Gene Action and Linkage of Avirulence Genes to DNA Markers in the Rust Fungus *Puccinia graminis*

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ABSTRACT

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Two strains of the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici*, were crossed on barberry, and a single F_1 progeny strain was selfed. The parents, F_1, and 81 F_2 progeny were examined for virulence phenotypes on wheat differential cultivars carrying stem rust resistance (*Sr*) genes. For eight *Sr* differentials, phenotypic ratios are suggestive of single dominant avirulence genes *Avr*T6, *Avr*T8a, *Avr*T9a, *Avr*T10, *Avr*T21, *Avr*T28, *Avr*T30, and *Avr*TU. Avirulence on the *Sr*; (*Sr* 'fleck') differential showed phenotypic ratios of approximately 15:1, indicating epistatic interaction of two genes dominant for avirulence. Avirulence on *Sr*9d favored a 3:13 over a 1:3 ratio, possibly indicating two segregating genes--one dominant for avirulence and one dominant for avirulence inhibition. Linkage analysis of eight single dominant avirulence genes and 970 DNA markers identified DNA markers linked to each of these avirulence genes. The closest linkages between *Avr*T genes and DNA markers were between *Avr*T6 and the random amplified polymorphic DNA marker crl34-155 (6 centimorgans [cM]) *Avr*T8a and the amplified fragment length polymorphism marker eAC/mCT-197 (6 cM) and between *Avr*T9a and the amplified fragment length polymorphism marker eAC/mCT-184 (6 cM). *Avr*T10 and *Avr*TU are linked at distance of 9 cM.

Additional keywords: AFLP, avirulence gene nomenclature, molecular map, RAPD, *Triticum aestivum*, *T. durum*.

Puccinia graminis Pers. is a heteroecious rust fungus, with uredinial (asexual) and telial stages on cereal and forage grass species (Poaceae, tribe Hordeae) and pycnial and aecial stages on members of the barberry family (Berberidaceae). In the early part of the century, stem rust of wheat (*Triticum aestivum* L. em Thell. and *Triticum durum* L.), caused by *P. graminis* f. sp. *tritici*, was often the major limiting factor in North American wheat production. Disease losses have been greatly reduced by the introduction of wheat cultivars with stem rust resistance (*Sr*) genes and the near eradication of barberry (*Berberis vulgaris* L.), the alternate *P. graminis* f. sp. *tritici* host in the central Great Plains (37). The latter measure had two effects: it slowed the development of epidemics by reducing and delaying the arrival of inoculum to the fields, and it extended the useful life of wheat cultivars by minimizing sexual recombination in the rust fungus and the consequent development of races with virulence to new *Sr* gene combinations (38). Despite these measures, the evolution of *P. graminis* f. sp. *tritici* races with new virulence remains a challenge to wheat and barley production. New sexual races with new combinations of virulence still become established in the Great Plains; wheat stem rust race Pgt-QCC, which overcame 50 years of resistance in barley conferred by the barley T-gene (42), is a recent example. Furthermore, new patterns of virulence may originate in the asexually reproducing stem rust population during dikaryotic (functionally diploid) growth on its wheat host through mutation and other asexual processes, allowing changes in virulence to *Sr* genes to accumulate in clonally related rust fungus lineages (40).

Understanding the function and expression of rust fungus genes that control stem rust avirulence is important for understanding genetic changes that allow stem rust strains to overcome *Sr* resistance genes. Current interpretations of the gene-for-gene model of host parasite interactions (11,13) suggest that resistance to rust and other fungal diseases is caused by host recognition of one or more fungal gene products (elicitors) encoded at avirulence gene loci (9,10,14,19,23). In support of this model, avirulence on most wheat *Sr* genes is expressed as a dominant or partially dominant trait (15,16,20-22,31,32,36,46). However, avirulence on at least one wheat stem rust resistance gene, *Sr*9d, has been reported to be recessive (15,16,21,22,32) and, in one study, to segregate as if recessive in some and dominant in other crosses of the stem rust fungus (20).

Detailed analysis of unusual segregation of avirulence and studies on the effects of mutation on avirulence in the flax rust fungus, *Melampsora lini* (Ehrenb.) Lév. (12,13,28,48,49); the wheat leaf rust fungus, *P. triticina* Erikss. (formerly considered part of the *P. recondita* complex [2]) (17,47,50); and the rice blast pathogen, *Magnaporthe grisea* (Hebert) Barr (8,9,26), have indicated that in other fungal pathosystems avirulence may frequently be under the control of two types of pathogen genes: avirulence (elicitor) genes and inhibitor genes that suppress or inhibit the host's phenotypic response to fungal avirulence gene products by various mechanisms (4,14,18,19,23). Elicitor and suppressor compounds have been isolated from apoplastic fluids of wheat leaves infected with the stem rust fungus (4). It is not, however, currently known to what extent race-specific virulence and avirulence in the wheat stem rust system is mediated by production or lack of production of race-specific or general elicitors versus

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mechanisms that suppress, compete with, or modify the effects of such elicitors.

Understanding the functions that avirulence alleles of the rust fungus have in compatible interactions is important for understanding the nature of host-parasite interactions in wheat stem rust and assuring that efforts to maintain resistance to sexual and asexual populations of the fungus will be effective. Towards this goal the following need to be determined: the number of fungal genes that control avirulence and virulence on different *Sr* genes, the dominant versus recessive expression of avirulence, and genetic linkage. This paper reports the development of an F_2 population of the wheat stem rust fungus and the expression and estimation of the number of segregating genes controlling avirulence on 10 host-resistance differentials. We report the first random amplified polymorphic DNA (RAPD) (54) and amplified fragment length polymorphism (AFLP) (53) markers linked to avirulence loci in *P. graminis* and propose a new system of nomenclature for defining avirulence genes. The F_2 population will be used as a basis to positionally clone avirulence genes and develop a genetic map of the *P. graminis* genome.

MATERIALS AND METHODS

Parental strains. Parents for genetic crosses were selected from stored uredinial isolates of *P. graminis* f. sp. *tritici* at the USDA-ARS Cereal Disease Laboratory, St. Paul, MN, based on the following criteria: (i) differences in their avirulence versus virulence (avr/vir) phenotypes; (ii) their ability to complete the sexual cycle; and (iii) their ability to produce abundant teliospores on adult wheat plants. Each parental strain was obtained from a single uredinium, presumed to have originated from a single urediniospore. Parental strains P1 (CRL 78-21-BB463, race Pgt-DFBJ as designated according to the modified [42] system of Roelfs and Martens [43]) and P2 (CRL 75-36-700-3, race Pgt-SCCL) were from the sexually reproducing North American population of *P. graminis* f. sp. *tritici* and had recently arisen from natural or artificial infections on common barberry.

Crosses. Teliospores for inoculating barberry were obtained 28 to 30 days after injecting suspensions of urediniospores in sterile water into the immature boot (31) and between the leaf sheath and stem of 6-week-old plants of the susceptible wheat cvs. Morocco (PI 431591) and Line E (PI 357308). Teliospore dormancy was broken by cycling telia-laden straw between 7 and −4 °C three times daily and soaking in distilled water two to three times a week. A small piece of the straw was sampled every 2 to 3 weeks to assess whether teliospores would germinate at 18°C (1). Once dormancy was broken (3 to 5 months), the straw was soaked in distilled water for 7 days at 4°C, blotted partially dry, and suspended over barberry plants in sealed chambers at 18°C with 12 h of light and 100% relative humidity (RH). Pycnia began to form on barberry leaves after 5 to 8 days, at which time the straw and the most heavily infected barberry leaves were removed. The humidity was then reduced to ambient conditions. To prevent self-fertilization, leaves bearing multiple pycnia that might enlarge and coalesce were removed approximately 5 to 6 days after immature aecia from overlapping pycnia were first noted.

A sterile toothpick moistened in sterile water was used to transfer pycniospores between pairs of well-isolated pycnia from the two parental strains to produce F_1 progeny. Selfs from the single F_1 strain CRL ZX107-1 were recovered by inoculating barberry plants as mentioned above and harvesting aecia either from the confluent edge of pairs of adjacent pycnia that were well separated from other pycnia or from crosses between selected pycnia. Individual aecia from crosses and selfs were cut from barberry leaves with surrounding uninfected leaf tissue before aecia had begun to shed aeciospores and held in individual petri plates at 4°C until the aecia matured.

To obtain uredinial strains, excised aecia were brought to room temperature at 100% RH, and loose aeciospores from individual aecia were dabbed onto primary leaves of a pot of 7-day-old susceptible plants (cvs. Morocco and McNair 701) that had been treated after emergence with maleic hydrazide to enhance uredinial development (45). After inoculation, pots were individually bagged in clear plastic and incubated at 20°C under 12 h of illumination for 3 to 7 days, then transferred to isolation chambers in a greenhouse. Urediniospores collected 14 to 21 days after inoculation were suspended in light mineral oil (Soltrol 170, Phillips Petroleum Company, Bartlesville, OK), and several dilutions of the spores were sprayed onto fresh pots of maleic hydrazide-treated wheat plants. Infection occurred in an 18°C dew chamber (41). To ensure purity of uredinial strains, each was derived from a single uredinial pustule presumed to originate from a single urediniospore. Up to three single pustule isolates were obtained per strain and were checked on wheat stem rust differentials for purity. Strains suspected of being a mixture of genotypes, based on either pathogenicity or DNA marker data and for which duplicate isolates were not available, were not used for analysis.

Infection phenotypes on differential cultivars. Forty-six wheat cultivars for which avirulence and virulence infection phenotypes (avr/vir phenotypes) could be readily differentiated were used as stem rust differentials (Table 1). Twenty-five F_2 progeny were used in initial tests to determine if the stem rust F_2 population segregated for avr/vir phenotypes on any of these differential cultivars. Subsequent tests used 81 F_2 progeny on a smaller set of differentials that included 10 *Sr* genes (*Sr*6, *Sr*8a, *Sr*9a, *Sr*9d, *Sr*10, *Sr*21, *Sr*28, *Sr*30, *Sr*; [*Sr* 'fleck'], and *SrU*) on which the stem rust fungus F_2 population showed segregation for avirulence and *Sr* differentials that determine the standard 16-gene race designations used by the Cereal Disease Laboratory to categorize races of North American stem rust fungus (42). Because the avr/vir phenotypes of the parental, F_1, and F_2 strains used in this study all differ from prevalent North American races on several of these differentials, the purity of these strains was checked with each set of differentials.

The avr/vir phenotypes were judged by comparing uredinial pustule size on each differential cultivar with previously published reports of infection types for compatible (high-infection type) and incompatible (low-infection type) interactions (39,41,44). Differentials were inoculated as 7-day-old plants (not treated with maleic hydrazide) (44), using suspensions of urediniospores in mineral oil as previously described. After infection, plants were placed in a greenhouse at 18 to 28°C under 160 W VHO fluorescent tubes with a 12 h photoperiod. Infection types of 0 to 4 were assigned 14 to 21 days after inoculation based on the size and appearance of uredinia (34,43). Infection types of each strain were judged after comparing the results of two or more inoculations on sets of differential cultivars, with each replication inoculated on a different day and at least one replication grown in a separate greenhouse. Infection types of 0 (no observable infection), fleck (a small amount of necrosis without spore production), and 1, 2, and 3C (small uredinial pustules, often with chlorosis or necrosis) were considered avirulent (incompatible) phenotypes, while infection type 4 (large uredinial pustules lacking chlorosis and necrosis) was considered a virulent (compatible) phenotype. For a few rust fungus strains where fully compatible pustules developed slower than expected on McNair 701 and other standard differentials with known compatibility, infection types of size 3 to 3+ were equivalent to the fully compatible infection type 4.

DNA preparation and amplification. Urediniospores of *P. graminis* f. sp. *tritici* were germinated as described by Liu et al. (30). DNA was prepared as described by Kubelik and Szabo (24) from lyophilized mats of germinated urediniospores. RAPD analysis was performed using PTC-100 thermocyclers (MJ Research, Watertown, MA) as described by Kubelik and Szabo (24). RAPD oligonucleotide primer kits (10-mer) were purchased from Operon

Technologies (op) (Alameda, CA) and Genosys (gen) (The Woodlands, TX). Custom-designed RAPD primers (crl) with a guanine + cytosine content of 80 to 100% were as described (24). Amplification products were electrophoresed in 1.4% agarose gels containing ethidium bromide at 0.8 μg/ml. AFLP analysis was performed as described by Vos et al. (53) using a commercially available kit (Gibco BRL, Grand Island, NY) and primers with two base instead of three base extensions. Amplified products were separated in 5% Hydro-link Long Ranger acrylamide sequencing gels (FMC Bioproducts, Rockland, MD) using SequiGen electrophoresis units (Bio-Rad Laboratories, Hercules, CA).

Data analysis. Chi-square values corrected for continuity (51, 55) were calculated to compare observed ratios of avirulent versus virulent phenotypes against Mendelian ratios of 3:1, 15:1, 1:3, and 3:13 that would be expected if avirulence phenotypes were controlled by a single dominant gene for avirulence, two dominant genes for avirulence, a single recessive gene for avirulence, or an epistatic interaction between a dominant gene for avirulence and a dominant gene inhibiting avirulence, respectively (Table 2). The methods of NaNagara (35) and Mather (33) were employed to distinguish whether data better fit a 1:3 or 3:13 ratio when neither ratio could be rejected on the basis of *P* value. Chi-square was also used to compare the ratio between presence and absence of markers among strains to a 3:1 ratio expected for dominant markers. In these chi-square analyses, *P* values identified the probability that even greater deviations from the test ratio than those observed could be expected by chance alone (51). $P < 0.05$ was selected as the level for rejecting the null hypothesis that data approximated a tested ratio.

The program Mapmaker, version 3.09 (Whitehead Institute for Biomedical Research, Cambridge, MA) (25) was used for multipoint linkage analysis of eight avirulence genes and 970 DNA markers. This set of DNA markers included 354 RAPD markers (348 dominant and 6 codominant markers) and 616 AFLP markers (607 dominant and 9 codominant markers). Each marker considered codominant satisfied three criteria: (i) there were no recombinant genotypes in repulsion (i.e., absence of both amplified products) identified in the F_2 population; (ii) each pair of amplified products was amplified by a single RAPD primer or AFLP primer pair; and (iii) at least one of the amplified products (allele) was in each of the two parents. Data was scored as an F_2 intercross and a threshold $\log_{10}$ of the likelihood ratio (lod) score of 3.0 was used. Genetic distance, expressed in centimorgans, was calculated using the Haldane mapping function (6). Given the size of the F_2 population, all distance values (centimorgans) have been rounded to the nearest whole number. In addition, two-point recombinant fraction values (*r*) were calculated for *Avr*T loci and closely linked DNA markers from the same parent and between two linked *Avr*T loci. Chi-square was used to test the null hypothesis of random assortment for pairs of loci, with $P < 0.05$ selected as the level for rejecting the null hypothesis, indicating statistically significant linkage.

Avirulence gene and DNA marker designations. For discussion and mapping purposes, each single gene presumed to control an avr/vir phenotype has been designated with the prefix "*Avr*T" indicating avirulence on *Triticum* stem rust differential and a suffix corresponding to the name of an *Sr* gene published in McIntosh (34) and Roelfs and Groth (41). Rust fungus genes presumed to inhibit or suppress expression of avirulence are designated with a prefix of "*I*" plus the *Avr* abbreviation for the avirulence gene(s) whose avirulence phenotype is presumed to be inhibited–a convention previously used in the flax rust fungus (28). Each DNA marker has been designated by the name of the RAPD primer or AFLP primer pair used and the size(s) of the amplified fragment(s) to the nearest unit of 10 bp for RAPDs and single bases for AFLPs. For example, the RAPD marker opAJ18-090 is a 0.9-kb DNA segment amplified using the AJ18 primer from Operon, and codominant AFLP marker eAG/mCC-173/247 is a pair of DNA segments of 173 and 247 bases amplified using primers *Eco*R1-AG and *Mse*1-CC.

RESULTS

Genetic analysis of avir/vi phenotypes. To determine if any of the genes controlling the avirulence phenotype were segregating in the *P. graminis* f. sp. *tritici* F_2 population, we tested 25 F_2 progeny on 46 wheat stem rust differential cultivars (Table 1). These F_2 progeny showed segregation for avr/vir phenotype on 10 *Sr* differential cultivars but had a nonvariable avirulence phenotype on 22 *Sr* differentials and a nonvariable virulence phenotype on 14 additional *Sr* differentials. To obtain F_2 segregation data, we tested 81 F_2 progeny on a smaller set of differentials that included the 10 *Sr* genes on which the *P. graminis* f. sp. *tritici* F_2 population had shown segregation for avirulence.

Although different sizes of avirulent infection types have been suggested as an indication of whether avirulence alleles are present in the homozygous versus heterozygous state (39,41), the differences are often minute. In some cases intermediate infection types were observed, which may represent the heterozygotes. However, there was enough variation to make it difficult to reliably score the intermediate classes. Therefore, we categorized infection phenotypes of *P. graminis* f. sp. *tritici* strains into only two classes: fully compatible interactions that produced large uredinial pustules, indicating virulence phenotypes versus interactions that were not fully compatible and produced smaller and more chlorotic or necrotic pustules, indicating avirulence phenotypes (39,41,44).

On most *Sr* differentials, the avirulence and virulence phenotypes of parents, F_1, and F_2 progeny fit patterns that would be expected if the avirulence phenotype were dominant, e.g., when an F_1 was virulent, all F_2 progeny were virulent, and when an F_1 was avirulent, F_2 progeny would either be avirulent or segregate for avirulence (Table 1). Exceptions were found with the *Sr*9d and *Sr*Tt-3 differentials. On *Sr*9d, the F_1 was virulent but the F_2 progeny segregated for avr/vir phenotypes. On *Sr*Tt-3, parent P2 was virulent, but parent P1, F_1, and F_2 progeny were all avirulent. In both of these cases, the data suggests the possibility of a dominant gene, that suppresses the expression of avirulence. For example, the data indicate that parent P2 was heterozygous for a dominant suppressor of avirulence on *Sr*Tt-3, and the F_1 is homozygous for the recessive allele of this gene.

Segregation data from eight differentials supported 3:1 avr/vir phenotype ratios, indicating eight single *P. graminis* f. sp. *tritici* avirulence genes with dominant gene expression (*Avr*T6, *Avr*T8a, *Avr*T9a, *Avr*T10, *Avr*T21, *Avr*T28, *Avr*T30, and *Avr*TU; Table 2). Segregation data from the *Sr*; (*Sr*'fleck') differential supported a 15:1 ratio (avr/vir) ($P > 0.8$), but not a 3:1 ratio ($P < 0.05$), indicating the likelihood that two dominant unlinked *P. graminis* f. sp. *tritici* avirulence genes interact epistatically to control phenotype on the *Sr*; differential cv. 8N221Sr;RHR. For segregation data from the *Sr*9d differential, chi-square tests of 1:3 (one recessive gene conditioning avirulence) and 3:13 ratios (epistatic interaction of one gene dominant for avirulence and one gene dominant for suppression of avirulence) gave *P* values of 0.19 and 0.95, respectively. Since the results from the two ratios did not reach the $P < 0.05$ needed to reject the null hypothesis, additional tests were used to determine whether the data better fit a 1:3 or 3:13 ratio. Although the F_2 population size is not large enough to unequivocally differentiate between these hypotheses, both Mather's (33) "ambiguous ratio" criterion and NaNagara's (35) "dividing line" favor the 3:13 ratio over the one gene 1:3 ratio.

Identification of DNA markers linked to avirulence genes. To identify DNA markers linked to the eight single dominant avirulence genes segregating in this F_2 population, RAPD and AFLP analyses were employed. Using 694 RAPD oligonucleotide primers (620 op, 40 gen, and 34 crl) yielded 360 reproducible amplification products that were detected in one or both of the

parents and segregated in the F_2 population. In addition, 21 AFLP primer pairs produced 625 polymorphic amplification products that segregated. In this set of RAPD and AFLP amplification products, 30 were identified that behaved as pairs of alleles to 15 codominant loci and were scored as codominant markers. Twenty-nine percent of the segregating DNA markers were found in both parents, indicating that at least one of the parents was heterozygous for each of these markers. This heterokaryotic nature was also observed with respect to the avirulence loci (Table 2) and indicates the high level of genetic heterogeneity that may be obtained between the two nuclei in the dikaryotic asexual stage of this fungus.

Linkage analysis of the 970 DNA markers and 8 avirulence genes identified DNA markers linked to each of the avirulence genes (Fig. 1). The closest DNA markers to avirulence genes are crl34-155, 6 cM from *Avr*T6; eAC/mCT-197, 6 cM from *Avr*T8a; and eAC/mCT-184, 6 cM from *Avr*T9a (Table 3). Two avirulence genes, *Avr*T10 and *Avr*TU, were linked (9 cM). Occasional minor differences in distance values given in Table 3 versus those given in Figure 1 are due to differences in methods of calculating distances. Two-point linkage analysis was used for data in Table 3, but multipoint linkage analysis was used for data in Figure 1. For example, the distance between the RAPD marker crl134-155 and *Avr*T6 is 5 cM on the genetic linkage map (Fig. 1) and 6 cM in the linkage data (Table 3).

For three of the avirulence loci (*Avr*T6, *Avr*T28, and *Avr*T30), each of the parents carry at least one avirulent allele (Table 2). This provides an opportunity to test whether the closest linked DNA marker provides a useful tag for these avirulence genes rather than an indication of parentage. In the case of the *Avr*T6

TABLE 1. Avirulence and virulence phenotypes of parental, F_1, and F_2 progeny strains from a cross of *Puccinia graminis* f. sp. *tritici* on wheat stem rust differential cultivars

Sr gene[a]	Cultivar	Accession no.[b]	Phenotypes of strains[c]			
			P1	P2	F_1	F_2
*Sr*5	LCSr5R1	CRL 1-1	Avr	Vir	Vir	Vir
*Sr*6	Isr6-Ra, W2691Sr6	CItr 14163, CRL 1-22	Avr	Avr	Avr	SEG
*Sr*7b	Isr7b-Ra	CItr 14165	Avr	Avr	Avr	Avr
*Sr*8a	Isr8-Ra	CItr 14167	Vir	Avr	Avr	SEG
*Sr*8b	Barletta Benvenuto	CItr 14196	Vir	Avr	Vir	Vir
*Sr*9a	Isr9a-Ra	CItr 14169	Avr	Vir	Avr	SEG
*Sr*9b	W2691Sr9b	Citr 17386	Avr	Avr	Avr	Avr
*Sr*9d	Isr9d-Ra	CItr 14177	Vir	Avr	Vir	SEG
*Sr*9e	Vernal	CItr 3686	Vir	Vir	Vir	Vir
*Sr*9g	CnsSr9g	CRL 5-63	Vir	Vir	Vir	Vir
*Sr*10	W2691Sr10	CItr 17388	Vir	Avr	Avr	SEG
*Sr*11	Isr11-Ra	CItr 14171	Avr	Avr	Avr	Avr
*Sr*13	W2691Sr13	CItr 17387	Avr	Avr	Avr	Avr
*Sr*14	W2691Sr14, Line A sel.	CRL 5-187, CRL 5147B	Avr	Avr	Avr	Avr
*Sr*15	W2691Sr15 Nk	CRL 1-13	Vir	Vir	Vir	Vir
*Sr*18	LCSr18 RI	CRL 5-20	Vir	Vir	Vir	Vir
*Sr*20	LCSr20Mq, LCSr20 RI	CRL 5-22, CRL 5-46	Vir	Vir	Vir	Vir
*Sr*21	Einkorn	CIr 2433, W 3586	Avr	Vir	Avr	SEG
*Sr*22	SwSr22T.B.	CRL 3-21	Avr	Avr	Avr	Avr
*Sr*24	BtSr24Ag	CRL 3-22	Avr	Avr	Avr	Avr
*Sr*25	LCSr25Ars	CItr 17474	Avr	Avr	Avr	Avr
*Sr*26	Eagle (A)	PI 365582	Avr	Avr	Avr	Avr
*Sr*27	WRT 238-5	CItr 14141	Avr	Avr	Avr	Avr
*Sr*28	W2691Sr28Kt	CRL 1-20	Avr	Avr	Avr	SEG
*Sr*29	Pusa/Etiole de Choisy	CRL 5-55B	Avr	Avr	Avr	Avr
*Sr*30	BtSr30Wst	PI 442897	Avr	Avr	Avr	SEG
*Sr*31	Line E/Kavkaz	CRL 5-161B	Avr	Avr	Avr	Avr
*Sr*32	Er5155	CRL 5-203	Avr	Avr	Avr	Avr
*Sr*33	Tetra Canthatch	RL 5405	Avr	Avr	Avr	Avr
*Sr*35	Mq(2)5XG2919	CRL 5-234	Vir	Avr	Vir	Vir
*Sr*36	W2691SrTt-1	CItr 17385	Avr	Avr	Avr	Avr
*Sr*37	W2691SrTt-2	CRL 5-171B	Avr	Avr	Avr	Avr
Srdp 2	Media Ag9d	CItr 3255	Avr	Avr	Avr	Avr
Sr;	8N221Sr;RHR	CRL 8-80	Avr	Avr	Avr	SEG
*Sr*Gt	BtSrGtGt	CRL 3-5	Avr	Avr	Avr	Avr
*Sr*H	H44 deriv.	CRL 5-167	Avr	Avr	Avr	Avr
*Sr*Kt2	Line AE sel.	CRL 1-20	Vir	Vir	Vir	Vir
*Sr*LC	Little Club	CItr 4066	Vir	Vir	Vir	Vir
*Sr*McN	McNair 701	CItr 15288	Vir	Vir	Vir	Vir
*Sr*Pl	Peliss	CItr 1584	Vir	Vir	Vir	Vir
*Sr*Pt	Peterson ML68-14	PI 355090	Vir	Vir	Vir	Vir
*Sr*Tmp	Triumph 64	CItr 13679	Avr	Avr	Avr	Avr
*Sr*Tt-3	Federation *2/SrTt-3	CRL5-64D	Avr	Vir	Avr	Avr
*Sr*U	SrUAc	CRL 5-182	Vir	Avr	Avr	SEG
*Sr*Wld	BtSrWld-1	CRL 3-25	Avr	Avr	Avr	Avr
*Sr*Wst-2	LcSrWst-2Wst	CRL 5-228	Vir	Vir	Vir	Vir

[a] *Sr* = Stem rust resistance.

[b] Original sources for stored derivative lines used as differentials: CRL = USDA, ARS, Cereal Disease (Rust) Laboratory, St. Paul, MN; CItr and PI = USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network, National Germplasm Resources Laboratory (Beltsville, MD); W = Plant Breeding Institute, The University of Sydney, Sydney, Australia; RL = Agriculture Canada, Winnipeg.

[c] Parental srains: P1 = CRL 78-21-BB463, P2 = CRL 75-36-700-3. F_1 strain = CRL ZX107-1. Avirulence (Avr) and virulence (Vir) determined by comparison with previously reported compatible and incompatible infection types (39,41,44). SEG indicates mixtures of avirulent and virulent phenotypes among the F_2 progeny strains.

locus, the closest DNA marker, crl34-155 (6 cM), was also found in both parents (Table 3). This was also true for the *Avr*T28 locus, in that both parents carry the DNA marker eAC/mTA-107 (9 cM). However, in the case of the *Avr*T30 locus, the closest DNA marker (eAC/mCT-448, 12 cM) is found in only one parent (P2). Although these DNA markers (crl34-155 and eAC/mCT-488) are not very tightly linked to the corresponding avirulence genes, it is possible that these markers may be useful for determining the presence of avirulent alleles for *Avr*T6 and *Avr*T28 loci.

Overall, the dominant DNA markers and the individual alleles of the codominant markers showed normal segregation. A frequency distribution, plotting the individual segregation ratios for all of the dominant DNA markers approximated a monomodal curve fitting the expected 3:1 ratio (data not shown). Approximately 16% of the markers (42 RAPDs and 113 AFLPs) showed segregation ratios that fell within the tails of the curve and would be rejected at the $P < 0.05$ level for fitting a 3:1 ratio. Linkage analysis of these markers failed to identify any specific regions of the genome that showed distorted segregation. In addition, none of the markers having skewed segregation ratios showed linkage to genes conditioning avirulence on *Sr*9d and *Sr*; differentials.

DISCUSSION

In this study, we developed a segregating F_2 population for *P. graminis* f. sp. *tritici* consisting of 81 F_2 progeny of a single cross. Of the 10 avr/vir phenotypes segregating on *Sr* differentials, segregation analysis indicated that 8 were due to single, dominant *Avr*T genes (Table 2). Thus, the results of this study support observations that avirulence is dominant for most *P. graminis* f. sp. *tritici* genes involved in avirulence. We confirmed previous reports of dominant avirulence for each of the following genes: *Avr*T6 (as avirulent on Eureka [22,32]); *Avr*T8a (synonym avirulence on *Sr*8 [15,16,22]); *Avr*T9a (15,16,22); *Avr*T10 (15); and *Avr*T21 (synonym avirulence on Einkorn [CItr2433]; 20,31,32). *Avr*T28, dominant for avirulence in this study, may have been referred to by Loegering and Powers (31) when they mentioned two dominant genes for avirulence detected in progeny of a rust cross using the differential cv. Kota (CItr5878), a cultivar later determined to carry resistance genes *Sr*28 and *Sr*'Kt2'. Dominant avirulence of *Avr*T30 and *Avr*TU had not been previously documented.

A 15:1 avr/vir segregation ratio indicated that two dominant unlinked genes control avirulence on *Sr*; (*Sr* 'fleck') differential cv. 8N221Sr;RHR. This most likely indicates that the *Sr*; differential carries two or more *Sr* genes that have not been previously differentiated–a problem frequently encountered in the development of stem rust differentials (31). The *Sr*; differential was derived from a cross between 'Kenya 58' (CItr12471) and susceptible cv. Baart (PI5078) but had not been selected as a single gene line (A. P. Roelfs, retired, *personal communication).* However, an alternative explanation is that the gene products of two unlinked *P. graminis* f. sp. *tritici* avirulence loci are recognized by the gene product of a single resistance gene (*Sr*;) and are acting as elicitors of the host resistance response. If the latter is true, there are two possibilities: (i) there could be duplicate, unlinked copies of an avirulence gene in *P. graminis* f. sp. *tritici* mediating host recognition, or (ii) two different unlinked avirulence genes could each be producing an elicitor that is recognized by the *Sr*; gene product. The exact nature of the *Avr*T genes controlling avirulence on the *Sr*; differential will remain unknown until the two genes are studied separately.

The observed ratio of avirulent to virulent phenotypes that was obtained on *Sr*9d (synonyms *Sr*1 resistance and resistance of Arnautka [CItr1493], Mindum [CItr5296)], and Spelmar [CItr6236]; [34]) was of special interest, because it provides evidence that avirulence and inhibitor genes might interact to control avir/vir phenotypes in *P. graminis*. Segregation on this *Sr* differential does not fit the 3:1 ratio expected of a dominant avirulence gene but fits 3:13 and 1:3 ratios, with a very close approximation to the 3:13 ratio. There are several possible explanations for the data: (i) two genes are segregating, one dominant for avirulence (an *Avr*T gene) and one dominant for the suppression of avirulence (an *IAvr*T inhibitor gene); (ii) a single dominant gene for suppression of avirulence (an *IAvr*T inhibitor gene) is segregating in a background fixed for avirulence; and (iii) a single avirulence gene that is genuinely recessive for avirulence is segregating. Distortions in segregation caused by linkage to lethal mutations or mating type genes might have caused a single gene, suggested in hypotheses two and three, to appear to fit a 3:13 two-gene ratio. However, regions of linkage having distorted segregation have not been identified in the current study. This and the very close approximation of the data to the 3:13 ratio suggest that the most likely explanation for the observed pattern of avir/vir phenotypes on *Sr*9d is the two-gene (avirulence gene plus inhibitor gene) hypothesis. Selfs of the avirulent F_2 progeny or backcrosses will need to be done to unequivocably determine the number and type of genes that control this avirulence phenotype.

Previous studies provide additional, albeit weak, support for an inhibitor gene affecting avirulence on the *Sr*9d gene. In one study,

TABLE 2. Segregation of avirulence and virulence phenotypes (avr and vir respectively) in a cross of *Puccinia graminis* f. sp. *tritici*

	Infection types of strains[b]					Analysis of Segregation Ratios in F_2[c]				
Sr gene[a]	P1	P2	F_1	F_2 avr	F_2 vir	Observed Ratio (avr/vir)	Expected Ratio (avr/vir)	χ^2	*P*	Model for avirulence
*Sr*6	0–;1	0–;1	0–;1	0–;1˚	4	58:21	3:1	0.038	0.867	*Avr*T6
*Sr*8a	4	2˜–2	;1–2	1–2˚	(4˚)–4	55:19	3:1	0.000	1.000	*Avr*T8a
*Sr*9a	2˚–2	4	;2–2	1–2	4	54:24	3:1	1.094	0.245	*Avr*T9a
*Sr*9d	4	;–2	4	;–2	4	14:64	1:3	1.709	0.194	1 gene
							3:13	0.001	0.975	2 genes
*Sr*10	4	0–13c	;–31c	;–31c	4	53:24	3:1	1.251	0.268	*Avr*T10
*Sr*21	1–2	4	2–2˚	1˜–2	(3c)–4	63:14	3:1	1.562	0.213	*Avr*T21
*Sr*28	4	0–0;	0–0;	0–0;	4	60:19	3:1	0.004	0.949	*Avr*T28
*Sr*30	2˜–2	2c–23	2˜–2	1–2˚	4	63:17	3:1	0.417	0.528	*Avr*T30
*Sr*U	4	;1c–23c	2c–23c	;–23c	4	55:19	3:1	0.000	1.000	*Avr*TU
Sr; ('fleck')	;2–23	2˚–2˚	;–2	;–2˚	(3c)–4	74:6	15:1	0.053	0.844	2 genes

[a] *Sr* = stem rust resistance.

[b] Parental strains: P1 = CRL 78-21-BB463, P2 = CRL 75-36-700-3; F1 strain: ZX107-1. Infection types are according to Roelfs (39), Roelfs and Groth (41) and Roelfs and McVey (44). Infection type: 0 = no observable symptoms; ; (i.e. 'fleck') = small amount of necrosis without spore production; 1, 2, and 3 = pustules of increasing size that produce urediniopsores with or without chlorosis (c); 4 = fully compatible infection type with large uredinial pustules lacking chlorosis and necrosis. +, –, and = indicate pustule sizes slightly larger and smaller than standard infection types, respectively. Parentheses indicate rare infection types.

[c] Chi-square values corrected for continuity (51,55). *P* value represents probability of greater chi-square. The null hypothesis (that the ratio is correct) is rejected at $P < 0.05$. Only ratios that cannot be rejected by chi-square analysis are presented.

small numbers of progeny were obtained from each of four selfed strains (20). Segregation for avirulence on *Sr*9d from three strains appeared to approximate a 1:3 ratio expected for the recessive avirulence phenotype, but the fourth approximated a 3:1 ratio for the dominant avirulence phenotype, indicating segregation of a second gene. Other genetic studies suggested that the avirulence phenotype on *Sr*9d was a recessive trait controlled by a single gene (15,16,21,32). Kao and Knott (22) suggested that avirulence on cv. Mq-Sr1, which carries *Sr*9d and another uncharacterized resistance gene, was a recessive trait controlled by two genes. In each case, the observed ratio of 1 avirulent/3 virulent could be explained by either a dominant inhibitor of avirulence or a recessive avirulence gene.

The discovery of inhibitor genes in fungal pathosystems has provided new insights into host-parasite interactions at a molecular level for some pathosystems. Inhibitor genes that control avirulence have been demonstrated in wheat leaf rust (17,48,50), flax rust (12,13,27,49), and rice blast disease caused by the ascomycetous fungus *Magnaporthe grisea* (8,9,26). For example, it has been postulated that there are separate inhibitor genes in *M. grisea* for each of 17 avirulence loci (9) and that some inhibitor genes in the flax rust fungus (28) and the wheat leaf rust fungus (47) affect the phenotypic expression of several avirulence genes. It is likely that there are genes that inhibit the expression of avirulence in *P. graminis*. However, the high frequency with which avirulence behaves as a single dominant trait in *P. graminis* indicates that inhibitor genes may be more rare or affect fewer avirulence genes than in other fungi.

The seven genetic linkage groups, containing 52 DNA markers and 8 avirulence genes, represent the first partial genetic map of *P. graminis*. These seven linkage groups cover only a portion of the 18 chromosomes contained in the haploid genome (5), which is estimated to contain 67 million base pairs (3). Development of a complete genetic map has been hindered by several factors, including the heterokaryotic nature of the asexual uredinial stage of *P. graminis* and the dominant nature of the markers scored. To complete this genetic map, a polymerase chain reaction-based, codominant marker system such as simple sequence length polymorphisms or single nucleotide polymorphisms will need to be developed. We have begun to clone and characterize RADP and AFLP markers linked to these eight avirulence genes, which will be used to develop codominant markers and physical maps of these regions. Given the difficulty of making crosses, it is unlikely that a significantly larger F_2 population will be generated. Therefore, the exact location of the avirulence genes on a physical map may depend on characterization of induced virulent mutants as well as transformation experiments with cloned segments of *P. graminis* f. sp. *tritici* DNA.

Though most avirulence genes did not appear to be linked to each other, *Avr*T10 and *Avr*TU were linked at ≈ 9 cM. Green (15) previously suggested that both *Avr*T7a and *Avr*T11 (as virulence to cv. Lee) are also linked to *Avr*T10, although this could not be

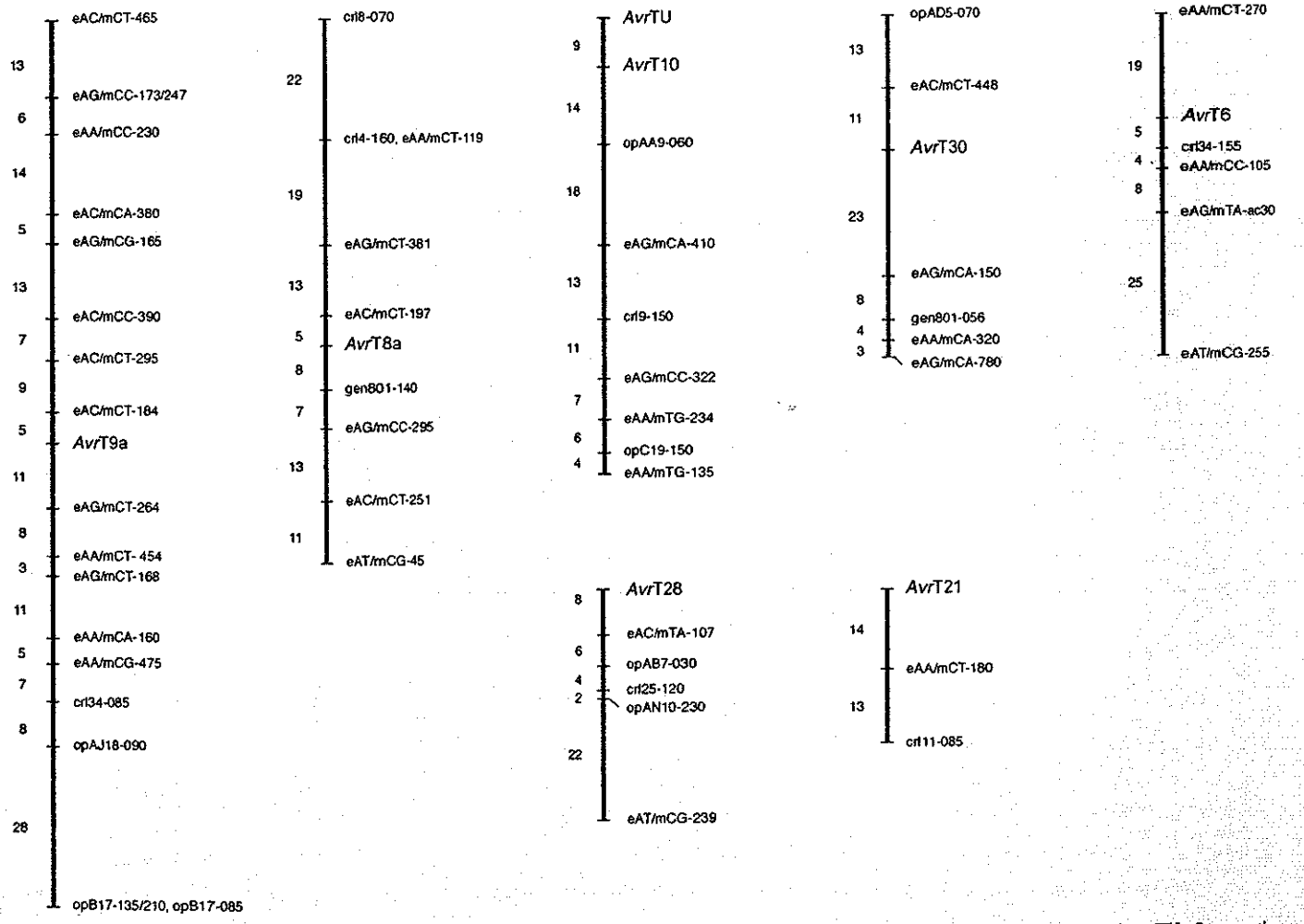

Fig. 1. Genetic maps of chromosomal regions containing avirulence genes *Avr*T6, *Avr*T8a, *Avr*T9a, *Avr*T10, *Avr*T21, *Avr*T28, *Avr*T30, and *Avr*TU. Segregation data were obtained from an F_2 intercross of *Puccinia graminis* f. sp. *tritici*. Markers are indicated on the right; distances between markers (in centimorgans) are indicated on the left. Markers prefixed with op, gen, and crl indicate random amplified polymorphic DNA (RAPD) markers obtained using RAPD primer kits from Operon and Genosys, and primers designed at the Cereal Disease Laboratory (24), respectively. Markers prefixed with eNN/mNN (e.g., eAG/mCG) indicate amplified fragment length polymorphism markers obtained with *Eco*RI and *Mse*I primers with two base extensions. Mapmaker (25) version 3.09 was used for multipoint linkage analysis.

tested in this study. Genetic linkage, with distances of 20 cM or more (7,29,52), has been shown between avirulence genes in *Magnaporthe grisea*. Linkages between avirulence genes have also been observed in *Melampsora lini*, which in some cases are tightly linked (28), indicating the possibility that avirulence genes in fungal genomes, like resistance genes in plants, may be clustered. At present only a few of the more than 60 wheat stem rust avirulence genes have been genetically analyzed. Therefore, the distribution of these genes within the *P. graminis* genome remains unknown.

We have utilized a new system of nomenclature to provide a uniform and flexible system for naming and mapping genes involved in avirulence in all formae speciales of *P. graminis* and fostering discussions of these genes. In this system, avirulence genes are named using a four letter prefix that indicates avirulence on a particular host, such as '*Avr*T', '*Avr*S', '*Avr*H', and '*Avr*A' for wheat (*Triticum*), rye (*Secalis*), barley (*Hordeum*), and oats (*Avena*), respectively, and a suffix that indicates the name of the stem rust resistance gene on which compatibility is assessed. In cases where a resistance gene has its origin in another host genus, e.g., *Sr*27 and *Sr*31, translocated from rye (34), the prefix would indicate the host in which compatibility is being assessed, rather than its host of origin, i.e., *Avr*T instead of *Avr*S.

A second feature of the proposed system is that it can be used to provide different names for two types of rust fungus genes that may condition avr/vir in race-specific interactions: (i) avirulence (*Avr*) genes that may be presumed to cause avirulence by their production of an elicitor of a host resistance response and show evidence of dominant or partially dominant gene expression of avirulence; and (ii) inhibitor (*IAvr*) genes that inhibit or suppress avirulence conditioned at one or more *Avr* loci, whether the *Avr* locus whose effect it moderates is itself fixed or variable for avirulence in the rust fungus population. Such inhibitor genes might be variously and tentatively identified in the stem rust fungus by dominant expression of avirulence suppression, loss of such suppression or inhibition after mutation, or crosses that prove epistatic interactions with *Avr* genes. Providing a prefix of *I* before the list of *Avr* designations for the avirulence gene(s) whose avirulence phenotype(s) are being inhibited follows current nomenclature in the flax rust system in which avirulence suppression was first discovered (28).

Inasmuch as the proposed system accepts the proposition that more than one rust fungus gene may affect the avr/vir phenotype on an *Sr* gene stem rust differential, it is in contrast to the previous gene naming systems for stem rust that are based on a strict gene-for-gene hypothesis that refers to *P. graminis* genes that control avr/vir phenotypes as *P* genes or pathogen genes (22,31,39,41,46). The ability of the new system to consolidate information from crosses of the rust fungus originating from the same or different host genera and to recognize potential elicitor versus inhibitor genes will allow many genes involved in avirulence to be incorporated into the same genetic map. The system, thus, may provide a platform for discussion of whether rust fungus genes can condition avirulence in more than one host genus.

In conclusion, this study has demonstrated that of the rust fungus genes affecting the avr/vir phenotype in wheat stem rust, most are dominant for avirulence and unlinked to each other. Moreover, the set of materials provided by this study will be useful for investigations of the genetic control of avirulence phenotypes in the host-parasite interactions of the wheat stem rust fungus, *P. graminis* f. sp. *tritici*. The F_2 intercross population, RAPD and AFLP markers may enable cloning and examination of gene structure of up to eight avirulence loci that may eventually provide answers to questions of how the gene products of avirulence loci function in the fungus and elicit resistance in the host.

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TABLE 3. Genetic linkage analysis of DNA markers and avirulence genes

Avirulence gene	Marker gene[a]	r[b]	Distance (cM)	(χ^2)[c]	P	lod score[d]	P1	P2
*Avr*T6	crl34–155	0.05	6	57.7	3.1 E –14	12.8	+/ Avr[e]	+/ Avr
*Avr*T6	eAA/mCC–114	0.07	12	32.3	1.3 E –8	6.5	–/ Avr	+/ Avr
*Avr*T8a	eAC/mCT–194	0.05	6	48.0	4.3 E –12	10.8	–/ Vir	+/ Avr
*Avr*T8a	gen801–140	0.08	9	40.0	2.5 E –10	9.5	–/ Vir	+/ Avr
*Avr*T9a	eAC/mCT–182	0.06	6	50.6	1.1 E –12	12.9	+/ Avr	–/ Vir
*Avr*T9a	eAG/mCT–264	0.09	11	40.8	1.7 E –10	9.3	+/ Avr	–/ Vir
*Avr*T10	*Avr*TU	0.09	9	39.3	3.6 E –10	10.2	Vir / Vir	Avr / Avr
*Avr*T10	opAA9–060	0.12	15	36.1	1.9 E –9	7.4	–/ Vir	+/ Avr
*Avr*T21	eAA/mCT–180	0.12	15	22.2	5.0 E –6	5.8	+/ Avr	–/ Vir
*Avr*T28	eAC/mTA–107	0.07	9	40.3	2.2 E –10	8.9	+/ Avr	+/ Avr
*Avr*T28	opAB7–030	0.12	15	25.4	4.7 E –7	6.1	+/ Avr	+/ Avr
*Avr*T30	eAC/mCT–448	0.10	12	39.8	2.8 E –10	8.6	+/ Avr	–/ Avr

[a] Markers prefixed with op, gen or crl indicate random amplified polymorphic DNA markers; markers prefixed with eNN/mNN indicate amplified fragment length polymorphism markers.
[b] Recombination fraction.
[c] Chi-square values corrected for continuity (51,55), testing the null hypothesis of no linkage. Values of $P < 0.05$ reject the null hypothesis.
[d] Log_{10} of the likelihood ratio.
[e] +: Parental isolate amplifying the DNA marker; –: Parental isolate not amplifying the DNA marker; Avr: parental isolate showing an avirulent phenotype on wheat stem rust differential with the corresponding stem rust (*Sr*) resistance gene; Vir: parental isolate showing a virulent phenotype wheat stem rust differential with the corresponding *Sr* resistance gene.

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