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Permalink

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Journal

Phytopathology, 94(11)

ISSN

0031-949X

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Publication Date

2004-11-01

DOI

10.1094/phyto.2004.94.11.1198

Peer reviewed

Molecular Mapping of the *Stb4* Gene for Resistance to *Septoria tritici* Blotch in Wheat

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Accepted for publication 19 July 2004.

ABSTRACT

Adhikari, T. B., Cavaletto, J. R., Dubcovsky, J., Gieco, J. O., Schlatter, A. R., and Goodwin, S. B. 2004. Molecular mapping of the *Stb4* gene for resistance to *Septoria tritici* blotch in wheat. *Phytopathology* 94:1198-1206.

Breeding wheat for resistance is the most effective means to control *Septoria tritici* blotch (STB), caused by the ascomycete *Mycosphaerella graminicola* (anamorph *Septoria tritici*). At least eight genes that confer resistance to STB in wheat have been identified. Among them, the *Stb4* locus from the wheat cv. Tadinia showed resistance to *M. graminicola* at both seedling and adult-plant stages. However, no attempt has been made to map the *Stb4* locus in the wheat genome. A mapping population of 77 F₁₀ recombinant-inbred lines (RILs) derived from a three-way cross between the resistant cv. Tadinia and the susceptible parent (Yecora Rojo × UC554) was evaluated for disease resistance and molecular mapping. The RILs were tested with Argentina isolate I 89 of *M. graminicola* for one greenhouse season in Brazil during 1999, with an isolate from Brazil (IPBr1) for one field season in Piracicaba (Brazil) during 2000, and with Indiana tester isolate IN95-Lafayette-1196-WW-1-4 in the greenhouse during 2000 and 2001. The ratio of resistant:susceptible RILs was 1:1 in all three tests, confirming the single-gene model for control of resistance to STB in Tadinia. However, the patterns of resistance and susceptibility

were different between the Indiana isolate and those from South America. For example, the ratio of RILs resistant to both the Indiana and Argentina isolates, resistant to one but susceptible to the other, and susceptible to both isolates was approximately 1:1:1, indicating that Tadinia may contain at least two genes for resistance to STB. A similar pattern was observed between the Indiana and Brazil isolates. The gene identified with the Indiana tester isolate was assumed to be the same as *Stb4*, whereas that revealed by the South American isolates may be new. Bulk-segregant analysis was used to identify amplified fragment length polymorphism (AFLP) and microsatellite markers linked to the presumed *Stb4* gene. The AFLP marker *EcoRI*-ACTG/*MseI*-CAAA5 and microsatellite *Xgwm111* were closely linked to the *Stb4* locus in coupling at distances of 2.1 and 0.7 centimorgans (cM), respectively. A flanking marker, AFLP E-AGG/M-CAT10, was 4 cM from *Stb4*. The *Stb4* gene was in a potential supercluster of resistance genes near the centromere on the short arm of wheat chromosome 7D that also contained *Stb5* plus five previously identified genes for resistance to Russian wheat aphid. The microsatellite marker *Xgwm111* identified in this study may be useful for facilitating the transfer of *Stb4* into improved cultivars of wheat.

Additional keywords: marker-assisted selection, *Triticum aestivum*.

Septoria tritici blotch (STB), caused by the ascomycete fungus *Mycosphaerella graminicola* (asexual stage: *Septoria tritici*), is one of the most destructive foliar diseases of wheat (*Triticum aestivum* L. em. Thell) worldwide (15,16). Severity levels of this disease have increased in many parts of the world due to replacement of traditional tall cultivars by high-yielding, semi-dwarf wheat cultivars (15). In the United States, STB was first reported in 1929 from California (28). Subsequently, it was found to be widespread in the Pacific Northwest (10), northern Great Plains (51), and eastern soft-wheat regions (43). Frequent rainfall and moderate temperatures are the major factors contributing to the

development of STB epidemics (15,19,42). Severe epidemics reduce both quality and yield, and losses of up to 50% have been reported (16,44). Deployment of resistant cultivars is the only economical method for controlling this disease in most parts of the world (15).

An understanding of the inheritance and map locations of resistance genes will improve the efficiency of breeding for increased resistance to STB. Genetic resistance to STB can be expressed as a reduction in lesion size, as reduced percentage of leaf area covered by pycnidia, or both (15,23,47). The inheritance of resistance to STB has been reported to be conditioned by additive, dominant, partially dominant, or recessive gene action (41,49). Until now, eight major genes for resistance to STB have been identified in wheat: *Stb1* (38), *Stb2* and *Stb3* (49), *Stb4* (47), *Stb5* (4), *Stb6* (9), *Stb7* (30), and *Stb8* (1). Molecular markers such as restriction fragment length polymorphisms (RFLPs), simple-sequence repeats (SSRs) or microsatellite markers, and amplified fragment length polymorphisms (AFLPs) (48) recently have been used to tag seven of the STB resistance genes in wheat.

The spring wheat cv. Tadinia (20) possesses the single dominant gene *Stb4* for resistance (47). This gene has been useful for controlling *M. graminicola* in California since 1975 (47), yet its

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Names are necessary to report factually on available data. However, the United States Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name implies no approval of the product to the exclusion of others that also may be suitable.

Publication no. P-2004-0913-04R

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map location and relationship to other mapped genes for resistance to STB are not known. The utility of this gene in breeding programs would be increased greatly by the availability of linked molecular markers. Development of such markers would expedite the selection of favorable gene combinations by marker-assisted selection to pyramid this gene with others in wheat breeding programs. The objectives of this study were to analyze the inheritance of *Stb4* resistance to *M. graminicola*, identify molecular markers closely linked to the gene, and map its chromosomal location in the wheat genome.

MATERIALS AND METHODS

Plant materials. Tadinia is an STB-resistant cultivar derived from the cross Tadorna × Inia 66. It has the *Stb4* gene for resistance that is expressed in both seedling and adult-plant stages under both field and greenhouse conditions (47). The susceptible parent used in this study was an F₁ derived from the cross between Yecora Rojo and UC554 (hereafter referred to as the susceptible parent). Both Yecora Rojo and UC554 are highly susceptible to STB. A three-way cross was made between the resistant Tadinia and the susceptible parent, and 77 F₁₀ recombinant-inbred lines (RILs) were derived after 10 generations of self pollination without selection at the University of California, Davis.

STB evaluation in the greenhouse. The F₁₀ RILs were evaluated for resistance to *M. graminicola* in a greenhouse of the Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN during 2000 and 2001. Inoculum preparation, inoculation with field isolate IN95-Lafayette-1196-WW-1-4 of *M. graminicola*, experimental design, and disease assessment were as described previously (1). Plants were inoculated once the flag leaves were expanded fully, approximately 7 weeks after transplanting, in a randomized, complete-block design. The 77 F₁₀ RILs, Tadinia, and Yecora Rojo were included in all tests. The third parent, UC554, was included in most, but not all, of the tests. Each pot contained a single plant of a parent or RIL and each inoculated plant was treated as an experimental unit. Each treatment was replicated three to six times during both years.

Symptoms of STB were assessed from 21 to 28 days after inoculation. Inoculated plants were rated by two disease-assessment methods (17,23,41): (i) disease severity (DS) was based on the visually estimated percentage of leaf area of necrotic lesions containing pycnidia, and (ii) average pycnidial density was rated within necrotic lesions with a score ranging from 0 to 5, where 0 = no pycnidia and 5 = maximum pycnidial production (1). Means of DS and pycnidial density were calculated over all plants tested for each year. A disease index was calculated for each parent and RIL by multiplying DS by pycnidial density, and a mean disease index was calculated over both years. A few lines with poor seed germination or that gave inconclusive results were retested with parental controls during 2002. The experimental entries were classified as R = resistant or S = susceptible based on the mean disease index, and these classifications were used for genetic and linkage analyses. The disease index was used because it combined severity and pycnidial density into a single number and it provided a clear separation between resistant and susceptible lines in preliminary testing, and χ^2 statistics were used to determine goodness of fit with expected segregation ratios.

A second greenhouse test was done in Piracicaba, São Paulo, Brazil, during 1999. Seeds of Tadinia, Yecora Rojo, the 77 F₁₀ RILs, and the additional resistant control UC1041 were planted in pots (eight pots per parent and RIL/replication) and arranged in a randomized block design with three replications. Plants were inoculated as described above with isolate I 89 of *M. graminicola*, which was collected originally from cv. Klein Cacique grown in Barrow, Buenos Aires, Argentina. Two inoculations were made, the first at tillering and the second at the flag-leaf stage. The percent leaf area covered by lesions was estimated by visual inspec-

tion when symptoms had developed after each inoculation. The two scores were averaged to derive the overall mean for each parent and RIL.

STB evaluation in the field. Monopycnidial isolate IPBr1 was obtained from wheat leaves of the susceptible cv. Cooperación Calquin that were infected naturally with *M. graminicola* in Piracicaba, Brazil. Fungal isolation was made by placing leaf segments bearing pycnidia in petri plates lined with moistened filter paper overnight, and transferring cirrhi individually with a sterile needle to plates of yeast-malt agar amended with streptomycin at 50 mg/liter. After isolation, the fungus was maintained on petri plates containing AMYS medium (14 g of agar, 4 g of malt extract, 4 g of yeast extract, and 4 g of sucrose in 1 liter of distilled water) in a growth chamber at 16 ± 1°C with a 12-h photoperiod, and transferred every 60 days. Inoculum was prepared by inoculating 250 ml of liquid yeast-glucose medium in 500-ml Erlenmeyer flasks with fresh pieces of *M. graminicola* colonies from agar plates. Erlenmeyer flasks were incubated for 7 days in a temperature-controlled shaker at 16°C. After sporulation, the liquid media were filtered and adjusted to a density of 10⁷ spores/ml with distilled water.

Field data were from a previously published experiment (18) conducted in Piracicaba, using a randomized block design with four replications. The 77 F₁₀ lines plus the three parental cultivars were planted in 1-m rows of eight plants, each spaced 0.17 m apart. The susceptible control “Cooperación Calquin” was used as a spreader row around each block. The experiment was planted during June 2000 and inoculated 60 days after planting at the flag-leaf stage as described previously (18). At 30 days after inoculation, disease was evaluated by visual estimation of damaged leaf area (DLA). DLA was assessed as the percentage of the flag leaf area covered with pycnidia using a rating scale where 0 = absence of pycnidia, 1 = 12, 2 = 25, 3 = 50, 4 = 75, and 5 = 87% of the leaf area covered with pycnidia (14).

DNA extraction. Young wheat leaves were harvested from the three parents and 70 of the F₁₀ RILs 1 week after seed germination. The harvested leaves were frozen at -80°C, lyophilized for 72 h, and stored at -80°C. Genomic DNA was isolated from lyophilized tissue with the DNeasy Plant Mini kit according to the manufacturer’s directions (Qiagen, Valencia, CA) or by the cetyltrimethylammonium bromide (CTAB) method (34). Each DNA sample was quantified by fluorometer (Hoefer Scientific Instruments, San Francisco) and adjusted to 25 ng/μl or 6 ng/μl for AFLP and microsatellite analysis, respectively. Seed germination for seven lines was poor and these were excluded from the molecular analyses.

Bulked-segregant analysis. Bulk-segment analysis (BSA) (33) was performed based on the Indiana greenhouse phenotypic data. For AFLP and microsatellite analysis, two DNA bulks were assembled by using equal amounts of DNA from resistant and susceptible F₁₀ RILs. The number of individuals in each bulk was 10 for AFLP analysis and 6 for the microsatellite markers. Band patterns of the bulks were compared with those from the three parents (Tadinia, Yecora Rojo, and UC554) to identify potential markers linked to the resistance locus. A total of 80 AFLP primer combinations and 240 microsatellite primer pairs from different wheat genomes was screened using BSA.

AFLP analysis. AFLP analysis was conducted using AFLP Analysis System I, AFLP Start Primer Kit (Invitrogen Life Technologies, Carlsbad, CA), as described by Vos et al. (48) with the modifications of Adhikari et al. (1). Briefly, 10 μl of genomic DNA (25 ng/μl) of each parent and all 70 F₁₀ RILs were digested simultaneously in a reaction volume of 25 μl with 2.5 units each of *EcoRI* and *MseI* restriction enzymes, and ligated to *EcoRI* and *MseI* adapters. The digested DNA, together with 2 pmol of *EcoRI* adapters and 20 pmol of *MseI* adapters, were ligated with 1 unit of T4 ligase. The ligation mix was incubated at 20°C for 2 h, then the enzyme was inactivated at 70°C for 15 min. The ligated

product was diluted 10 fold in Tris-EDTA (TE) (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). Pre-amplification, primer labeling, selective amplification, electrophoresis, and photography were as described previously (1). The following AFLP primer extensions were used for the selective amplification: AAC, AAG, ACA, ACC, ACG, AGC, AGG, ACT, ACTT, ACTA, ACTG, and ACTC for the *EcoRI* primer; and CAC, CAG, CAT, CTA, CTC, CTG, CTT, CAA, CAAA, CAAT, CAAG, and CAAC for *MseI*. The AFLP bands were scored by visual inspection, and AFLP loci were named based on the combination of selective nucleotides in each primer (*EcoRI* and *MseI*) and the relative size of the band. For example, AGG/CAT10 refers to the tenth polymorphic band (bands numbered from high to low molecular weight) amplified with the primer combination *EcoRI*-AGG and *MseI*-CAT. Estimating the size of the bands was difficult due to problems with the marker standard.

Cloning and sequencing of AFLP fragments. The two AFLP fragments closest to the resistance gene (*EcoRI*-AGG/*MseI*-CAT10 and *EcoRI*-ACTG/*MseI*-CAAA5) were isolated, re-amplified to confirm the correct size, and cloned with the TA cloning kit (Invitrogen, Carlsbad, CA). Ten independent clones from each band were sequenced with an ALF Express System automated sequencer (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

Microsatellite analysis. A total of 240 microsatellite primer pairs specific for all wheat chromosomes was selected (36,39) and evaluated for linkage with the *Stb4* gene. In addition, two random amplified polymorphic DNA primers (UBC219 and UBC261) linked to the leaf rust resistance gene *Lr29* (37) also were evaluated for linkage to *Stb4*. All primers were synthesized by MWG Biotech Inc. (Charlotte, NC). Polymerase chain reactions (PCRs) were performed as described previously (1,2) with the Hot Star *Taq* DNA polymerase kit (Qiagen Inc., Valencia, CA). Each PCR (25 μ l) contained 5.8 μ l of sterile deionized water, 4 μ l of 10 \times Q-solution, 2 μ l of 10 \times PCR buffer, 1 μ l of 25 mM MgCl₂, 2 μ l of 10 mM dNTPs, 4 μ l of primer solution (4 μ M each), 0.2 μ l of Hot

Star *Taq* DNA polymerase (5 units/ μ l), and 6 μ l (6 ng/ μ l) of template DNA. PCR reactions were performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA) at standard amplifications of 94°C for 3 min, followed by 44 cycles of 94°C for 1 min; 50, 55, or 60°C (based on primer annealing temperature) for 1 min; and 72°C for 2 min, with a final extension at 72°C for 7 min before cooling to 4°C. PCR products were separated by electrophoresis in 3% agarose gels (Amresco, Solon, OH) at 4 V/cm in 0.5 \times Tris-acetate EDTA (TAE) buffer (0.02 M Tris, 0.0095 M glacial acetic acid, and 0.005 M EDTA).

To further confirm polymorphism, PCR products were separated on 12% (29:1 acrylamide/Bis) nondenaturing polyacrylamide gels (398 by 338 by 0.4 mm) as described by the manufacturer (Promega Corp., Madison, WI). Band sizes were estimated relative to a 25-bp DNA size-standard ladder (Invitrogen, Carlsbad, CA). After electrophoresis at 60 W for 2 h, PCR products were detected by silver staining as described previously (1,2). The gel was dried and photographs were taken with a Polaroid camera (Fotodyne Inc., Hartland, WI).

Aneuploid analysis. To confirm the chromosomal location of the *Stb4* gene, genomic DNA from wheat cv. Chinese Spring (CS), six nulli-tetrasomic (NT) lines of CS (N7AT7B, N7AT7D, N7BT7D, N7BT7A, N7DT7A, and N7DT7B), and the resistant and susceptible parents was analyzed with the five microsatellite primer pairs for loci *Xgwm44*, *Xgwm111*, *Xgwm295*, *Xgwm428*, and *Xgwm437*. The presence or absence of the corresponding characteristic PCR band was compared among the NT lines and three parents. Further amplification with microsatellite primer *Xgwm111* and the two AFLP primer combinations AGG/CAT10 and ACTG/CAAA5 was performed using DNA from Thatcher (TC), one CS ditelosomic line CS DT7DS (missing the long arm of chromosome 7D), and the two Canthatch (CTH) ditelosomic lines DT7DS (missing the long arm of chromosome 7D) and CTH DT7DL (missing the short arm of 7D).

Linkage analysis. Segregating molecular markers were scored in each RIL as either (i) Tadinia-type for resistance or (ii) Yecora Rojo \times UC554-type for susceptibility. Then, χ^2 values were used to test the hypothesis of segregation of resistance versus susceptibility and presence and absence of AFLP or microsatellite markers for a 1:1 ratio. Goodness-of-fit values were calculated for all segregation ratios and $P < 0.05$ was selected as the level for rejecting the null hypothesis. Linkage analysis was performed with the MAPMAKER program (version 2.0 for Macintosh) (25). The Kosambi (24) mapping function was used to convert recombination data into centimorgans (cM). The marker order was obtained by a multipoint analysis. The linkage map was plotted by selecting 0.4 as the maximum recombination fraction and 3.0 as the minimum logarithm of the odds ratio.

Analysis of disease scores and molecular markers. The relationship between measures of disease and the SSR marker *Xgwm111* was visualized with paired scatter plots made with DeltaGraph (version 4.0.5). Consistency between the two greenhouse experiments and the field data was analyzed similarly. This approach tested how well the molecular markers predicted resistance or susceptibility in the greenhouse and field experiments.

RESULTS

Inheritance of STB resistance. In the Indiana greenhouse tests, the phenotypes of the RILs were either highly resistant like Tadinia or very susceptible like Yecora Rojo and UC554. The mean disease index separated the parents and all of the RILs into resistant (R) or susceptible (S) classes (Fig. 1); parents and RILs with a disease index of 20 or less were considered resistant, while those with a mean index of more than 55 were scored as susceptible. All but one of the susceptible RILs had mean disease index greater than 70, whereas all but six of the resistant RILs had an index of 5 or less (Fig. 1). The mean disease index values for the

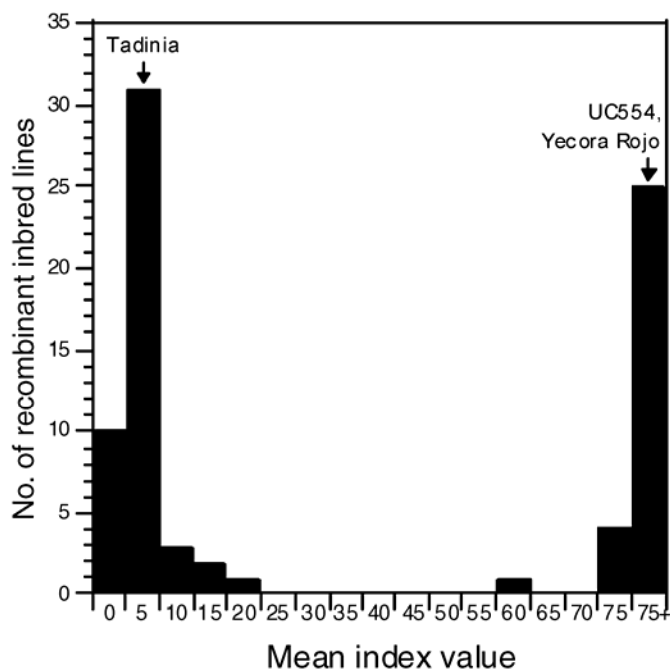


Fig. 1. Segregation of resistance to *Mycosphaerella graminicola* isolate IN95-Lafayette-1196-WW-1-4 in progeny of the wheat cross Tadinia \times (Yecora Rojo \times UC554). Histogram showing the frequency distribution of a multiplicative index of *Septoria tritici* blotch severity and pycnidial density averaged over 2 years of greenhouse testing among 77 F₁₀ recombinant-inbred lines. Each bar of the histogram is labeled with the maximum value for that class. The classes containing the mean index values of the three parents are indicated by vertical arrows.

parents were 1.4 for Tadinia (R), 160.0 for Yecora Rojo (S), and 215.1 for UC554 (S). The observed segregation ratio of resistant ($n = 47$) to susceptible ($n = 30$) RILs in the Indiana greenhouse tests had more resistant lines than expected but was not different from 1:1 ($\chi^2 = 3.75$, $0.05 < P < 0.10$), supporting segregation of a single locus. Segregation of the RILs did not fit the 3:1 ($\chi^2 = 8.00$, $P < 0.005$) ratio expected for two independent genes.

In the greenhouse tests with the Argentina isolate, RILs with disease scores from 0.3 to 1.5 were considered resistant, whereas those with scores from 1.8 to 4.6 were classified as susceptible due to a gap from 1.5 to 1.8 in the histogram of the results (data not shown). Resistance to STB segregated among the 77 F_{10} RILs in a 1:1 ratio (R:S = 35:42, $\chi^2 = 0.64$, $0.25 < P < 0.50$), confirming the existence of a single gene.

The field trials with the Brazil isolate also gave a good discrimination between RILs that were resistant (DLA from 0 to 1.3) versus those that were susceptible (DLA from 2.0 to 5.1) (data not shown). The 30:47 ratio of R:S RILs showed a slight excess of susceptible plants, but was not significantly different from 1:1 ($\chi^2 = 3.75$, $0.05 < P < 0.10$). No heterogeneity in response to the disease was observed within the rows indicating homozygosity of the resistance gene.

AFLP analysis. In all, $\approx 90\%$ of the 80 AFLP primer combinations tested produced clearly separated band patterns. On average, each primer combination produced ≈ 145 scorable bands; therefore, the total number of loci assayed over the 72 combinations that gave good amplification was more than 10,000. Among these, 26 primer combinations gave one or more bands in BSA that potentially were linked to *Stb4*. When tested with individual DNA of the 10 STB-resistant and 10 STB-susceptible lines used to make the resistant and susceptible bulks, more than half (14 of 26) of these potential linkages were confirmed and these selected primer combinations were used to determine the association between molecular markers and phenotypes in 70 F_{10} RILs (Fig. 2). Amplified DNA fragments ranged in size from 30 to 800 bp, but most polymorphic bands were fewer than 500 bp in size. Each polymorphic primer combination yielded from 112 to 177 amplified DNA bands, with 2 to 15 bands appearing polymorphic between the parents and the bulks. To simplify the banding patterns, some primers that gave potentially useful amplification profiles were extended by adding an additional selective nucleotide to their 3' ends. AFLP primer combinations with four selective nucleotides amplified fewer bands than those with three selective nucleotides, which gave better separation on the gels. However, AFLP amplification with four selective nucleotides produced a slightly different profile of polymorphism compared with the same primers with only three selective nucleotides (data not shown).

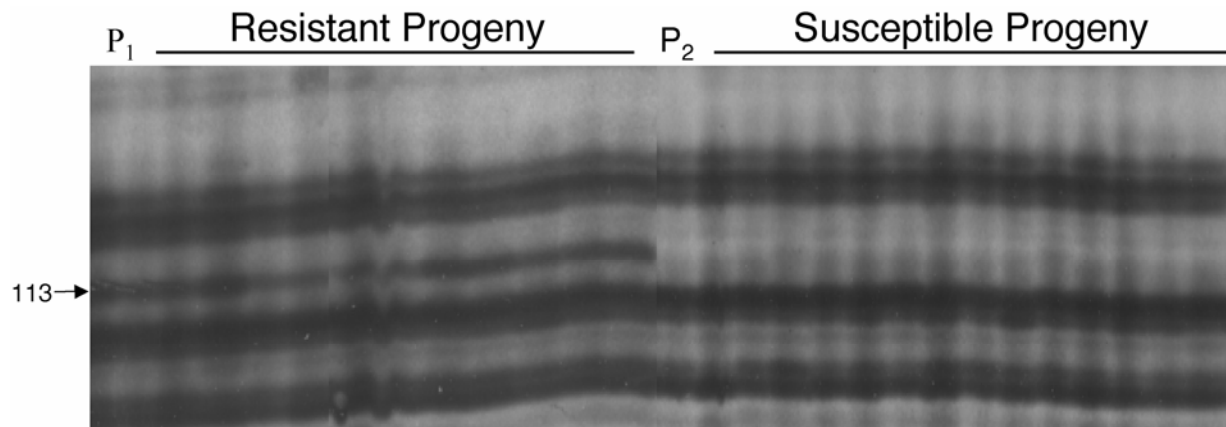


Fig. 2. Amplified fragment length polymorphism marker *EcoRI-ACTG/MseI-CAAA5* segregating among the F_{10} recombinant-inbred lines derived from a cross between the resistant wheat cv. Tadinia and the susceptible parent (Yecora Rojo \times UC554). Progeny resistant and susceptible to isolate IN95-Lafayette-1196-WW-1-4 of *Mycosphaerella graminicola* are indicated by horizontal lines. P_1 and P_2 denote the resistant and susceptible parents, respectively. Both Yecora Rojo and UC554 lacked the 113-bp band indicated by the arrow on the left.

Microsatellite analysis. Among the 240 microsatellite primers tested, only the primer pair *Xgwm111* amplified a specific DNA fragment related to STB resistance, based on polymorphism between the DNA of resistant and susceptible plants. An ≈ 210 -bp DNA fragment from the resistant parent Tadinia and resistant progeny appeared to be linked to the *Stb4* gene (Fig. 3). Other microsatellite loci linked to *Xgwm111* were either monomorphic or, for *Xgwm44*, gave segregation patterns with bands that could not be interpreted accurately.

Chromosomal location of *Stb4*. The 7D location of microsatellite locus *Xgwm111* and the positive controls *Xgwm44*, *Xgwm295*, *Xgwm428*, and *Xgwm437* (39) was confirmed using CS NT lines. These microsatellites amplified the specific DNA fragment from CS and all of the NT lines except for N7DT7A and N7DT7B. Because there has been some confusion about the arm location of *Xgwm111* (27,39), we retested this microsatellite with CS and CTH ditelosomic stocks. An ≈ 210 -bp-specific PCR band was amplified by the *Xgwm111* primers in TC, CS, CS DT7DS, and CTH DT7DS, but was absent in CTH DT7DL (Fig. 4). These results confirmed the mapping of *Xgwm111* to the short arm of chromosome 7D. However, both AFLP markers (AGG/CAT10 and ACTG/CAA5) amplified a fragment from both CS DT7DS

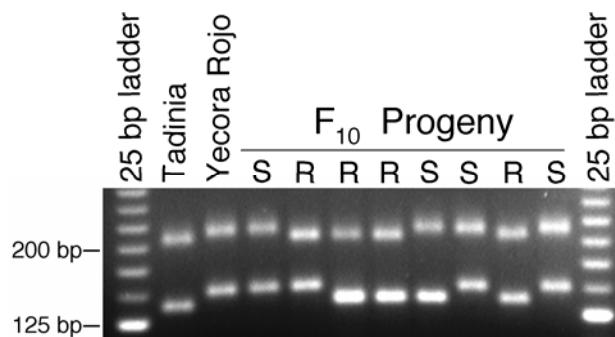


Fig. 3. DNA bands amplified from two parents and eight F_{10} recombinant-inbred lines derived from a cross between the resistant wheat cv. Tadinia (*Stb4* gene for resistance to isolate IN95-Lafayette-1196-WW-1-4 of *Mycosphaerella graminicola*) and the susceptible parent (Yecora Rojo \times UC554) with the microsatellite primer pair for locus *Xgwm111* shown in a 3% agarose gel. A 25-bp DNA ladder was used as a standard size marker. Resistant and susceptible progeny are indicated by R and S, respectively. An ≈ 210 -bp DNA fragment is amplified from the resistant parent Tadinia and resistant progeny compared with a band of ≈ 220 bp in the susceptible parent and progeny. Locations of 125- and 200-bp size-standard bands are indicated on the left. A second locus segregates among these progeny but is not linked to the resistance gene.

and CTH DT7DL; therefore, they could not be located to a specific chromosome arm.

Linkage analysis and genetic mapping. A total of 15 molecular markers was used for linkage analysis. Among these, 12 markers (11 AFLP and 1 microsatellite) formed a linkage group

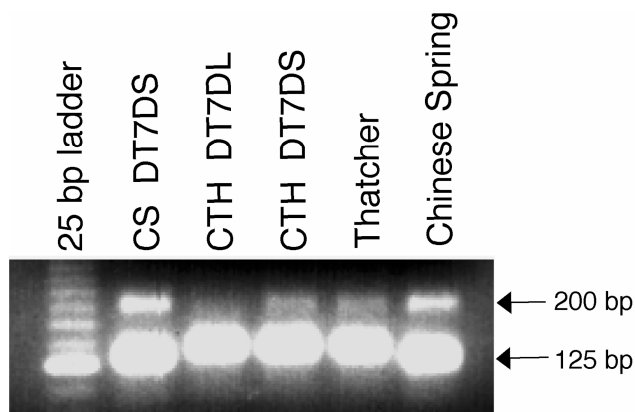


Fig. 4. Comparison of polymerase chain reaction (PCR) bands amplified with chromosome-specific microsatellite primer pair *Xgwm111* using ditelosomic wheat stocks. PCR products were separated by electrophoresis in a 3% agarose gel. A specific band of ≈ 210 bp (indicated by the arrow) present in ditelosomic stocks Chinese Spring (CS) DT7DS, Canthatch (CTH) DT7DS, Thatcher, and CS but absent in CTH DT7DL, confirms that *Xgwm111* is located on the short arm of chromosome 7D.

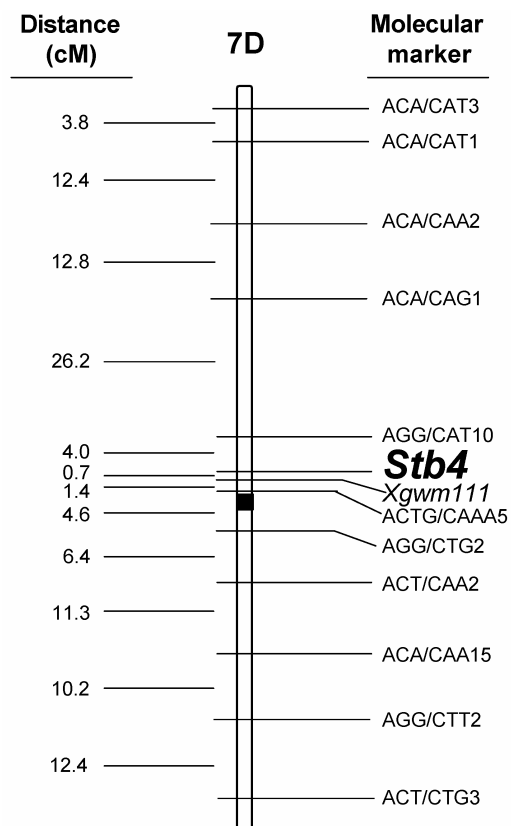


Fig. 5. Amplified fragment length polymorphism (AFLP) and microsatellite linkage map constructed from the F_{10} recombinant-inbred lines derived from the resistant cv. Tadinia crossed with the susceptible parent (Yecora Rojo \times UC554). Distances in centimorgans (cM) are indicated on the left; molecular markers and the resistance locus are on the right. Gene *Stb4* controls resistance to isolate IN95-Lafayette-1196-WW-1-4 of the Septoria tritici blotch pathogen *Mycosphaerella graminicola*. The black area denotes one possible position for the centromere. However, the orientation of *Stb4* with regard to *Xgwm111* is uncertain and could be the reverse of what is shown here. Map distances are proportional to the distances in cM.

with *Stb4* (Fig. 5). The microsatellite and four of the five AFLP markers that were most closely linked to *Stb4* gave segregations that were consistent with a 1:1 ratio (Table 1). All six of the most closely linked molecular markers were in coupling with the resistance allele. The 113-bp AFLP marker *EcoRI*-ACTG/*MseI*-CAA5 and microsatellite marker *Xgwm111* were on one side of the *Stb4* gene at distances of 2.1 and 0.7 cM, respectively, while AFLP AGG/CAT10 was 4 cM from *Stb4* on the flanking side (Fig. 5). Analysis of these markers mapped *Stb4* near the centromere on the short arm of chromosome 7D.

Analysis of disease scores relative to molecular markers. The plot of pycnidial density versus disease severity revealed two main clusters of RILs near the values for the resistant and susceptible parents (Fig. 6A). All RILs with a severity $>10\%$ also had the same allele as the susceptible parent at SSR locus *Xgwm111*, whereas those with a severity $<10\%$ had the Tadinia allele, with only four exceptions (Fig. 6A). Three of these exceptions had been scored as possibly susceptible during the 2000 test but, due to poor germination, were not included during the 2001 test in which severity values generally were higher. These lines were retested during 2002 when severity levels also generally were low. The fourth exceptional line consistently was scored as resistant even though it had the susceptible-associated allele at locus *Xgwm111* and the other molecular markers tested. Four of the RILs were recombinant for one or more of the molecular marker loci, but always had the phenotype predicted by marker *Xgwm111* (data not shown). All RILs scored as susceptible had a pycnidial density greater than 1.7 (Fig. 6A).

The plot of mean pycnidial density in the Indiana greenhouse test versus mean severity in the Brazil greenhouse test with an isolate from Argentina showed a different response (Fig. 6B). Many RILs that were scored as resistant to the Indiana isolate and had the resistance-associated marker allele were susceptible to the Argentina isolate, and the converse also occurred; many RILs that were susceptible to the Indiana isolate and had the susceptible-associated marker allele were very resistant to the Argentina isolate (Fig. 6B). When the plot was divided into quadrats based on a value of 1.7 to distinguish between resistant and susceptible responses for both the Indiana and Argentina isolates, the number of RILs in each quadrat was (clockwise, beginning in the upper left) 19:21:13:17, which fits a ratio of 1:1:1:1 ($\chi^2 = 2.0$, $0.5 < P < 0.75$).

A similar pattern was seen in the plot between the Indiana greenhouse pycnidial density data and the mean severity with a Brazil isolate in the field (Fig. 6C). When divided into quadrats as described above, the ratio of the RILs was 20:24:10:16, which is consistent with a 1:1:1:1 ratio ($\chi^2 = 6.11$, $0.10 < P < 0.25$).

The pattern was different when the Argentina isolate greenhouse data were plotted against those for the Brazil isolate from

TABLE 1. Segregation of the six molecular markers closest to the *Stb4* gene for resistance to isolate IN95-Lafayette-1196-WW-1-4 of *Mycosphaerella graminicola* among 70 F_{10} recombinant-inbred lines of wheat derived from the three-way cross Tadinia \times (Yecora Rojo \times UC554)

Marker ^a	Tadinia	Yecora Rojo \times UC554 ^b	χ^2 ^c
E-AGG/M-CAT10	37	33	0.23
E-ACTG/MCAA5	37	33	0.23
E-AGG/M-CTG2	37	33	0.23
E-ACT/M-CAA2	41	29	2.06
E-ACA/M-CAA15	44	26	4.66
<i>Xgwm111</i>	36	34	0.06

^a Names of amplified fragment length polymorphism markers consist of selective nucleotides from primers for *EcoRI* and for *MseI* separated by a slash; the microsatellite marker is indicated by italics. All markers are linked in coupling with the resistance gene.

^b The patterns for Yecora Rojo and UC554 were distinct from Tadinia and scored as a single marker.

^c Goodness of fit (χ^2) of the ratios of the molecular markers among 70 F_{10} recombinant-inbred lines expected for a single gene (1:1); χ^2 values greater than 3.84 indicate a poor fit to a 1:1 segregation ratio ($P < 0.05$).

the field (Fig. 6D). Although some RILs occurred in all four quadrats, the ratio was significantly different from 1:1:1:1 ($\chi^2 = 26.69$, $P < 0.005$). Most RILs were in the upper right or lower left quadrats, as expected if both isolates identify the same gene. The ratio of RILs in these two quadrats was 34:20, consistent with a 1:1 ratio ($\chi^2 = 3.63$, $0.05 < P < 0.10$). In all, $\approx 50\%$ of the RILs in each quadrat had the susceptible-associated marker allele, as

expected if the resistance gene identified with the Argentina and Brazil isolates is different from *Stb4*.

DISCUSSION

The multiplicative disease index combined data for both severity and pycnidial density into a single number and allowed easy

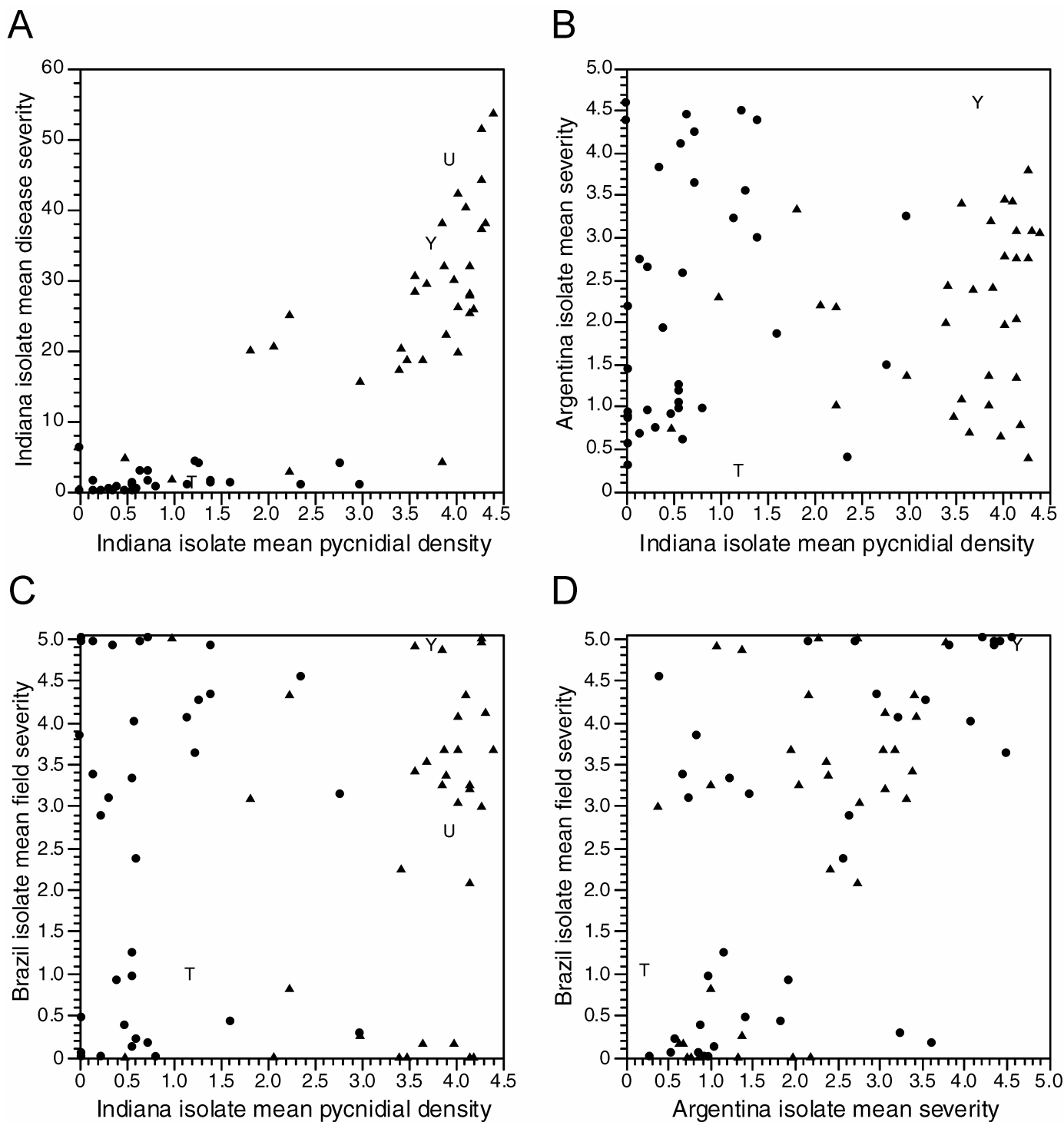


Fig. 6. Relationships between phenotypic measures of disease and molecular markers among the F_1 recombinant-inbred lines (RILs) derived from the three-way cross between the resistant wheat cv. Tadinia and the susceptible parent (Yecora Rojo \times UC554) inoculated with three isolates of *Mycosphaerella graminicola*. Scatter plots between **A**, greenhouse measures of mean pycnidial density versus mean disease severity in response to inoculation with Indiana isolate IN95-Lafayette-1196-WW-1-4 of *M. graminicola*; **B**, mean pycnidial density in the Indiana greenhouse test versus mean severity with Argentina isolate I 89 in a greenhouse in Brazil; **C**, mean pycnidial density in the Indiana greenhouse test versus mean field severity with a Brazil isolate in Brazil; and **D**, mean greenhouse severity with Argentina isolate I 89 versus mean field severity with a Brazil isolate in Brazil. RILs with the susceptible-associated allele at microsatellite locus *Xgwm111* are indicated by black triangles; those with the resistance-associated allele are shown as gray circles. T = resistant parent Tadinia, U = susceptible parent UC554, and Y = susceptible parent Yecora Rojo.

separation of resistant and susceptible RILs. The accuracy of the index prediction was confirmed with the molecular markers. Previous studies had shown that pycnidial production alone is a better indicator of resistance or susceptibility than severity, because some lines can give large lesions with little or no sporulation (23). However, in our analysis, either measure could have provided effective separation of resistant and susceptible RILs. Only four lines had a different phenotype than predicted by the molecular markers, and three of these may have been because they were not tested during 2001 when severity values were highest. The fourth line could have been a rare double recombinant with the resistance allele inserted among the susceptible-associated marker alleles. A more likely explanation may be that this sample was mislabeled during the testing process. Unfortunately, the four exceptional RILs had all of the susceptible-associated marker alleles and, therefore, could not have been single recombinants. The low number of exceptional RILs makes the interaction of additional genetic factors an unlikely explanation.

The resistance locus in *Tadinia*, identified with an Indiana isolate of *M. graminicola*, mapped very near the centromere on the short arm of chromosome 7D. This resistance is assumed to be the same as the *Stb4* gene in *Tadinia* named previously (47). However, the greenhouse test with an isolate of *M. graminicola* from Argentina and the field experiment with an isolate from Brazil identified a second likely major gene for resistance to STB in this cultivar. With the present data, it is impossible to be certain which gene is the same as *Stb4* and which may represent a new gene. Therefore, we arbitrarily designate the gene identified with the Indiana tester isolate as *Stb4* and assume the additional gene is either new or the same as one of those named previously. Additional testing with the same California isolate used originally to name *Stb4* will be required to answer this question definitively.

Mapping of the *Stb4* locus was expedited by the use of BSA with AFLPs and microsatellites. The AFLP primer pairs with three selective nucleotides at the 3' end generated complicated banding patterns that were difficult to score accurately compared with those with four selective nucleotides. Furthermore, the polymorphisms obtained from the AFLP primers with three and four nucleotides at the 3' end were different and were difficult to compare due to the large numbers of bands involved (data not shown). Similar results were reported previously by Bai et al. (5), who recommended combinations of primers with three and four, or four and four, selective nucleotides for wheat AFLP analysis. Using four and four AFLP selective nucleotides allowed us to identify a 113-bp AFLP marker, *EcoRI*-ACTG/*MseI*-CAAA5, that was closely linked to the *Stb4* locus in wheat.

In this study, the mapping population used was a three-way cross generated by the breeding program at the University of California. The susceptible parent was an F₁ from the cross Yecora Rojo × UC554. Thus, we might expect only 25% of the polymorphic bands in the RIL population to have been derived from Yecora Rojo and another 25% to have originated from UC554. The resistant parent *Tadinia* should be the origin for the remaining 50% of the polymorphic bands. With some exceptions in microsatellite analysis, both susceptible cvs. Yecora Rojo and UC554 contained similar-sized bands, and all three parents often were monomorphic. This low level of DNA polymorphism can be attributed to the high degree of relatedness among the three parental wheat cultivars, which are all elite germ plasm in the breeding program for improved red spring wheat cultivars at the University of California, Davis.

Our attempts to convert AFLP bands into easily assayed PCR-based markers were not successful, presumably because the polymorphism was near the 3' end of the original primer sequence or was within one of the restriction-enzyme recognition sites. To test this hypothesis, inverse PCR (8) was used to extend the sequences in both directions. Unfortunately, primers designed

within the flanking regions also gave a product of similar size, which could not discriminate between resistant and susceptible individuals.

Problems in converting these AFLP markers are compounded by the use of *EcoRI* as the six-base cutter in the AFLP analysis. This enzyme is insensitive to cytosine methylation and often gives AFLP fragments corresponding to repetitive sequences that cannot be used as RFLP markers (6,7). In contrast, methylation-sensitive enzymes such as *PstI* give a higher frequency of polymorphisms in hypomethylated regions of the genome which are more likely to be single copy rather than repetitive (50). AFLP analysis with *PstI* rather than *EcoRI* as the six-base cutter may give bands with a higher probability of being converted into a simpler type of marker system.

Among the seven STB resistance loci mapped previously, all but one are on different chromosomes from *Stb4*. Gene *Stb1* mapped to the long arm of chromosome 5B (3), *Stb2* is on the distal region of the short arm of chromosome 3B, and the *Stb3* gene is on the short arm of chromosome 6D (2). The *Stb6* gene was mapped to the distal part of the short arm of chromosome 3A (9) and gene *Stb7* is located on the long arm of chromosome 4A (30). The most recently identified gene, *Stb8*, was mapped to the long arm of chromosome 7B (1). All of these must be distinct from *Stb4* on chromosome 7D.

The seventh mapped gene, *Stb5*, was located to the short arm of chromosome 7D with microsatellite, RFLP, and morphological markers (4). The closest loci were microsatellite *Xgwm44* and the morphological marker for red coleoptile, *Rc3*, which flanked the *Stb5* gene at distances of 7.2 and 6.6 cM, respectively. Microsatellite *Xgwm111* also was linked to *Stb5*, but at a distance of 11.9 cM (4), and was indicated as mapping to the other side of the centromere (on the long arm) of chromosome 7D opposite *Stb5*. More recent analyses have shown that *Xgwm111* maps to the short arm rather than the long arm of chromosome 7D (27). This conclusion was supported by our analysis of ditelosomic stocks, indicating that *Stb4* and *Stb5* both are probably on the short arm of chromosome 7D.

It is intriguing that both *Stb4* and *Stb5* are located near the centromere of wheat chromosome 7D. These genes could be identical, allelic, or different but linked. The only marker in common between the two maps is microsatellite *Xgwm111*, which is 0.7 cM from *Stb4* but 11.9 cM from *Stb5*. The difference between these distances is significant ($P < 0.05$); however, because they were calculated from different mapping populations, it is not clear if these loci really are distinct. Unfortunately, none of the AFLP markers was mapped in the *Stb5* population, and loci *Xgwm44* and *Rc3* were not informative in our *Stb4* mapping population; therefore, it is not possible to determine the map order between the other linked markers and the resistance loci *Stb4* and *Stb5*. A California isolate of *M. graminicola* was unable to differentiate wheat lines containing *Stb4* and *Stb5* in limited inoculation tests (S. B. Goodwin, unpublished data), but it is not known whether that means the resistance genes are the same or if the California isolate simply is avirulent to both. Therefore, with the present data, it is not possible to determine with certainty if *Stb4* and *Stb5* are allelic variants of the same gene or different but closely linked genes. These competing hypotheses must be tested by additional genetic analyses and by reciprocal pathogenicity tests with the isolates used here and by Arraiano et al. (4).

In addition to *Stb4* and *Stb5*, this region of chromosome 7DS also contains several genes for resistance to the Russian wheat aphid, *Diuraphis noxia* (26,27). Genes *Dn1*, *Dn2*, *Dn5*, *Dn6*, and *Dnx* are located from 1.5 to 3.8 cM from microsatellite locus *Xgwm111*. These distances are intermediate between those estimated for *Stb4* and *Stb5* from the same microsatellite, which might indicate that the genes for resistance to STB flank those for resistance to Russian wheat aphid. Therefore, this region of chromosome 7DS may contain a large cluster of resistances and could

be a good location to search for additional genes with specificities for diverse pests and pathogens.

Other interesting genes located on chromosome 7DS include several for resistance to leaf rust, such as *Lr29* (37,40), *Lr34* (32,35), and *Lr43* (21), the stripe rust resistance gene *Yr18* (45), and the *Barley yellow dwarf virus* resistance gene *Bdv1* (46), which all are closely linked to *Lr34*. However, *Lr34* mapped approximately 30 cM distal to *Rc3* and was unlinked to the centromere (12); therefore, these additional genes are not part of the same cluster as *Stb4* and the *Dn* genes. At least two resistance gene clusters occur on the short arm of chromosome 7D, one (containing *Stb4*) near the centromere and the second (with the rust resistance genes) located much more distally.

The resistance conferred by the *Stb4* gene was effective in the field in California from 1975 through the late 1990s (47). However, the effectiveness of this gene decreased dramatically recently. Some resistant cultivars developed from Tadinia (e.g., RSI5 and Kern) were susceptible, with up to 25% leaf area infected in parts of the Sacramento Valley of California during 2000 (22). This indicates that the pathogen is capable of overcoming this single-gene resistance. Sexual reproduction of *M. graminicola* is known in California (29) along with high levels of genetic diversity for molecular markers within populations (11,31). It is likely that variability also occurs for pathogenicity, which should allow for rapid shifts in virulence of the populations (23). On the other hand, pathogenic specialization of this fungus is less distinct (13) than that of some other wheat pathogens, even though gene-for-gene interactions are known to occur in this pathosystem (9). The use of this gene in wheat improvement may still be valuable in California and other parts of the United States when in combination with other STB resistance genes.

Breeding for resistance to STB cannot be performed in areas where weather conditions are adverse for disease development. In addition, environmental influences on symptom expression may result in inaccurate disease assessments. Introgression of genes for resistance against *M. graminicola* into new wheat cultivars is further compounded by the long latent period of the disease (typically at least 18 days in the greenhouse, usually longer), and the lack of defined tester isolates to identify specific resistance genes in gene pyramids. Microsatellite *Xgwm111* is a PCR-based, genome-specific, co-dominant marker that displays a specific allele linked to *Stb4* at a distance of only 0.7 cM. This marker will facilitate pyramiding *Stb4* with other STB resistance genes into a single wheat cultivar for more durable resistance to *M. graminicola*.

ACKNOWLEDGMENTS

Published as paper 17421, Purdue University Agricultural Research Programs. This work was supported by USDA CRIS project 3602-22000-013-00D and USDA-IFAFS competitive grant 2001-04462. The research in Brazil was supported by FAPESP (*Fundação de Amparo a Pesquisa do Estado de São Paulo*). We thank F. Urmeev, B. Roberts, J. Breeden, B. Wallace, and K. Brikmanis for performing the greenhouse experiments; and B. Gill for providing seeds of the Canthatch ditelosomic stocks.

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