,150
22
0 AEGAMpAG,300
✪ 2 Avirulence
2 AVIR1
2 AVIR2
14 BEGAMpCC,426
23
0 AEATMpAG,328
8 AEGGMpCG,192
8 AEGAMpCC,64
8 BEGAMpAC,334

TABLE 2

(A)virulence phenotypes and *MAT* genotypes in the *M. graminicola* F_1 mapping population ($N = 61$) derived from a cross between isolates IPO323 and IPO94269

(A)virulence phenotypes	*MAT* genotypes[a]	N[b]	F_1 isolates
Virulent	*MAT1-1*	16	1, 22, 23, 24, 27, 51,[c] 83, 90, 91, 134, 136, 158, 167, 180, 182, 184
	MAT1-2	16	12,[c] 40, 47, 62, 68, 73, 87, 124, 126, 142,[c] 176, 179, 183, 192,[c] 198,[c] 202
Avirulent	*MAT1-1*	19	14, 50, 88, 94,[c] 100, 115, 117, 119, 125, 131, 132, 137, 147, 157, 160, 164, 174, 193, 197
	MAT1-2	10	10, 29, 84, 95, 109, 111, 118, 144, 173, 200

[a] Mating types were identified by backcrossing to *MAT1-1* (IPO323) and *MAT1-2* (IPO94269) tester isolates, as well as by PCR amplification of *MAT*-specific sequences (Waalwijk *et al.* 2002).

[b] Segregation ratios for virulent:avirulent (32:29; $\chi^2 = 0.15$, $P = 0.95$–0.90) and *MAT1-1*:*MAT1-2* (35:26; $\chi^2 = 2.20$, $P = 0.5$–0.3) accord with the expected 1:1 ratio for a single segregating character.

[c] *MAT* genotypes were determined by PCR amplification only.

cies (Corlett 1991), most of which are pathogenic on one or more plant hosts, including both monocots and dicots, gymnosperms, ferns, horsetails, and lycopods (Farr *et al.* 1995). As mentioned before, *M. graminicola* is a major wheat disease with a large annual economic impact in Western Europe. A linkage map of this organism facilitates the elucidation of crucial biological aspects of the pathosystem, such as avirulence, pathogenicity, and mating. This map, together with other indispensable tools, such as a crossing and transformation protocol as well as high throughput genomic analyses, make *M. graminicola* a useful model for other host-pathogen relationships in the Dothideales.

AFLP analysis was a fast way to generate a large number of polymorphic markers that provided the overall framework for the map. However, these markers were often clustered, which reduced the overall length of the final map. Similar clustering of AFLP markers has been reported in many other species, including animals (Young *et al.* 1998), plants (Spada *et al.* 1998; Boivin *et al.* 1999; Remington *et al.* 1999), and oomycetes (Van der Lee *et al.* 1997). Clustering of AFLP markers in plants is often localized at centromeric regions (Qi *et al.* 1998; Haanstra *et al.* 1999). If the same phenomenon occurs in *M. graminicola*, then the probable positions of the centromeres can be inferred for about half of the 23 linkage groups.

The large number of AFLP markers permitted easy identification of identical progeny that subsequently could be removed from the analysis. In *M. graminicola* twin progeny isolates may arise from the mitotic division that follows meiosis II in the ascus, giving four pairs of genetically identical ascospores. Genetically identical isolates according to the AFLP analyses in this study usually had consecutive or near-consecutive numbers, indicating that they were isolated from the same part of the water-agar plate. This is consistent with the suggested simultaneous ejection of ascospores from the same ascus (Kema *et al.* 1996b).

In our study, AFLP markers appeared to be more reliable than RAPD markers. Therefore, RAPD markers are less likely to cluster and JoinMap tends to position

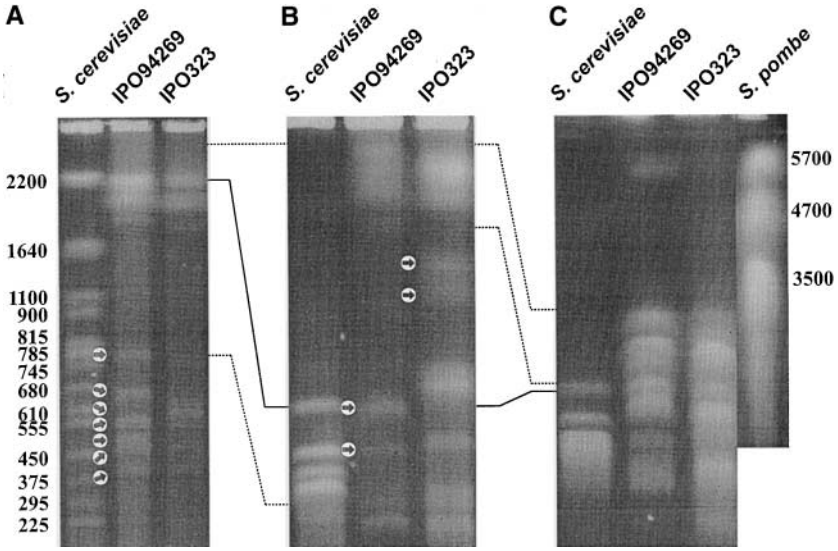

Figure 2.—Estimation of the number of chromosomes of the *M. graminicola* genome by pulsed-field gel electrophoresis. (A) Small, (B) medium, and (C) large chromosomes were separated as described in materials and methods.

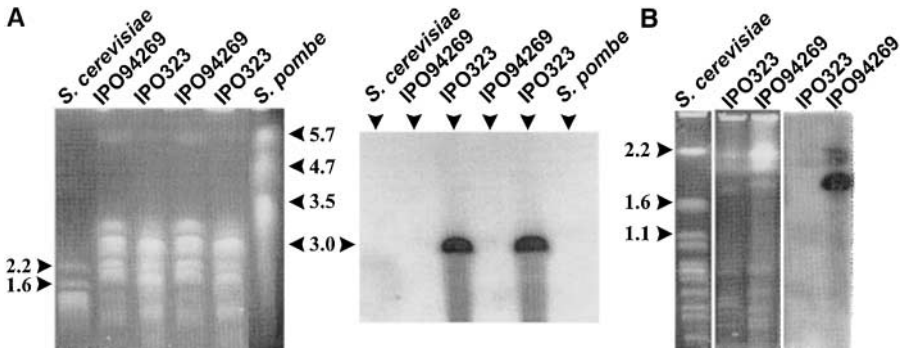

FIGURE 3.—Physical mapping of *AVR* and *MAT* loci to some of the largest *M. graminicola* chromosomes by Southern analysis of PFGE filters. (A) Hybridization with probe AVIR2, which is derived from a perfectly cosegregating AFLP marker, is shown. The probe hybridizes to a chromosome of ~3.0 Mb in *M. graminicola* isolate IPO323 but not to any chromosome in isolate IPO94269. (B) Hybridization with a heterologous probe from the fungus *T. yallundae* specific for the HMG box of the *MAT1-2* allele is shown. The probe hybridizes to a chromosome of ~1.8 Mb in size.

them at the end of linkage groups to cope with the friction created by the scoring errors. Indeed, we observed that RAPD markers were at the ends of 13 of the 18 linkage groups in which they occurred. This suggests that RAPDs inflated the total mapped region of the genome by ~22%, from 995 cM with the AFLP markers alone to 1216 cM for the combined map. Hence, differences in the reliability of different marker types may lead to an overestimation of the mapped genomic region. Nevertheless, inclusion of multiple marker types may be critical for thorough genome coverage. Although almost four times as many AFLP as RAPD markers were on the map, RAPD markers were present on the 17 largest linkage groups and on 18 of the 23 groups in total.

We observed a significant discrepancy between the number of linkage groups and the estimated number of chromosomes. The total number of chromosomes in *M. graminicola* is not known for certain. In our study 13–15 chromosomes could be discerned, whereas 17 or 18 chromosomes were observed by McDONALD and MARTINEZ (1991). Although comigration of similar-sized chromosomes is a legitimate explanation of the observed discrepancy, we expect that some of the smallest linkage groups probably will be joined with others once additional markers are added to the map. This was supported by Southern analyses of the PFGE gels using probes for the mating type and avirulence loci that hybridized to two of the larger chromosome bands, despite these loci being mapped on two of the smallest linkage groups. Differences may exist among electrophoretic karyotypes of individual isolates within species (COOLEY and CATEN 1991; KEMA *et al.* 1999). However, in *M. graminicola* the difference in numbers and sizes of the chromosomes apparently does not preclude sexual recombination and viability of the offspring. So far, generated segregating populations only erratically contained few nongerminating ascospores (this study and KEMA *et al.* 2000).

The current map density in the relatively small *M. graminicola* genome is sufficiently high to identify genetic and physical linkages with the biological traits analyzed in this study. On average, the current map contains one marker every 4.3 cM. However, this overestimates the functional density of the map. If cosegregating markers are reduced to 1 per cluster, the number of independent markers on the map is 229, or 1 every 5.3 cM. Also, 39 of the 302 markers (12%) were unlinked to the linkage groups, indicating that parts of the genome contain low marker densities. Due to the strong clustering of markers it is difficult to calculate the total genome size on the basis of these mapping data.

The mapping process effectively identified molecular markers linked to the mating type and avirulence loci. Subsequent bulked segregant analysis identified several additional markers. Inclusion of >30 progeny in each bulk resulted in markers cosegregating with avirulence. The recent cloning of the mapped mating type gene (WAALWIJK *et al.* 2002) and the progress in cloning the avirulence locus demonstrate the potential utility of the map for map-based cloning of selected genes. This approach now can be used to identify markers linked to any gene that segregates in this cross.

Little is known about syntenic relationships among fungal genetic maps. This is mainly due to the small number of species that have been compared and the lack of common marker sets mapped in multiple species. A common set of markers is greatly needed to test for synteny among fungal genomes. Recent sequence analyses have shown a high degree of synteny between the genetic maps of *S. cerevisiae* and *Ashbya gossypii* in the Saccharomycetales (DIETRICH *et al.* 1999). The Neurospora genome project resulted in the first entire DNA sequence of a filamentous fungus, which is of huge interest for synteny studies among this group of organisms (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). As synteny among fungal species in the same order may be high, the current map of *M. graminicola* can be used to develop anchor points for comparative mapping in other species within the Dothideales. Knowledge of syntenic relationships provides information about gene clusters in general, but synteny near the avirulence locus of *M. graminicola* might particularly provide information about the evolution of host-patho-

gen relationships in closely related species that are destructive pathogens of important crop hosts.

The results of this study confirmed that avirulence to the cultivars Kavkaz, Shafir, and Veranopolis is under single-gene control and that a single locus may control pathogenicity to all three (Kema *et al.* 2000). The ability to perform genetic analyses in the pathogen will complement analyses of resistance in wheat and for the first time will permit thorough analyses of host-pathogen interactions in this economically important pathosystem (Brading *et al.* 2002).

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