Identification of Molecular Genetic Markers in *Pyrenophora teres* f. *teres* Associated with Low Virulence on 'Harbin' Barley

John J. Weiland, Brian J. Steffenson, Richard D. Cartwright, and Robert K. Webster

First and second authors: Department of Plant Pathology, North Dakota State University, Fargo 58105-5677; third author: Cooperative Extension Service, University of Arkansas, Little Rock 72203; and fourth author, Department of Plant Pathology, University of California, Davis 95616. Current address of J. J. Weiland: Sugarbeet and Potato Research, USDA-ARS, Fargo, ND 58105-5677. Accepted for publication 6 November 1998.

ABSTRACT

Weiland, J. J., Steffenson, B. J., Cartwright, R. D., and Webster, R. K. 1999. Identification of molecular genetic markers in *Pyrenophora teres* f. *teres* associated with low virulence on 'Harbin' barley. *Phytopathology* 89:176-181.

Two isolates of the barley net blotch pathogen (*Pyrenophora teres* f. *teres*), one possessing high virulence (0-1) and the other possessing low virulence (15A) on the barley cultivar Harbin, were crossed and the progeny of the mating were isolated. Conidia from cultures of the parent and progeny isolates were used as inoculum to determine the inheritance of virulence in the pathogen. Of the 82 progeny tested, 42 exhibited high viru-

lence and 40 exhibited low virulence on 'Harbin' barley. The data support a model in which a single, major gene controls virulence in *P. teres* f. *teres* on this barley cultivar (1:1 ratio; $\chi^2 = 0.05$, $P = 0.83$). Preparations of DNA were made from parental and progeny isolates, and the DNA was subjected to the random amplified polymorphic DNA (RAPD) technique in a search for molecular genetic markers associated with the virulence phenotype. Five RAPD markers were obtained that were associated in coupling with low virulence. The data indicate that the RAPD technique can be used to tag genetic determinants for virulence in *P. teres* f. *teres*.

Additional keywords: avirulence, bulked segregant analysis, gene tagging.

Net blotch of barley (*Hordeum vulgare* L.) is caused by the ascomycete *Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* (Sacc.) Shoemaker). The disease is distributed worldwide and is particularly prevalent in cool, wet growing regions (26). Although seed and wild gramineous species can harbor the pathogen, infected barley stubble from previous crops is the most important source of inoculum for initiating epidemics (4). In seasons or production regions that favor net blotch disease development, yield losses in barley can reach 40% (26).

Numerous reports have documented the reactions that occur between *P. teres* isolates and various barley lines. Barley lines range from being highly resistant to highly susceptible to this pathogen (7,12,25,29), and pathotype diversity exists within *P. teres* (29). Moreover, two distinct pathogenic forms of *P. teres* exist, one inducing a net form of net blotch (caused by *P. teres* f. *teres*) and a second inducing a spot form of net blotch (caused by *P. teres* f. *maculata*) (27). The wide range of symptoms observed in the interaction of *P. teres* with barley suggests that this pathosystem might be ideal for investigations into the inheritance of pathogen virulence and host plant resistance (27).

Previous work has shown that the ability to produce net versus spot-type symptoms is conditioned in *P. teres* by two independently segregating genes (27). Similarly, studies with the progeny of a mating between *P. teres* f. *maculata* and *P. graminea* (the barley leaf stripe pathogen) have shown that the leaf-stripping symptom segregates from the spot symptom as if conditioned by two independent genes (27). No previous report, however, has addressed

the manner in which virulence in *P. teres* is inherited. Steffenson and Webster (29) reported on the low virulence of *P. teres* f. *teres* isolate 15A (pathotype 10-15-19) to 'Harbin' and 'Ming' barleys, whereas an isolate virulent to these lines (0-1) was obtained after that study. The current study was undertaken to investigate the genetics of virulence in *P. teres* f. *teres* using the progeny of a mating between isolates 15A and 0-1. In addition, the random amplified polymorphic DNA (RAPD) technique was applied to this population in an effort to identify molecular genetic markers linked with pathogen virulence.

MATERIALS AND METHODS

Fungal isolates and mating protocol. *P. teres* f. *teres* isolate 0-1 was collected from a barley field in Ontario, Canada (provided by A. Tekauz, Agriculture and Agri-Food Canada, Winnipeg, Canada), and isolate 15A was collected from a barley field in Solano County, California; both isolates were single-spored and stored on silica gel (11) until needed. The isolates were determined to be of opposite mating type by pairing in culture.

The procedure for the mating between *P. teres* f. *teres* isolates 0-1 and 15A was a modification of that described by Smedegård-Petersen (28). Barley straw, including the leaf sheath, was cut into pieces of 3 to 4 cm in length. The tissue was autoclaved in a glass petri dish at 44 kg/m² for 40 min just prior to use. Straw pieces were transferred to plastic petri dishes containing Sach's nutrient agar (35 to 40 ml per plate) (32) with ~1 cm separating each straw piece. Isolates 0-1 and 15A were cultured separately on V8 juice agar (V8A) (29) in an incubator at 22°C with a 12-h photoperiod until abundant conidia were produced (~10 days). Conidia were scraped from plates after adding sterile water, and the suspension was adjusted to 10,000 conidia per ml. A 0.1-ml aliquot of the suspension of each isolate was pipetted onto the straw pieces to initiate the mating. Plates were sealed with Parafilm (American Can Co., Greenwich, CT) and were placed in a dark incubator at 15°C.

Corresponding author: B. J. Steffenson
E-mail address: bsteffen@badlands.nodak.edu

Publication no. P-1998-1222-03R

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1999.

After 4 weeks, several pseudothecia were removed at weekly intervals and crushed under a slide cover for microscopic examination. Mature ascospores were present 6 to 8 weeks after initiating the mating. When mature ascospores were found, single pseudothecia were individually transferred to agar blocks (1 cm²), which then were adhered to the lid of a petri plate containing water agar. After replacing the lid onto the plate, the covered dish was incubated under fluorescent light at room temperature. Over the course of several hours, ascospores were ejected from asci within the pseudothecia down onto the water agar. By turning the lid every 15 min, the separation of individual ascospores on the agar surface was achieved. Ascospores were transferred to V8A for the production of conidia that subsequently were stored on silica gel (11) until needed.

Inoculation and evaluation of barley germ plasm. 'Harbin' (CI 4929), 'Ming' (CI 4797), and 'Kombar' (CI 15694) barley previously were used for the evaluation of pathotypes of *P. teres* (29). 'Harbin' and 'Ming' are known to possess at least one gene for resistance to *P. teres* f. *teres* (19), whereas 'Kombar' is susceptible to many pathotypes of the fungus (29). Conidia produced from the ascospore progeny were used for the inoculation of these barley accessions. Distilled water was added to 14-day-old cultures grown on V8A, and conidia were scraped from the plates and filtered

through two layers of cheesecloth, after which the suspension was adjusted to a concentration of 10,000 conidia per ml using a haemocytometer. The conidial suspension was transferred to atomizer vessels (model 15; DeVilbiss Co., Somerset, PA) and one drop of a 0.1% solution of polyoxyethylenesorbitan monolaurate (Tween-20) was added as a wetting agent. Seed of 'Harbin', 'Ming', and 'Kombar' was sown in a peat moss/perlite (3:1) potting mix at a rate of 3 to 4 seeds per container (cones 4 cm in diameter by 20.5 cm long) and were grown in a greenhouse (16-h photoperiod using supplemental lighting, average temperature 25°C) both before and after inoculation.

The inoculum prepared from each of the parent and progeny isolates was applied to 12-day-old plants (second leaf fully expanded) in two replicate containers per experiment, with an application rate of ~0.7 ml per plant. Following inoculation, plants were incubated for 20 h in a dark chamber (100% relative humidity, 20°C) maintained near saturation by periodic misting with an ultrasonic humidifier (model 460; Holmes Products, Milford, MA). Inoculated plants were allowed to air dry before being transferred to the greenhouse. At 12 to 14 days postinoculation, plants were assessed for symptoms and rated according to a 1 to 10 scale developed by Tekauz (31). At least three independent inoculations on different inoculation dates were performed with each *P. teres* f.

TABLE 1. Reaction of 'Harbin' barley to *Pyrenophora teres* f. *teres* parental and progeny isolates and association of random amplified polymorphic DNA (RAPD) markers with low virulence

Isolate	IR ^a	I01/I07 ^b	I01/I13	P01/P09	I03/I06	I07/I16	Number	BSA ^c	Isolate	IR ^a	I01/I07 ^b	I01/I13	P01/P09	I03/I06	I07/I16	Number	BSA ^c
Parent 15A	2 (1,3)	+	+	+	+	+	...	...	52	9 (6,10)	-	-	-	-	-	41	...
Parent 0-1	8 (7,9)	-	-	-	-	-	...	...	55	4 (4)	+	+	+	+	+	42	...
Progeny # 2	7 (6,10)	+	+	+	+	-	1	H1	57	3 (2,6)	+	+	+	+	+	43	...
4	3 (1,4)	+	+	+	+	+	2	...	61	3 (4,6)	+	+	+	+	+	44	...
6	2 (1,3)	+	+	+	+	+	3	L1	62	3 (1,4)	+	+	+	+	+	45	L2
7	8 (7,10)	-	-	-	-	-	4	...	64	8 (9,10)	-	-	-	-	-	46	H2
8	7 (6,10)	-	-	-	-	+	5	...	65	2 (1,3)	+	+	+	+	+	47	L2
9	3 (2,4)	+	+	+	+	+	6	...	67	3 (2,4)	+	+	+	+	+	48	...
10	3 (1,4)	+	+	+	+	+	7	...	68	8 (10)	-	-	-	-	-	49	...
11	3 (2,4)	+	+	+	+	+	8	L1	69	3 (2,4)	-	-	-	-	+	50	...
12	3 (2,4)	+	+	+	+	+	9	L1	70	2 (1,3)	+	+	+	+	-	51	L2
13	3 (2,5)	+	+	+	+	+	10	L1	72	8 (7,10)	-	-	-	-	-	52	H2
14	2 (3,5)	+	+	+	+	+	11	L1	73	3 (2)	+	+	+	+	-	53	L3
15	2 (1,3)	+	+	+	+	+	12	L1	74	8 (6,10)	-	-	-	-	-	54	H2
16	4 (3,5)	+	+	+	+	+	13	L1	76	8 (7,10)	-	-	-	-	-	55	...
17	8 (6,9)	-	-	-	-	-	14	...	77	7 (6,10)	-	-	-	+	-	56	...
18	8 (7,9)	-	-	-	-	-	15	H1	78	8 (7,10)	-	-	-	+	-	57	H2
19	3 (2,4)	+	+	+	+	+	16	L2	79	2 (3)	+	+	+	+	-	58	...
20	8 (6,10)	-	-	-	-	-	17	H1	82	7 (6,10)	-	-	-	-	-	60	...
21	3 (2,4)	+	+	+	+	+	18	...	83	2 (1,3)	+	+	+	+	+	61	L3
22	3 (1,4)	+	+	+	+	+	19	L2	84	2 (1,4)	+	+	+	+	+	62	L3
23	3 (4)	+	+	+	+	+	20	...	85	2 (3)	+	+	+	+	+	63	L3
27	8 (6,10)	-	-	-	-	-	21	...	86	8 (6,10)	-	-	-	-	-	64	H2
28	8 (6,10)	-	-	-	-	-	22	H1	87	8 (6,10)	-	-	-	-	-	65	H2
29	8 (7,10)	-	-	-	-	-	23	...	88	3 (2)	+	+	+	+	+	66	...
30	8 (6,10)	-	-	-	-	-	24	H1	89	8 (6,10)	-	-	-	-	-	67	H3
32	7 (6,10)	-	-	-	-	-	25	...	91	3 (4,6)	+	+	+	+	+	68	...
33	8 (9,10)	-	-	-	-	-	26	H1	92	3 (2,4)	+	+	+	+	+	69	L3
34	3 (2,4)	+	+	+	+	+	27	...	93	7 (8,10)	+	+	+	+	+	70	...
35	7 (6,10)	-	-	-	-	-	28	...	94	2 (1,3)	+	+	+	+	+	71	L3
36	8 (6,10)	-	-	-	-	-	29	...	95	8 (7,10)	-	-	-	-	-	72	H3
37	8 (6,10)	-	-	-	-	-	30	...	96	8 (6,10)	-	-	-	-	-	73	H3
38	2 (3)	+	+	+	+	+	31	...	97	3 (2,4)	+	+	+	+	+	74	...
41	3 (1,4)	+	+	+	+	+	32	...	99	8 (6,10)	-	-	-	-	-	75	H3
42	8 (7,10)	-	-	-	-	-	33	...	100	8 (7,10)	-	-	-	-	-	76	...
45	6 (5,10)	-	-	-	-	-	34	...	101	8 (7)	-	-	-	-	-	77	H3
46	3 (2,4)	+	+	+	+	+	35	...	102	8 (6,9)	-	-	-	-	-	78	...
47	2 (1,3)	+	+	+	+	+	36	L2	104	8 (9)	-	-	-	-	-	79	...
48	3 (2,4)	+	+	+	+	+	37	L2	105	8 (6,9)	-	-	-	-	-	80	H3
49	8 (6,10)	-	-	-	-	-	38	H1	107	8 (9)	-	-	-	-	-	81	...
50	3 (2,4)	+	+	+	+	+	39	...	108	8 (7,9)	-	-	-	-	-	82	H3
51	8 (7,10)	-	-	-	-	-	40	H2									

^a Numbers reflect the most common infection response (IR) induced by an isolate with the range of observed IRs shown in parentheses.

^b Markers produced by the indicated primer pair were either present (+) or absent (-) after applying RAPD to the parent and progeny DNA.

^c Progeny isolates with high (H) and low (L) virulence used to generate bulked DNA samples numbered 1 to 3 (e.g., H1 was used in the number 1 DNA bulk of high virulence progeny).

teres isolate. Segregation in the progeny for low virulence and high virulence was subjected to chi-square analysis (30).

DNA preparations and RAPD analysis. Parent and progeny isolates were grown on V8A in petri dishes until the plate was nearly covered with the culture (~14 days). Distilled water (0.5 ml) was pipetted onto a plate and the spore-mycelial mass was scraped into a mortar using a clean razor blade. Quartz sand (-50 +70 mesh; Sigma Chemical Co., St. Louis) was added to the mortar until the liquid was just absorbed, and then 1 ml of extraction buffer (EB; 2% cetyltrimethylammonium bromide, 1.4 M sodium chloride, 20 mM disodium EDTA, and 0.1 M Tris-HCl, pH 8.0) was added to the suspension. The mixture was ground to a thick paste and was mixed with an additional 3 ml of EB. The suspension was transferred to a graduated polypropylene tube, adjusted to 0.2% β -mercaptoethanol, and incubated at 65°C for 1 h. After incubation, 2 ml of water-saturated chloroform/isoamyl alcohol (24:1) was added to the tube and the solution was shaken by hand. The phases were separated by centrifugation ($5,000 \times g$ for 10 min), and the aqueous phase was transferred to a new tube. Nucleic acids were precipitated from the aqueous phase by the addition of 0.6 volumes of isopropanol. The precipitate was recovered by centrifugation ($16,000 \times g$ for 15 min at 4°C) and washed with 95% ethanol before drying. The dried nucleic acid pellet was resuspended in TE (10 mM Tris-HCl and 1 mM disodium EDTA, pH 8.0) that contained ribonuclease A at 50 μ g/ml. Following a 30-min incubation at 37°C, the mixture was extracted once with TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1), and the aqueous phase was precipitated by the addition of 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol. The final DNA pellet was resuspended in 100 μ l of TE and stored at 4°C.

Template DNA of the parent and progeny isolates was adjusted to 0.1 μ g/ml for use in the polymerase chain reactions (PCRs). Bulked segregant analysis (BSA) (18) was used in the search for markers linked to virulence traits in *P. teres* f. *teres*, since it permits the screening of several unique primers per experiment. For BSA, DNA preparations of 21 progeny from within each of the two phenotype classes (low virulence and high virulence) were combined so that three separate DNA pools (seven progeny per pool; Table 1) were formed. Each 50- μ l reaction contained 1 ng of template DNA; 0.2 mM each of two arbitrary decamer oligonucleotides (Operon, Inc., Alameda, CA); 50 mM potassium chloride; 10 mM

Tris-HCl (pH 9.0); 0.1% TRITON-X100; 2.0 mM magnesium chloride; and 0.1 mM each of dGTP, dATP, dTTP, and dCTP. Reactions were incubated in a thermocycler (PTC-100V; MJ Research Inc., Watertown, MA) with temperature and time parameters of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min (40 cycles total). Loading dye ($10\times = 0.45$ M Tris base, 0.45 M boric acid, 1 mM disodium EDTA [pH 8.9], 50% glycerol, 0.1% bromophenol blue, and 0.1% xylene cyanol) was added to 10 ml of each reaction before loading the samples onto a 1.5% agarose gel (Tris-borate electrophoresis buffer) (1). Gels were subsequently stained with ethidium bromide, transilluminated by UV light, and photographed using Polaroid 667 film in a Polaroid camera (model MP4; Polaroid Corp., Penfield, NY). Genetic linkage data were analyzed using the MAPMAKER software package (16) for the Macintosh computer in which centimorgan values were derived from recombination data using the Kosambi function (14).

RESULTS

Isolation of ascospores and evaluation of virulence in *P. teres* f. *teres*. A total of 110 ascospore progeny was obtained from the mating of *P. teres* isolates 0-1 and 15A. After 5 years in storage, 82 progeny were recovered after plating and were used in the current study. Inoculation of isolates 15A and 0-1 to barley lines

Fig. 1. Frequency distribution of modal values for the infection response (IR) of 'Harbin' barley to *Pyrenophora teres* f. *teres* 15A and 0-1 progeny isolates. The 1 to 10 scale along the horizontal axis is based on the reaction scale of Tekauz (31). The number of progeny-inducing responses within the specific IR classes is represented by the height of the bars. Modal IR values for parental isolates 15A and 0-1 are directly below the arrows.

Fig. 2. Products of the application of random amplified polymorphic DNA analysis to *Pyrenophora teres* f. *teres* parental isolate DNA and pooled DNA samples. Markers identified using the primer pairs of I01/I07, I01/I13, I03/I06, I07/I16, and P01/P09 are shown. Bulked DNA from progeny that induced reactions of high virulence (lanes marked high) and low virulence (lanes marked low) on 'Harbin' barley was used as a template in the polymerase chain reactions. Reactions using DNA prepared from the parental isolates (0-1 and 15A as marked) were performed in parallel. Of each 50- μ l reaction, 10 μ l was electrophoresed on a 1.6% agarose gel and photographed after staining with ethidium bromide. Arrows point to the amplified products that are common to the parent 15A and the progeny isolates with low virulence. The relative size of each marker band (indicated at the right of each photograph) was determined by comparison with the 1 kb standards (Life Technologies, Inc., Gaithersburg, MD).

'Harbin' and 'Ming' induced the formation on leaves of a pinpoint necrotic fleck surrounded by a faint chlorotic halo at 3 to 4 days postinoculation. By 12 days postinoculation, only a necrotic fleck was visible on 'Harbin' and 'Ming' barley plants inoculated with isolate 15A; however, isolate 0-1 induced severe net blotch symptoms. From several preliminary experiments involving isolate 0-1, higher infection responses were observed on 'Harbin' than on 'Ming'. Isolates 0-1 and 15A both were highly virulent on 'Kombar' barley, with isolate 15A inducing slightly more severe leaf chlorosis than isolate 0-1. Based on these findings, the inheritance of virulence in the 15A × 0-1 progeny population was examined on 'Harbin', with the inclusion of 'Kombar' as a susceptible check.

Infection responses (IRs) of 'Harbin' to *P. teres* f. *teres* isolates 0-1, 15A, and their progeny are summarized in Table 1. The IRs were expressed as a mode and range; the most prevalent IR (typifying >90% of the lesions observed on leaves) is presented as the mode, whereas the lowest and highest IR recorded represent the range. Many of the progeny exhibited the same IR exhibited by the parent isolates (i.e., mode of 2 for 15A and 8 for 0-1; Fig. 1). The most common IRs observed among the progeny were for those exhibiting a mode of 3 (low virulence; 26 progeny) or a mode of 8 (high virulence; 33 progeny).

Progeny that produced IRs in the 2 to 4 range were considered to possess low virulence, whereas those that produced IRs in the 6 to 9 range were considered to possess high virulence. The ratio of progeny exhibiting low virulence (*n* = 40) to those exhibiting high virulence (*n* = 42) was near 1:1 (χ^2 = 0.05; *P* = 0.83), suggesting that a single, major gene controls virulence in *P. teres* f. *teres* on 'Harbin' barley.

Identification of RAPD markers linked to low virulence in *P. teres* f. *teres*. The combination of BSA and RAPD marker generation were used to obtain molecular genetic markers associated with low virulence in *P. teres* f. *teres* to 'Harbin' barley. Previous studies have demonstrated the utility of RAPDs in the detection of genetic variation that exists in natural populations of *P. teres* (17,23,24). Initial experiments indicated that the use of two arbitrary oligonucleotides in the RAPD analysis generated more electrophoretic bands from the DNA of isolates 0-1 and 15A (data not shown) than did use of a single primer. Two arbitrary decamer primers were subsequently used in each PCR reaction, which further increased the number of screening reactions that could be derived from a single primer set. Primers and associated markers are referred to throughout the text according to the alphanumeric designators provided by Operon, Inc.

From the screening of 86 diprimer combinations, five unique DNA bands were amplified from DNA of the 15A parent and the pooled DNA of the low virulence isolates that were absent from the DNA fingerprints of the 0-1 parent DNA and the pooled DNA of the high virulence isolates. No markers were obtained from the 86 combinations that were uniquely associated with the high virulence phenotype. Fractionation of the PCR products after BSA-RAPD analysis illustrating the detection of the five markers is shown in Figure 2. Subsequent tests confirmed that the amplified DNA band associated with low virulence was dependent on the presence of both arbitrary primers in the reaction; use of the indicated primers individually in the PCR reaction failed to generate the observed band (data not shown). Markers were designated I03/I06, I01/I07, I01/I13, P01/P09, and I07/I16 (Table 2), according to the decamer primer pair used to generate each marker DNA product.

Mapping of markers around the locus conditioning low virulence on 'Harbin' barley. Markers obtained by BSA were

evaluated for linkage to low virulence by testing progeny individually for the presence or absence of the markers. Using the same primer pairs that generated markers from the bulked DNA samples, PCR was performed on DNA prepared from individual progeny isolates. All progeny DNA that failed to produce the marker band of interest was retested to ensure that marker absence was not artifactual. The results of these tests are summarized in Tables 1 and 2. Tabular representation of the data clearly indicates an association between the five markers and low virulence in the progeny on 'Harbin' barley. All of the markers obtained were associated with low virulence; therefore, we tentatively designate the locus conferring the trait *avrHar*, denoting avirulence to 'Harbin' barley.

Marker data were then examined to determine the linkage relationship of the markers with respect to themselves and to the *avrHar* locus. Progeny were scored as possessing either high virulence or low virulence and as either possessing or lacking the markers generated by RAPD analysis. Genetic linkage analysis performed by the MAPMAKER computer program is summarized in Figure 3. The RAPD markers that map closest to the *avrHar* locus were I01/I13, I01/I07, and P01/P09, which cosegregated with each other and were 3.8 cM from *avrHar*.

DISCUSSION

The data presented in this study indicate that a single major gene controls virulence in *P. teres* f. *teres* on 'Harbin' barley. A near 1:1 segregation of high to low virulence to 'Harbin' in the progeny of the 15A × 0-1 cross is consistent for such a model, because *P. teres* is a haploid fungus. Cosegregation of molecular genetic markers with each other and with low virulence further support this model. Many of the progeny induced IRs that were not the same as that of the parents. This may be due to the presence of additional genes that modify the expression of the major viru-

Fig 3. Map of the linkage of the molecular genetic markers to the putative *avrHar* locus. The phenotype (low virulence or high virulence) and association with a molecular marker (+ or -) of each of the progeny of the mating between *Pyrenophora teres* f. *teres* isolates 0-1 and 15A were entered into the program MAPMAKER for the Macintosh computer. Markers I01/I07, I01/I13, and P01/P09 cosegregated with each other and mapped 3.8 cM from *avrHar* (log of the likelihood ratio = 4.0; θ = 0.4).

TABLE 2. Number of progeny associated with indicated random amplified polymorphic DNA marker

	I01/I07	I01/I13	I03/I06	I07/I16	P01/P09
Low virulence progeny (40 total)	39	39	39	36	39
High virulence progeny (42 total)	2	2	4	2	2

lence gene or to variation inherent in the disease phenotyping. The simple inheritance pattern is similar to the reported segregation of loci controlling the production of net symptoms versus spot symptoms in the progeny of crosses between *P. teres* f. *teres* (net form) and *P. teres* f. *maculata* (spot form) (27). Recently, a single gene also was found to control the virulence of *Cochliobolus sativus* on barley (34). The molecular tagging of genes that influence symptom type and virulence in these examples may lead to their cloning using map-based strategies (6,13,20).

For other pathosystems involving the genera of *Pyrenophora* and *Cochliobolus*, the involvement of host-specific toxins (HSTs) in pathogenicity and virulence is well documented (5,21,35–37). Production of such HSTs in many of these cases appear to be controlled by major genes (5,36). In the wheat-tan spot pathosystem, pathotypes of *P. tritici-repentis* have been characterized that produce both chlorosis and necrosis, chlorosis only, necrosis only, or neither of these symptom types on wheat (15). A protein produced by this organism has been characterized recently that appears to be the necrosis-producing toxin (3,33,37), whereas a compound of greater than 3,500 molecular weight is proposed to be the chlorosis toxin (21). Furthermore, the *ToxA* locus of *P. tritici-repentis*, which encodes a protein phytotoxin of a similar size to that reported by a number of laboratories and is capable of inducing tan necrosis when infiltrated into leaves, has been determined to be the major pathogenicity determinant in the infection of wheat by this fungus (5). In experiments with maize, disruption of the genes for the production of T-toxin and HC-toxin have been correlated with decreased virulence in *C. heterostrophus* (36) and loss of pathogenicity in *C. carbonum* (22), respectively. Results from the recent gene disruption experiments in *Cochliobolus* represent the culmination of decades of research on the biochemistry of the T- and HC-toxins (35).

Nonspecific peptide and protein toxins have been implicated in the production of disease symptoms in *P. teres* (2,9) and *P. graminea* (10), respectively, and as such cannot explain the differential virulence observed in the current study. It is possible, however, that the segregation of the five RAPD markers with low virulence to 'Harbin' barley may reflect their linkage in repulsion to a locus specifying the production of a cultivar-specific virulence factor such as a HST. The data also support a model whereby a suppressor of a virulence gene has been tagged by the markers reported here. Biochemical analysis of fungal culture filtrates and evaluation of additional crosses among different isolates of *P. teres* could resolve this question. The segregation of all RAPD markers obtained in coupling with the low virulence character nevertheless prompted us to denote the locus as *avrHar* for avirulence to 'Harbin' barley. The existence of *avrHar* would imply that 'Harbin' possesses a corresponding dominant resistance gene, in agreement with the gene-for-gene hypothesis for the race-specific interaction of pathogens with host plants (8). Indeed, barley lines 'Harbin' and 'Ming' previously were reported to carry dominant genes for resistance to *P. teres* f. *teres* (19). The possibility that *avrHar* is the avirulence counterpart to these resistance genes will be reinvestigated in inoculations to barley populations derived from crosses between 'Harbin' and other lines that are susceptible to *P. teres* isolate 15A.

Segregating progeny populations derived from crosses between fungal isolates within the genera *Pyrenophora* and *Cochliobolus* are being used in our laboratory to examine the inheritance of virulence and symptom production on barley (34; J. J. Weiland, Y. Sun, and B. J. Steffenson, unpublished data). The heterothallic mating system, haploid genetics, and distinct pathogenic characteristics of *P. teres*, *P. graminea*, and *C. sativus*, combined with the diploid genetics of the barley host, make these pathosystems attractive for the study of host-pathogen genetics. By integrating the biochemistry of toxin production with molecular genetics, the significant advances made in the *P. tritici-repentis*-wheat and *Cochliobolus*-maize-oat pathosystems could be applied to the

study of *Pyrenophora*-barley and *Cochliobolus*-barley interactions. Such studies should clarify the nature of virulence and avirulence in these interactions and reveal the importance of toxin production in the pathogenesis of barley by these pathogens.

ACKNOWLEDGMENTS

This work was supported by USDA-NRI-CGP post-doctoral fellowship project #NDR-9401291 awarded to J. J. Weiland. We thank T. Fetch for technical expertise and R. Senn for greenhouse assistance.

LITERATURE CITED

1. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1989. Short Protocols in Molecular Biology. John Wiley & Sons, New York.
2. Bach, E., Christensen, S., Dalgaard, L., Larsen, P. O., Olsen, C. E., and Smedegård-Petersen, V. 1979. Structures, properties and relationships to the aspergillomarasmine of toxins produced by *Pyrenophora teres*. *Physiol. Plant Pathol.* 14:41-46.
3. Ballance, G. M., Lamari, L., and Bernier, C. C. 1989. Purification and characterization of a host-selective toxin from *Pyrenophora tritici-repentis*. *Physiol. Mol. Plant Pathol.* 35:203-213.
4. Brown, M. P., Steffenson, B. J., and Webster, R. K. 1993. Host-range of *Pyrenophora teres* isolates from California. *Plant Dis.* 77:942-947.
5. Ciuffetti, L. M., Tuori, R. P., and Gavena, J. M. 1997. A single gene encodes a selective toxin causal to the development of tan spot of wheat. *Plant Cell* 9:135-144.
6. De Wit, P. J. G. M. 1997. Pathogen avirulence and plant resistance: A key role for recognition. *Trends Plant Sci.* 2:452-458.
7. Douiyssi, A., Rasmusson, D. C., and Roelfs, A. P. 1998. Responses of barley cultivars and lines to isolates of *Pyrenophora teres*. *Plant Dis.* 82:316-321.
8. Flor, H. H. 1956. The complementary genetic systems in flax and flax rust. *Adv. Genet.* 8:29-54.
9. Friis, P., Olsen, C. E., and Møller, B. L. 1991. Toxin production in *Pyrenophora teres*, the ascomycete causing the net blotch disease of barley (*Hordeum vulgare* L.). *J. Biol. Chem.* 266:13329-13335.
10. Haegi, A., and Porta-Puglia, A. 1995. Purification and partial characterization of a toxic compound produced by *Pyrenophora graminea*. *Physiol. Mol. Plant Pathol.* 46:429-444.
11. Hargreaves, J., and Turner, G. 1992. Genetic transformation in plant pathogenic fungi. Pages 79-96 in: *Molecular Plant Pathology: A Practical Approach*. S. J. Gurr, M. J. McPherson, and D. J. Bowles, eds. IRL Press, Oxford, United Kingdom.
12. Harrabi, M., and Kamel, A. 1990. Virulence spectrum to barley in some isolates of *Pyrenophora teres* from the Mediterranean region. *Plant Dis.* 74:230-232.
13. Knogge, W. 1996. Molecular basis of specificity in host/fungus interactions. *Eur. J. Plant Pathol.* 102:807-816.
14. Kosambi, D. D. 1944. The estimation of map distances from recombination values. *Ann. Eugen.* 12:172-175.
15. Lamari, L., Sayoud, R., Boulif, M., and Bernier, C. C. 1995. Identification of a new race in *Pyrenophora tritici-repentis*: Implications for the current pathotype classification system. *Can. J. Plant Pathol.* 17:312-318.
16. Lander, E. S., Green, P., Abrahamson, J., Barlow, A., Daly, M. J., Lincoln, S. E., and Newburg, L. 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1:174-181.
17. Louw, L. P. J., Victor, D., Crous, P. W., Holz, G., and Janse, B. J. H. 1995. Characterization of *Pyrenophora* isolates associated with spot and net type lesions on barley in South Africa. *J. Phytopathol.* 143:129-134.
18. Michelmore, R. W., Paran, I., and Kesseli, R. V. 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers for specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. U.S.A.* 88:9828-9832.
19. Mode, C. J., and Schaller, C. W. 1958. Two additional factors for host resistance to net blotch in barley. *Agron. J.* 50:15-18.
20. Oliver, R., and Osbourn, A. 1995. Molecular dissection of fungal phytopathogenicity. *Microbiology (UK)* 141:1-9.
21. Orolaza, N. P., Lamari, L., and Ballance, G. M. 1995. Evidence of a host-specific chlorosis toxin from *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat. *Phytopathology* 85:1282-1287.
22. Panaccione, D. G., Scott-Craig, J. S., Pocard, J.-A., and Walton, J. D. 1992. A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. *Proc. Natl. Acad. Sci. U.S.A.* 89:6590-6594.
23. Peever, T. L., and Milgroom, M. G. 1994. Genetic structure of *Pyreno-*

- phora teres* populations determined with random amplified polymorphic DNA markers. Can. J. Bot. 72:915-923.
24. Peltonen, S., Jalli, M., Kammiovirta, K., and Karjalainen, R. 1996. Genetic variation in *Drechslera teres* populations as indicated by RAPD markers. Ann. Appl. Biol. 128:465-477.
 25. Robinson, J., and Jalli, M. 1996. Diversity among Finnish net blotch isolates and resistance in barley. Euphytica 92:81-87.
 26. Shipton, W. A., Khan, T. N., and Boyd, W. J. R. 1973. Net blotch of barley. Rev. Plant Pathol. 52:269-290.
 27. Smedegård-Petersen, V. 1977. Inheritance of genetic factors for symptoms and pathogenicity in hybrids of *Pyrenophora teres* and *Pyrenophora graminea*. Phytopathol. Z. 89:193-202.
 28. Smedegård-Petersen, V. 1978. Genetics of heterothallism in *Pyrenophora graminea* and *P. teres*. Trans. Br. Mycol. Soc. 70:99-102.
 29. Steffenson, B. J., and Webster, R. K. 1992. Pathotype diversity of *Pyrenophora teres* f. *teres* on barley. Phytopathology 82:170-177.
 30. Suzuki, D. T., Griffiths, A. J. F., Miller, J. H., and Lewontin, R. C. 1986. An Introduction to Genetic Analysis, 3rd ed. W. H. Freeman and Company, New York.
 31. Tekauz, A. 1985. A numerical scale to classify reactions of barley to *Pyrenophora teres*. Can. J. Plant Pathol. 7:181-183.
 32. Tinline, R. D. 1951. Studies on the perfect stage of *Helminthosporium sativum*. Can. J. Bot. 29:467-480.
 33. Tuori, R. P., Wolpert, T. J., and Ciuffetti, L. M. 1995. Purification and immunological characterization of toxic components from cultures of *Pyrenophora tritici-repentis*. Mol. Plant-Microbe Interact. 8:41-48.
 34. Valjavec-Gratian, M., and Steffenson, B. J. 1997. Genetics of virulence in *Cochliobolus sativus* and resistance in barley. Phytopathology 87:1140-1143.
 35. Walton, J. D. 1996. Host-selective toxins: Agents of compatibility. Plant Cell 8:1723-1733.
 36. Yang, G., Rose, M. S., Turgeon, B. G., and Yoder, O. C. 1996. A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. Plant Cell 8:2139-2150.
 37. Zhang, H.-F., Franc, L. J., Jordahl, J. G., and Meinhardt, S. W. 1997. Structural and physical properties of a necrosis-inducing toxin from *Pyrenophora tritici-repentis*. Phytopathology 87:154-160.