Identification of Molecular Markers Linked to a *Pyrenophora teres* Avirulence Gene

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ABSTRACT

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Genetic control of avirulence in the net blotch pathogen, *Pyrenophora teres*, was investigated. To establish an appropriate study system, a collection of 10 net form (*P. teres* f. *teres*) and spot form (*P. teres* f. *maculata*) isolates were evaluated on a set of eight barley lines to identify two isolates with differential virulence on an individual host line. Two net form isolates, WRS 1906, exhibiting avirulence on the cv. Heartland, and WRS

1607, exhibiting high virulence, were mated and 67 progeny were isolated and phenotyped for reaction on Heartland. The population segregated in a 1:1 ratio, 34 avirulent to 33 virulent ($\chi^2 = 0.0$, $P = 1.0$), indicating single gene control of WRS 1906 avirulence on Heartland. Bulk segregant analysis was used to identify six amplified fragment length polymorphism markers closely linked to the avirulence gene (*Avr_{Heartland}*). This work provides evidence that the *P. teres*–barley pathosystem conforms to the gene-for-gene model and represents an initial step toward map-based cloning of this gene.

Pyrenophora teres Drechsler (anamorph: *Drechslera teres* (Sacc.) Shoemaker) is a haploid, ascomycete pathogen which is the causal agent of barley net blotch. Net blotch is a common, persistent disease found in all barley-producing regions of the world and is of economic concern to the barley industry. Financial losses to this foliar pathogen result from both yield reduction, ranging from 15 to 35% (13,18,37), and diminished grain quality (19).

A large number of *P. teres* pathotypes have been reported. For example, Tekauz (40) identified 45 net form (*P. teres* f. *teres*) and 20 spot form (*P. teres* f. *maculata*) pathotypes using a set of 9 and 12 barley lines, respectively. Similarly extensive pathotype variability has been described from California (36) and the former Soviet Union (2). Within the barley genome, loci controlling net blotch resistance have been identified on every chromosome. Although several studies have located quantitative trait loci (QTL) associated with resistance (26,35), a number of single, major genes controlling resistance have been identified (1), with several mapped (10,17,48).

The numerous *P. teres* pathotypes and barley resistance genes suggest that this pathosystem likely conforms to the classic gene-for-gene model (8). Much progress in understanding the molecular basis of this model has been made via the cloning of many plant resistance (*R*) genes and their cognate pathogen avirulence (*Avr*) genes. One significant aspect of this work has been the discovery that pathogen *Avr* gene products (effectors) contribute to a pathogen's ability to infect its host. For example, *Avr4* from the tomato leaf mold pathogen, *Cladosporium fulvum*, is a chitin-binding lectin that binds to and protects the fungal cell wall from plant chitinases (41). Another important finding is that many *R*

proteins do not interact directly with pathogen effectors, but rather monitor changes to the effector virulence target. This has become known as the guard model (6). For example, *Cf-2* from tomato initiates a hypersensitive response only after the *C. fulvum* *Avr2* effector has been bound to the tomato cysteine protease *Rcr3* (32).

A majority of *Avr* genes cloned are from bacterial pathogens belonging to the genera *Pseudomonas* and *Xanthomonas*. However, a large number of important plant diseases are caused by fungal and oomycete pathogens, which present a greater cloning challenge than bacteria due to their larger genomes (typically 10 to 40 Mb), difficulty in culturing (as with the obligate biotrophs), and lack of simple transformation systems (which hinders functional characterization of putative *Avr* genes). Despite this, a number of *Avr* genes have been cloned from the oomycetes *Peronospora parasitica* (3,29), *Phytophthora infestans* (4), and *P. sojae* (33), as well as from the fungal pathogens *Melampsora lini* (7), *C. fulvum* (12,16,42,47), *Magnaporthe grisea* (23), and *Rhynchosporium secalis* (31).

A variety of methods have been used to clone these genes. Reverse genetics has been used successfully to clone the *Avr4*, *Avr4E*, and *Avr9* effectors from *C. fulvum* (12,42,47) and the *Nip1* effector from *R. secalis* (31), whereas a functional screen identified *Avr2* from *C. fulvum* (16). Armstrong et al. (4) used a candidate gene approach in combination with association mapping to isolate *Avr3a* from *P. infestans*. The remaining fungal and oomycete *Avr* genes have been isolated using a map-based cloning approach. *Pyrenophora teres* also is amenable to map-based cloning because it has a heterothallic mating system, controlled by alternate alleles of the *MAT* gene (28), that is helpful for creating the mapping population necessary for map-based cloning.

This study reports the identification of amplified fragment length polymorphism (AFLP) markers linked to an *Avr* gene in the *P. teres* isolate WRS 1906 that limits the ability of this isolate to infect the barley cv. Heartland. This and other work indicating that the *P. teres*–barley pathosystem conforms to the gene-for-gene model is discussed. Markers identified in this study will provide a starting point for map-based cloning of this *Avr* gene.

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MATERIALS AND METHODS

Barley lines and *P. teres* isolates. To establish the appropriate study system, it was necessary to identify two *P. teres* isolates exhibiting a differential ability to infect an individual host line. Seven cultivated barley (*Hordeum vulgare* subsp. *vulgare*) lines and one wild barley (*H. vulgare* subsp. *spontaneum*) accession were selected for screening. A ninth line ('Harbin'), used in a previous evaluation of avirulence in *P. teres* (46), was screened with the two parental isolates selected to form the mating population in this study, to determine whether the avirulence locus in this study was the same as that previously reported. The lines used are listed in Table 1. Eight net form (*P. teres* f. *teres*) and two spot form (*P. teres* f. *maculata*) isolates were chosen to evaluate virulence on the barley lines (Table 2).

Virulence phenotyping. *P. teres* cultures used for virulence evaluation initially were established from single spores collected from infected barley leaves. Dry leaves infected with *P. teres* were surface sterilized in 50% ethanol for 15 s and 2% sodium hypochlorite for 30 s, then rinsed in distilled water. Leaf sections were placed on dry filter paper in petri plates and a second, moistened piece of filter paper was attached to the lid. Plates were incubated at 21°C with a 12-h photoperiod under cool white light (15 W, 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux) (General Electric, Mississauga, ON, Canada) to promote sporulation. After 3 to 5 days, single conidia were transferred to petri plates (9 cm in diameter) containing V8A (18% V8 juice [vol/vol], 30 mM CaCO_3 , and 2% agar) using a sterile pipette tip (pulled to a fine point). Cultures were incubated for 10 days as above. Inoculum was prepared by washing conidia from plates with 10 ml of water and filtering through two layers of cheesecloth. Inoculum concentration was adjusted to 10^4 conidia/ml. A drop of Tween 20 was added to the suspension as a wetting agent. Subsequent cultures for inoculation were initiated by placing several crystals of silica gel-containing conidia onto V8A plates. Silica gel stocks were prepared from the initial single-spore-derived cultures using the method of Smith and Onions (34).

Barley seeds were surface sterilized in 0.12% formaldehyde for 1 h and then rinsed in running tap water for 30 min. Five seeds per line were planted in a group, with two groups per 15-cm pot. Pots contained Terra-lite Redi-Earth (W.R. Grace Ltd., Ajax, ON, Canada). Plants were grown at 21°C with a 16-h photoperiod (75% relative humidity) for 10 to 14 days and staked to prevent touching of leaves between pots. Inoculum then was applied to three-leaf-stage plants with a 50-ml spray bottle. Inoculum was applied to run-off, ≈ 7.5 ml per 15-cm pot. Plants then were incubated in darkness for 24 h at 100% relative humidity. Then, they were returned to the previous conditions for 1 week before disease symptoms on the second leaf were rated on a 1-to-10 scale (39). Isolates that produced a rating of ≥ 5 were classified as

virulent while those eliciting a reaction of ≤ 4 were considered avirulent. Four independent inoculations on each barley line were carried out on different inoculation dates with the eight isolates initially evaluated. Subsequently, three independent inoculations on different inoculation dates were carried out with each isolate in the mapping population selected for *Avr* gene identification on the corresponding barley line.

***P. teres* mating and mating-type determination.** Barley stem sections, including the leaf sheath, were cut into 3- to 4-cm pieces and autoclaved at 18 psi for 30 min. Stem pieces were transferred to petri plates containing 35 to 40 ml of Sach's nutrient agar (4.6 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 3.3 mM KCl, 1.4 mM K_2HPO_4 , 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 mM $\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5\text{H}_2\text{O}$, 40 mM CaCO_3 , 2% agar, pH to 6 with H_3PO_4) with 1 cm separating each piece (five to six pieces/plate). Isolates were cultured separately on V8A at 21°C with a 12-h photoperiod until abundant conidia were produced (≈ 10 days). Conidia were collected from the plates as previously described and the suspension was adjusted to 10^4 conidia/ml. A 25- μl aliquot of each isolate was pipetted onto opposite ends of each straw piece to initiate mating. Plates were sealed with parafilm and placed in the dark at 15°C. After 12 weeks, the plates were transferred to a 9-h photoperiod with an increase of 3 min/day for 3 weeks. Mature pseudothecia were produced at this point. Mature asci were released from the pseudothecia by crushing with a sterile needle, and ascospores were released from the asci by crushing with a finely drawn glass pipette tip. Individual ascospores (primarily random meiotic products along with complete tetrads when possible) were transferred to 5% water agar plates to germinate overnight at 25°C. Germinated ascospores were transferred to V8A plates to produce conidia that were stored as silica gel stocks.

Mating type was determined using polymerase chain reaction (PCR) primers specific to the *MAT-1* and *MAT-2* alleles (28). The *MAT-1* primers were 5'-AAC AGA CTC CTC TTG ACA ACC CG-3' (forward) and 5'-TGA CGA TGC ATA GTT TGT AAG GGT-3' (reverse), and the *MAT-2* primers were 5'-CAA CTT TTC TCT ACC ACA CGT ATC CC-3' (forward) and 5'-TGT GGC GAT GCA TAG TTC GTA C-3' (reverse). PCR reactions were carried out in 20- μl reactions and contained 1 $\times$ buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl), 2.5 mM MgCl_2 , 0.2 mM dNTPs, 4 pmol of each primer, 1 U of *Taq* polymerase, and 20 ng of DNA. PCR conditions consisted of 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, followed by a final hold at 72°C for 1 min. PCR products were loaded on 1% agarose gels containing ethidium bromide at 0.1 $\mu\text{g/ml}$ and electrophoresed in 0.5 $\times$ Tris-borate-EDTA (TBE) for 2 h at 115 V. Gels were photographed under UV light.

DNA extraction and bulked segregant analysis. Conidia were harvested from 10- to 14-day-old cultures grown on V8A and

TABLE 1. Barley lines used to evaluate virulence of net blotch isolates

Species, line	Origin ^a
<i>Hordeum vulgare</i>	
subsp. <i>vulgare</i>	
'Excel'	University of Minnesota
'CDC Dolly'	University of Saskatchewan
'Harbin'	Manchurian landrace
'Harrington'	University of Saskatchewan
'Heartland'	Brandon Research Centre, AAFC
'Norbert'	Cereal Research Centre, AAFC
TR251	Brandon Research Centre, AAFC
TR473	University of Saskatchewan
subsp. <i>spontaneum</i>	
Caesarea 26-24 ^b	Israeli landrace

^a AAFC = Agriculture and Agri-Food Canada.

^b Provided by P. Hayes (Oregon State University, Corvallis, OR), collected by E. Nevo (University of Haifa, Israel).

TABLE 2. Net blotch isolates used in this study

Form, isolate ^a	Collection location ^b	Collection year
Net		
SK 1	Scott, SK	2001
SK 2	Lashburn, SK	2001
SK 3	North Battleford, SK	2001
WRS 102	Indian Head, SK	1959
WRS 858	Teulon, MB	1973
WRS 1607	Prince Albert, SK	1985
WRS 1906	Fredricton, NB	1994
WRS 1907	PEI	1996
Spot		
SK 4	North Battleford, SK	2001
WRS 857	Oakbank, MB	1973

^a All WRS isolates were provided by A. Tekauz (Cereal Research Centre, Agriculture and Agri-Food Canada).

^b Provinces in Canada: SK = Saskatchewan, MB = Manitoba, NB = New Brunswick, and PEI = Prince Edward Island.

added to 75 ml of liquid medium (LM) (10 mM MgSO₄ · 7H₂O, 20 mM KH₂PO₄, 30 mM sucrose, 0.1% bacto-peptone, and 0.1% yeast extract) in a 250-ml flask. Flasks were shaken at 150 rpm at 25°C with constant light for 24 h. Mycelium was harvested by centrifugation at 2,800 × g for 8 min at 25°C, rinsed once with distilled water, spun again, and placed at –80°C overnight. Samples were freeze-dried and ground for 3 min with glass beads using an automated shaker. To each sample, 20 ml of warm (65°C) extraction buffer (EB) (0.5 M NaCl, 100 mM Tris-Cl [pH 8.0], 50 mM EDTA [pH 8.0], 1.25% sodium dodecyl sulfate, plus sodium bisulfite at 3.8 g/liter just before use) was added, mixed, and incubated at 55 to 60°C for 30 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the tubes, mixed vigorously, and centrifuged for 15 min at 250 × g at 25°C. The upper phase was transferred to a new 50-ml tube. Two volumes of (–20°C) 95% ethanol was added, mixed gently to precipitate the DNA, and placed at –20°C for 30 to 60 min. DNA was hooked on a Pasteur pipette, transferred to a new 50-ml tube, and washed with 30 ml of (–20°C) 70% ethanol. After removing the 70% ethanol, the pellet was dissolved in 2 ml of Tris-EDTA (pH 8.0) by incubation at 60°C. After adding 1 ml of 7.5 M ammonium acetate, the tubes were spun for 20 min at 16,000 × g. The supernatant was transferred to a new tube, 20 ml of (–20°C) 95% ethanol was added and left for 30 min at –20°C, and the DNA hooked into a microfuge tube. The tube was centrifuged for 10 min at 16,000 × g, after which the 95% ethanol removed, washed in 70% ethanol, and left to dry upside down for 30 min in a fumehood. The pellet was dissolved in 200 to 400 µl of sterile water. To quantify DNA concentrations, a 1:50 dilution of DNA was made and compared with a series of lambda DNA dilutions (5, 10, 20, 30, 40, and 50 ng/µl). Samples were run on 1.2% 0.5× TBE agarose gels for 2 h at 115 V.

Bulked segregant analysis (BSA) (21) was carried out with 30 progeny isolates derived from the cross WRS 1906 × WRS 1607. Equal concentrations of DNA from 15 virulent isolates were bulked into three pools containing 5 isolates each. Three similar pools also were constructed using DNA from 15 avirulent isolates. These six pools then were used to identify AFLP markers linked to the avirulence gene. Any markers found linked to the pools were screened against the entire mapping population.

AFLP analysis. AFLP was carried out using a method modified from Vos et al. (44), with 250 ng of DNA used as the starting template. Primers containing only one selective base (E-A, E-C, E-G, M-A, M-C, or M-T) were used for the preselective amplification. In all, 12 *Eco*RI primers containing two selective bases (E-AA, E-AC, E-AG, E-AT, E-CA, E-CC, E-CG, E-CT, E-GEA, E-GC, E-GG, or E-GT) and 12 *Mse*I primers containing two selective bases (M-AA, M-AC, M-AG, M-AT, M-CA, M-CC, M-CG, M-CT, M-TA, M-TC, M-TG, or M-TT) were used in all combinations for selective amplification reactions. Primer pairs showing a large number of polymorphisms between parental isolates were used to screen the bulked DNA pools. AFLP bands were run on 6% polyacrylamide gels and visualized by silver staining. Markers were named using the four extension letters from the selective primers and the band size. For example, marker AACT305 denotes a band of 305 bp produced with the primer pair E-AA/M-CT.

Data analysis and linkage group construction. All virulence phenotypic data and AFLP markers identified by BSA were tested for deviations from the expected 1:1 segregation ratio using the χ^2 test ($\alpha = 0.05$). For the purposes of mapping, virulence ratings similar to the avirulent parent and markers originating from this parent were scored as 'a' while virulence similar to the highly virulent parent and markers originating from this parent were scored as 'b.' JoinMap 3.0 (43) was used to group and order the markers and virulence phenotype data. Linkage was established with a log of the likelihood ratio (LOD) score of 4.0, ordered (χ^2 jump restriction = 5, maximum recombination value = 0.5, minimum LOD = 1.0), and a goodness-of-fit (ripple) performed after the addition of each new marker to the growing linkage group. Linkage between ordered loci was calculated using the Kosambi mapping function (14).

RESULTS

Virulence variability of *P. teres* isolates. Virulence of the *P. teres* isolates on the barley lines is summarized in Table 3. Reactions ranged from highly virulent to avirulent, with no one isolate being virulent or avirulent on all lines. Incompatible reactions were observed most often with Heartland (7 of 10 iso-

TABLE 3. Virulence phenotypes of 10 *Pyrenophora teres* isolates on nine barley lines

Isolate	Barley line ^a								
	Excel	CDC Dolly	Harbin	Harrington	Heartland	Norbert	TR251	TR473	Caesarea 26-24
SK 1	2 (3)	7	–	9	1 (2)	7 (8)	4 (5)	7 (6,8)	7 (6,8)
SK 2	2 (1,3)	8 (7)	–	9	1	8	7 (6,8)	7 (8)	4
SK 3	2 (1,3)	3 (4)	–	9	3 (5)	8	8 (6)	4 (6)	4
WRS 102	2 (3)	2	–	8 (7,9)	2 (3)	2 (4)	2	2 (3)	7
WRS 858	2 (1,3)	3 (4)	–	9	7 (6,8)	7 (6,8)	2 (1,3)	3 (4)	3
WRS 1607	6 (8)	5	8 (7)	9 (8)	8 (9)	5 (4,6)	1 (2)	3 (5)	8 (7,9)
WRS 1906	6 (5)	5	7 (8)	8 (9)	1 (2)	6 (5,7)	2 (3)	5 (4)	3
WRS 1907	5 (4)	1 (2)	–	8 (7,9)	2 (1)	1 (3)	2	2 (1)	3 (2,4)
SK 4	9 (8)	9	–	8 (7,9)	7	9	2 (1)	9	5 (4)
WRS 857	7 (6)	8 (7)	–	9 (8)	2 (1)	7	2 (1)	7 (6,8)	2 (1)

^a The most frequent rating (mode) is reported with other observed ratings in parentheses; – = virulence phenotype not evaluated.

TABLE 4. Mating-type determination and crossing results for *Pyrenophora teres* isolates used in this study

Isolate	Mating-type	Successful crosses	Self compatible
SK 1	MAT-2	SK 2, WRS 858, WRS 1607	No
SK 2	MAT-1	SK 1, SK 3, WRS 102, WRS 1906, WRS 1907	No
SK 3	MAT-2	SK 2, WRS 858, WRS 1607	No
WRS 102	MAT-2	SK 2, WRS 858, WRS 1607	No
WRS 858	MAT-1	SK 1, SK 3, WRS 102, WRS 1906, WRS 1907	No
WRS 1607	MAT-1	SK 1, SK 3, WRS 102, WRS 1906, WRS 1907	No
WRS 1906	MAT-2	SK 2, WRS 858, WRS 1607	No
WRS 1907	MAT-2	SK 2, WRS 858, WRS 1607	No
SK 4	MAT-2	None	No
WRS 857	MAT-2	None	No

lates), whereas compatible reactions were recorded on 'Harrington' with all isolates tested. Disease ratings across replications did not differ by more than two units.

***P. teres* mating and phenotyping of mapping population.** Matings between all possible biparental combinations of the same form were established. To confirm the self-incompatibility of this pathogen, crosses using a single isolate also were attempted. The production of pseudothecia and ascospores was observed in a number of net × net crosses, but no successful crosses involving a spot form isolate were observed (Table 4). None of the matings involving a single isolate produced any ascospores.

The *MAT-1/MAT-2* PCR assay confirmed that successful crosses occurred between isolates with alternate mating-type alleles, whereas unsuccessful crosses resulted from isolates having the same mating-type allele (Table 4).

The cross between WRS 1906 and WRS 1607 was selected to generate a mapping population to identify an avirulence gene. These parental isolates were selected because of their mating compatibility, the large number of ascospores produced from the cross, and their contrasting virulence on the barley line Heartland (Fig. 1A). Additionally, both isolates were highly virulent on Harbin (Table 3), indicating that the gene controlling avirulence in WRS 1906 against Heartland is not the same as the avirulence gene identified by Weiland et al. (46). Because both parents were highly virulent on Harrington (Fig. 1B), this barley line was used as a positive control when phenotyping the mapping population. In all, 93 single ascospore progeny were originally isolated from this cross, but this number was reduced to 67 after some ascospores failed to germinate and others were identified as clones (via identical AFLP banding patterns). The population segregated 34:33 avirulent/virulent ($\chi^2 = 0.0$, $P = 1.0$) (Fig. 2; Table 5) when phenotyped for virulence on Heartland, suggesting single gene control of the avirulent phenotype. This 1:1 ratio also was con-

firmed when a complete set of ascospores from the same ascus segregated 4:4 avirulent/virulent. All isolates were highly virulent on Harrington (data not shown).

AFLP markers linked to the *Avr* gene. Parental isolates WRS 1906 and WRS 1607 were screened with 144 AFLP primer pairs producing an average of 40 bands per reaction and 3.6 polymorphisms between the parents. Twenty-three primer pairs were selected based on the high number of scorable polymorphic bands produced and the diversity of selective bases in the primers. From these 23 primer pairs, six bands were identified by BSA as linked to the avirulence phenotype (Fig. 3). Five were derived from WRS 1607 and one from WRS 1906 (Table 6). When these bands (markers) were screened across the entire mapping population (Table 5), all segregated in a 1:1 Mendelian ratio (Table 6). The linkage group produced extends 20 centimorgans, with five of the markers located to one side of the *Avr* gene and a single marker on the other (Fig. 4). The avirulence locus was designated *Avr_{Heartland}*.

DISCUSSION

The 1:1 segregation ratio for virulence on Heartland observed for the mapping population derived from WRS 1906 × WRS 1607 provides evidence that a single gene (designated *Avr_{Heartland}*) in WRS 1906 is responsible for determining avirulence on this barley line. This observation is similar to a previous report by Weiland et al. (46), in which a single gene also was identified in a cross between isolates 0-1 and 15A controlling avirulence of 15A on the barley line Harbin. However, the gene identified in this study is not the same as that located by Weiland et al. (46) because both WRS 1906 and WRS 1607 are highly virulent on Harbin.

Identifying a second gene in *P. teres* which conditions interaction with a specific barley host provides support that the *P. teres*–barley pathosystem follows a gene-for-gene as opposed to a toxin model. To date, only one toxin (ToxC) has been isolated from *P. teres* cultures (9). Although three *P. teres* toxins were reported originally (5), subsequent work demonstrated that ToxA was a precursor of ToxC and ToxB was an artifact generated from ToxC when culture conditions changed (9).

The presence of two distinct genes controlling host line-specific interactions rules out the possibility that both produce toxins that could form the basis of a race system similar to the related tan spot pathogen of wheat, *P. tritici-repentis*, which follows a toxin model with its host (38). *P. tritici-repentis* is the only pathogen

Fig. 1. Virulence of *Pyrenophora teres* parental isolates, WRS 1906 and WRS 1607, used to create the mapping population. **A**, Differential reactions on the barley line 'Heartland'. **B**, Reactions on the barley control line 'Harrington'.

Fig. 2. Frequency of disease reactions incited on the barley line 'Heartland' by *Pyrenophora teres* progeny isolates of the mapping population derived from the WRS 1906 × WRS 1607 cross. Isolates that produced a rating of ≥5 were classified as virulent, whereas isolates eliciting a reaction of ≤4 were classified as avirulent. WRS 1906 elicited a rating of 1, whereas WRS 1607 elicited a rating of 8.

TABLE 5. Virulence phenotypes of the parental isolates, WRS 1906 and WRS 1607, and the 67 derived progeny on the barley line 'Heartland' and association of amplified fragment length polymorphism (AFLP) markers linked to the avirulence locus (*Avr_{Heartland}*)

Isolate	Virulence rating ^a	AFLP markers ^b						Bulk ^c
		GACT215	GATA182	GATG430	GACG308	CGAA1600	GTTA285	
1	3 (2,4)	–	–	–	–	+	–	...
2	8	+	+	+	+	–	+	V3
3	3 (4)	–	–	–	–	+	–	...
4	8	+	+	+	+	–	+	...
5	9 (8)	+	+	+	+	–	+	V1
6	3 (1)	–	–	–	–	+	–	...
7	3 (2,4)	–	–	–	–	+	–	...
8	2 (1)	–	–	–	–	+	+	A3
10	8 (7)	–	–	–	–	+	+	V3
35	8 (7)	+	+	+	+	–	+	V2
36	1 (2)	–	–	–	–	+	–	A3
39	9 (8)	+	+	+	+	–	+	V1
41	2 (5)	–	–	–	–	+	–	...
42	2 (3)	–	–	+	–	+	–	...
43	8 (9)	+	+	+	+	–	+	...
44	8 (7,9)	–	–	–	–	+	+	V3
45	4 (5)	–	–	–	–	+	–	...
46	8 (7,9)	+	+	+	+	–	+	V3
47	7 (6)	+	+	+	+	–	+	...
48	2	–	–	–	–	+	–	...
49	4 (5)	–	–	–	–	+	–	...
50	9	+	+	+	+	–	+	V1
51	7 (6)	+	+	+	+	–	+	...
52	2 (4)	–	–	–	–	+	–	...
53	7	–	+	+	+	–	+	...
54	8 (6)	+	+	+	+	–	+	...
55	2 (1,4)	–	–	–	–	+	–	...
56	2 (3)	+	+	–	–	+	–	...
57	7 (6)	+	+	+	+	–	+	...
58	2	–	–	–	–	+	–	...
59	9 (8)	+	+	+	+	–	+	V2
60	1	–	–	–	–	+	–	A2
61	9	+	+	+	+	–	+	V1
62	2 (1)	+	–	–	–	+	–	A2
65	8 (7)	–	+	+	+	–	+	V3
66	1 (3)	+	–	–	–	+	–	A1
68	1 (2)	–	–	–	–	+	+	A2
70	4 (3)	–	–	–	–	+	–	A3
73	7 (6)	+	+	+	+	–	+	...
74	7 (5)	+	+	+	+	–	+	...
75	7	+	+	+	+	–	+	...
77	4 (5)	–	–	–	–	+	–	...
79	7	–	+	+	+	–	+	...
81	8 (6,8)	+	+	+	+	–	+	...
83	3	–	–	–	–	+	–	...
84	8 (6,9)	+	+	+	+	–	+	V2
86	7 (6)	+	+	+	+	–	+	...
87	1	–	–	–	–	+	–	A1
88	1 (2)	–	–	–	–	+	–	A1
89	7	+	+	+	+	–	+	...
91	7	+	+	+	+	–	+	...
92	3 (2,5)	+	+	–	–	+	–	A3
93	7	–	–	+	+	–	+	...
94	2 (4)	–	–	–	–	+	–	...
95	7 (6)	+	+	+	+	–	+	V2
96	7 (8)	+	+	+	+	–	+	V1
97	2 (1,3)	–	–	–	–	+	–	A1
98	3	–	–	–	–	+	–	...
99	7	+	+	+	+	–	+	...
100	4	–	–	–	–	+	–	...
101	3 (4)	–	–	–	–	+	–	...
102	1 (3)	–	–	–	–	+	–	A2
103	9 (8)	+	+	+	+	–	+	V2
105	1 (2)	–	–	–	–	+	–	A3
107	2 (3)	+	+	+	+	+	–	A2
109	7 (5)	+	+	+	+	–	+	...
110	1 (2)	–	–	–	–	+	–	A1
1906	1	–	–	–	–	+	–	...
1607	8 (7)	+	+	+	+	–	+	...

^a The most frequent rating (mode) is reported with other observed ratings in parentheses.

^b Presence (+) or absence (–) of an AFLP marker.

^c Indicates isolates used to generate bulked DNA samples (e.g., V1 was bulk number one of highly virulent progeny).

which produces multiple host-selective toxins (HSTs) against a single host species (15). Three toxins have been identified in *P. tritici-repentis* which define eight races based on their virulence against three differentials (38).
 A defining characteristic of HSTs is their essential role in disease establishment, thus making them pathogenicity factors.

Fig. 3. Identification of amplified fragment length polymorphism markers by bulked segregant analysis linked to the *Pyrenophora teres* avirulence gene (*Avr_{Heartland}*). AB represent avirulent bulks, VB represent virulent bulks, and the name of the marker is listed to the left of each image.

Reiss and Bryngelsson (30) noted that, although *P. teres* toxin application to detached leaves mimicked net blotch symptoms, cultivar-specific resistance was lost, indicating that the toxins alone did not account for interaction specificity with barley. Therefore, it appears that this toxin is a virulence factor, not a pathogenicity factor, further support for the gene-for-gene model in this pathosystem.

It is conceivable that additional toxins could be identified, perhaps from apoplastic washes instead of artificial culture environments; however, the large number of *P. teres* pathotypes identified argues against this possibility. For example, 45 net form and 20 spot form pathotypes were identified on 9 and 12 barley lines, respectively, in western Canada (40); 80 net form pathotypes were reported in the former Soviet Union using 7 barley lines (2); and 13 pathotypes were identified in California using 22 barley lines (36). Even with the limited number of isolates used in this study, no two demonstrated the same compatibility or incompatibility pattern across the barley lines.

The diversity of *P. teres* pathotypes reported is likely the result of sexual reproduction. A recent study by Rau et al. (28) concluded that sexual reproduction was occurring in field populations of *P. teres*. They found that both mating types existed in a 1:1 Mendelian ratio in all six populations sampled. Such ratios, along with genotypic diversity (25) and gametic equilibrium (27) reported in *P. teres*, are typical indicators of randomly mating

TABLE 6. Data for amplified fragment length polymorphism markers linked to the *Pyrenophora teres* avirulence locus (*Avr_{Heartland}*)

Marker	Segregation ratio ^a	χ^2	<i>P</i>	Parental origin
GACT215	35:32	0.059	0.8069	WRS 1607
GATA182	34:33	0	1	WRS 1607
GATG430	34:33	0	1	WRS 1607
GACG308	35:32	0.059	0.8069	WRS 1607
CGAA1600	36:31	0.239	0.6250	WRS 1906
GTGA285	32:35	0.239	0.6250	WRS 1607

^a WRS 1906 genotype/WRS 1607 genotype.

Fig. 4. Linkage group encompassing the *Pyrenophora teres* avirulence gene (*Avr_{Heartland}*) controlling incompatibility on the barley line ‘Heartland’. Recombination units are indicated on the left (cM = centimorgans) and marker names are shown on the right.

populations (22). The implications of sexual reproduction as it applies to virulence and pathotype generation has not been investigated specifically in *P. teres*; however, pathogens which incorporate sexual reproduction as part of their lifecycle, combined with production of abundant asexual spores and high potential gene flow, are more likely to overcome host genetic resistance (20). These criteria are met by *P. teres*, which may explain the prevalence of net blotch throughout barley growing regions.

The presence of more than one *Avr* gene in *P. teres* is consistent with other pathogenic fungi and oomycetes which follow the gene-for-gene model. *Avr* genes have been reported as allelic forms in *P. parasitica* (29), as clustered genes in *M. lini* (7), and, like *P. teres*, as independent loci in *Blumeria graminis* (24). Numerous *R* loci also are observed in the corresponding hosts and often a small number of these account for a large number of resistance specificities. For example, five loci in flax define over 30 different rust resistance specificities (11), while the barley *Mla* locus controls specific interaction with 32 different *B. graminis* isolates (45). Determining whether the relatively limited number of major *R* loci identified in barley (1,10,17,48) are similarly diverse in their recognition of *P. teres* isolates would provide a stronger argument in support of the gene-for-gene model.

This study identified AFLP markers linked to a *P. teres* avirulence gene (*Avr_{Heartland}*) which will provide a starting point for map-based cloning of this gene. Characterization of this gene and its encoded protein would establish a role in virulence and provide a valuable comparison with the *P. tritici-repentis* toxins. In no other genera do distinct pathogenic species follow both the toxin and gene-for-gene models. This gene is being mapped onto a genetic map of *P. teres* so that future mapping of *Avr* genes can clearly establish their relationship to one another and more firmly determine the relationship between specific barley *R* genes or alleles and *P. teres* *Avr* genes.

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