Research Note

Avirulence in the Wheat *Septoria tritici* Leaf Blotch Fungus *Mycosphaerella graminicola* Is Controlled by a Single Locus

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Accepted 4 September 2000.

Segregation of avirulence in *Mycosphaerella graminicola*, a heterothallic ascomycete that causes wheat septoria tritici leaf blotch, was studied in F1, BC1, and F2 populations by inoculation assays on five wheat cultivars in the seedling stage and by amplified fragment length polymorphism and random amplified polymorphic DNA analyses. F1 was generated by crossing isolates IPO323 (avirulent) and IPO94269 (virulent). All F1, BC1, and F2 progeny isolates were virulent on the susceptible check cultivar Taichung 29 and were avirulent on the resistant check cultivar Kavkaz-K4500. Avirulence segregation was observed in F1 and in several BC1 and F2 generations on the differential cultivars Shafir, Kavkaz, and Veranopolis at a 1:1 ratio. Avirulence for the three differential cultivars always cosegregated. We conclude that avirulence in isolate IPO323 is controlled by a single, seemingly complex locus.

Additional keywords: gene-for-gene, specificity.

Mycosphaerella graminicola (Fuckel) J. Schröt. in Cohn is a major disease in northwestern Europe (Polley and Thomas 1991; Van Ginkel and Rajaram 1993). For many years, the emphasis of research into the wheat–*M. graminicola* pathosystem has been on fungus epidemiology (Royle et al. 1986; Royle 1994; Sanderson et al. 1983; Shaw et al. 1993; Thomas et al. 1989). More recently, McDonald and colleagues elucidated fundamental aspects of the population genetics of *M. graminicola* (McDonald and Martinez 1990; McDonald et al. 1999). There has been less progress in understanding the interaction between host and pathogen. A few papers addressed the pathogenesis of *M. graminicola* in wheat (Cohen and Eyal 1993; Duncan and Howard *in press*; Hilu and Bever 1957; Kema et al. 1996d), which revealed that the fungus penetrates the host largely through the stomates. Colonization is characterized by a hemibiotrophic nature with intercellular growth and quantitative and qualitative modes of resistance expression. Disease scoring under field conditions is hampered by the fact that pathogen populations are extremely diverse and hardly anything is known about their composition and dy-

namics with respect to pathogenicity factors. Hence, breeding for *M. graminicola* resistance has been only partially successful and, to date, only a few resistance genes have been identified (Somasco et al. 1996).

We studied diversity in *M. graminicola* (Kema and Van Silfhout 1997; Kema et al. 1996a; Kema et al. 1996b; Kema et al. 1999). One of the major inferences from our studies is the specificity of the host-pathogen interaction at the species and cultivar levels, suggesting a gene-for-gene relationship. This subject has been debated since Eyal et al. (1973) proposed physiologic specialization in *M. graminicola*. This ongoing debate as to whether specificity occurs in the *M. graminicola*–wheat pathosystem is primarily a result of a lack of formal genetic proof for the hypotheses that have been put forward over the years (Johnson 1992). We therefore focused on the genetics of *M. graminicola* in order to enhance an understanding of pathogenicity in this fungus.

We produced an F1 ($n = 202$) by crossing two Dutch field isolates, as previously described (Kema et al. 1996c). IPO323 (*Mat1-1*) was isolated in 1981 from the commercial cultivar Arminda (Kema and Van Silfhout 1997), and it is highly specific to a range of wheat cultivars in seedling and adult plant experiments (Brown et al. *in press*; Kema and Van Silfhout 1997). IPO94269 (*Mat1-2*) is a single ascospore-derived isolate (Kema et al. 1996c), which is highly virulent to a range of commercial cultivars (G. H. J. Kema, *unpublished*). Sixty-eight F1 isolates were backcrossed to both parent isolates to determine mating types. BC1 ($n = 73$) and F2 ($n = 70$) generations were subsequently produced to verify observed F1 segregation ratios. All progenies were entirely viable, although sometimes a slight delay in germination of the ascospores occurred, which was probably a result of discharge timing differences.

Although *M. graminicola* is a bipolar heterothallic ascomycete that theoretically prevents self-fertilization, all populations were checked for authenticity with routine random amplified polymorphic DNA (RAPD) analyses (Fig. 1). DNA from all isolates was extracted with a Puregene D-6000 DNA Isolation Kit (Gentra Systems Inc., Minneapolis, MN, U.S.A.), in accordance with the manufacturer's instructions. Polymerase chain reactions were used to generate RAPD markers in a PTC-200 Thermal Cycler (MJ Research Inc., Waltham, MA, U.S.A.) with primer OPB-6 (TGCTCTGCC)

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(Operon Technologies Inc., Alameda, CA, U.S.A.), following a protocol described previously (Kema et al. 1996c). Subsequent amplified fragment length polymorphism (AFLP) analyses of the F1 in the framework of a mapping project (G. H. J. Kema, *unpublished*) with primer combination E19M15 (*Eco*-GA/*Msp*-CA) followed a protocol that calls for fluorescently labeled primers (Bonants et al. 1999). The analyses

detected seven twins that were excluded from the inheritance study reported here. Eventually 221 isolates (F1: $n = 78$; BC1: $n = 73$; F2: $n = 70$) were tested for avirulence.

Inoculum (10^7 spores/ml) was produced and inoculations were conducted following a previously reported protocol (Kema et al. 1996a) that was slightly modified. The parents and all progeny isolates were tested on bread wheat cultivars

Fig. 1. Segregation for random amplified polymorphic DNA markers generated with primer OPB-6 in two *Mycosphaerella graminicola* backcross populations that do not segregate for phenotypes in inoculation assays (☆ = recombinant isolates for the indicated amplicons, bp). BC1-2 = IPO94269*IPO69323.24. BC1-3 = IPO323*IPO69323.84.

Fig. 2. Inoculation assays of *Mycosphaerella graminicola* on wheat. Phenotypes of Dutch *M. graminicola* isolates IPO323 and IPO94269 on five bread wheat cultivars at 21 days after inoculation. Bar = 1 mm.

Taichung 29, Kavkaz/K4500, Veranopolis, Kavkaz, and Shafir. The first two cultivars are susceptible and resistant to IPO323 and IPO94269, respectively, and the other three differentiate these isolates (Fig. 2). Primary leaves were inoculated 10 days after planting, and plants were incubated as reported previously (Kema et al. 1996a). Symptom development was typical in all inoculation assays, which were repeated two or more times. Necrosis usually started at the leaf tips 3 to 4 days postinoculation (dpi). At 9 to 10 dpi, expanding necrosis developed and the first pycnidia formed throughout the leaf. Pycnidia formation was restricted to the necrotic area and was completed at 16 to 21 dpi. Disease observations were conducted by scoring at 21 dpi to determine whether a cultivar-isolate combination was compatible (complete necrosis with an abundance of pycnidia) or incompatible (none or very limited [tip] necrosis, without pycnidia), which could be distinguished easily (Fig. 2). The parental and all F1, BC1, and F2 progeny isolates were virulent on cultivar Taichung 29 and avirulent on cultivar Kavkaz/K4500. Progeny isolates were either avirulent or virulent on the differential cultivars Veranopolis, Kavkaz, and Shafir (Table 1).

Avirulence for each differential cultivar segregated according to a 1:1 ratio in the F1 ($\chi^2 = 1.85$, $P = 0.2$ to 0.1), indicating that avirulence in IPO323 is controlled by a single locus.

Interestingly, all progeny isolates showed merely parental phenotypes in the inoculation assays (Fig. 2). Thus, they were either virulent or avirulent on cultivars Veranopolis, Kavkaz, and Shafir. Nevertheless, the AFLP analyses (Fig. 3) confirmed that the F1 is a regularly segregating population. Avirulence and mating type inherited independently, resulting in virulent and avirulent F1 individuals that were either *Mat1-1* or *Mat1-2* (not shown), which enabled the generation of BC1 and F2 populations to confirm F1 segregation ratios for avirulence.

We expected avirulence segregation when virulent and avirulent isolates were crossed and no segregation when either two virulent or two avirulent isolates were intercrossed. BC1-1, BC1-2, and BC1-3 did not segregate and were entirely virulent (BC1-1 and BC1-2) or avirulent (BC1-3) on the three differential cultivars (Table 1). For RAPD markers, however, BC1-2 and BC1-3 clearly segregated independently from avirulence (Fig. 1). BC1-4 was generated by backcrossing an avirulent F1 progeny isolate to the virulent parent, segregated in a 1:1 fashion ($\chi^2 = 0.29$, $P = 0.7$ to 0.5) and, similar to the F1, merely showed parental phenotypes. In order to attain a second confirmation of these results, we generated the F2 populations. F2-1 and F2-2 resulted from crosses among virulent F1 isolates and were entirely virulent. F2-3 and F2-4

Table 1. Segregation of avirulence and virulence on five bread wheat cultivars in F1, BC1, and F2 populations of *Mycosphaerella graminicola*

Generations ^a	Type of cross ^b	No. of progeny isolates tested	Cultivars				
			Taichung 29	Kavkaz/K4500	Veranopolis	Kavkaz	Shafir
F1	AV	78	0/78 ^c	78/0	45/33	45/33	45/33
BC1-1	VV	19	0/19	19/0	0/19	0/19	0/19
BC1-2	VV	20	0/20	20/0	0/20	0/20	0/20
BC1-3	AA	20	0/20	20/0	20/0	20/0	20/0
BC1-4	AV	14	0/14	14/0	6/8	6/8	6/8
F2-1	VV	17	0/17	17/0	0/17	0/17	0/17
F2-2	VV	16	0/16	16/0	0/16	0/16	0/16
F2-3	AV	10	0/10	10/0	5/5	5/5	5/5
F2-4	AV	27	0/27	27/0	15/12	15/12	15/12

^a F1 = IPO323 × IPO94269; BC1-1 = IPO94269 × IPO69323.23; BC1-2 = IPO94269 × IPO69323.24; BC1-3 = IPO323 × IPO69323.84; BC1-4 = IPO94269 × IPO69323.164; F2-1 = IPO69323.23 × IPO69323.178; F2-2 = IPO69323.24 × IPO69323.176; F2-3 = IPO69323.24 × IPO69323.144; F2-4 = IPO69323.133 × IPO69323.144.
^b AV = avirulent × virulent; VV = virulent × virulent; AA = avirulent × avirulent.
^c Number of avirulent/virulent progeny isolates.

Fig. 3. Example of segregation for amplified fragment length polymorphism (AFLP) markers generated with primer combination *Eco*-GA/*Msp*-CA on 34 individuals from the F1 population derived from a cross between *Mycosphaerella graminicola* isolates IPO323 and IPO94269 **A**, Interpretation of AFLP phenotypes in genotypes. **B**, All possible genotypes are identified. Markers 1 (1:1 = 15:19, $\chi^2 = 0.47$, $P = 0.5$ to 0.3) and 3 (1:1 = 16:18, $\chi^2 = 0.12$, $P = 0.8$ to 0.7) inherit independently from each other. Marker 2 (1:0 = 34:0, $\chi^2 = 0.0$, $P = 1.0$) occurs in both parents and in all progeny isolates. Marker 4 (3:1 = 26:8, $\chi^2 = 0.04$, $P = 0.95$ to 0.9) probably represents two comigrating but independently segregating AFLP markers.

resulted from intercrossing avirulent and virulent F1 progeny isolates and segregated 1:1 for avirulence (F2-3: $\chi^2 = 0$, $P = 1$; F2-4: $\chi^2 = 0.33$, $P = 0.7$ to 0.5), again without the occurrence of isolates that showed recombinant avirulence patterns. Reviewing these results, we addressed the most basic ingredient of the gene-for-gene relationship: specificity of interactions, changing from compatible to incompatible when a host genotype is challenged by different parasite genotypes or when different host genotypes are challenged by one parasite genotype (Thompson and Burdon 1992). We tested 221 isolates from several segregating generations and consistently observed monogenic inheritance of avirulence without the occurrence of any recombinant phenotype.

Our working hypothesis for the current experiments is that cultivars Veranopolis, Kavkaz, and Shafir each carry at least one resistance gene that is not present in the other cultivars. A single gene for resistance was determined in cultivar Veranopolis (Wilson 1979) and two additive resistance genes have been reported in cultivar Kavkaz (Danon and Eyal 1990), both conclusions depending on the type of isolates used. We previously showed that cultivar Shafir is generally susceptible and avirulence for it is rare and restricted to durum wheat-derived isolates as well as a few Dutch isolates such as IPO323. Cultivars Kavkaz and Veranopolis were clearly different in their response to a large number of isolates from diverse geographic origins. Additional statistical analyses indicated that cultivar Veranopolis, unlike cultivar Kavkaz, showed one of the highest differentiating responses to *M. graminicola* (Kema et al. 1996a; Kema et al. 1996b). Consequently, IPO323 was considered to carry at least three avirulence genes.

Previous suggestions for a gene-for-gene relationship between wheat and *M. graminicola* (Eyal et al. 1973; Kema et al. 1996a) are supported by the current data. The observed (co)segregation of avirulence for cultivars Veranopolis, Kavkaz, and Shafir in the F1, BC1, and F2 generations suggests that avirulence in IPO323 is controlled by a single, most likely complex locus that may comprise a cluster of tightly linked avirulence genes. Linkage of avirulence genes was observed previously but was generally loose in *M. grisea* (Leung et al. 1988; Valent et al. 1991), *Phytophthora sojae* (Gijzen et al. 1996), and *Bremia lactucae* (Ilott et al. 1989). A cluster of cosegregating avirulence genes also was reported recently in *M. grisea* (Dioh et al. 2000). Nonetheless, we considered several alternative explanations for our results: i) the three differential cultivars share a common gene for resistance, hence, IPO323 carries a single avirulence gene whose product is recognized by that resistance gene; ii) IPO323 carries a single avirulence gene, but its product is recognized by different resistance genes in the differentials; iii) the identified locus might not be involved in the perception of signals per se but might function downstream of a pathway, leading to a general defense response; and iv) the current data do not exclude the possibility that a virulence factor was identified.

Until now, evidence for specificity in the *M. graminicola*-wheat pathosystem was indirect and largely statistical in inoculation experiments or inheritance studies of the host (Kema et al. 1999). Despite the significance of these observations, it did not result in a general acceptance of this phenomenon. Evidently, adding more statistical evidence will not elucidate specificity. Genetic proof, therefore, is inevitable to enable the progress of knowledge and an enhanced understanding of

specificity in this pathosystem. Experiments to clone and characterize the identified putative avirulence locus are under way. Analyses of additional crosses between *M. graminicola* strains and wheat cultivars are being carried out to resolve whether the gene-for-gene relationship explains specificity in the *M. graminicola*-wheat pathosystem.

ACKNOWLEDGMENTS

We thank J. Koeken and S. Verhaegh for conducting some of the experiments. The Wageningen Mycosphaerella group is gratefully acknowledged for discussion and critical reading of the manuscript. Part of this work was supported by EU-Biotech grant BIO4-CT96-0352. This paper is dedicated to the memory of Z. Eyal, one of the pioneers of research on *M. graminicola*, who passed away on July 30, 1999.

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