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ORIGINAL PAPER

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Molecular mapping of *Stb1*, a potentially durable gene for resistance to septoria tritici blotch in wheat

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Abstract Septoria tritici blotch (STB), caused by the ascomycete *Mycosphaerella graminicola* (anamorph *Septoria tritici*), was the most destructive disease of wheat in Indiana and adjacent states before deployment of the resistance gene *Stb1* during the early 1970s. Since then, *Stb1* has provided durable protection against STB in widely grown wheat cultivars. However, its chromosomal location and allelic relationships to most other STB genes are not known, so the molecular mapping of *Stb1* is of great interest. Genetic analyses and molecular mapping were performed for two mapping populations. A total of 148 F_1 plants (mapping population I) were derived from a three-way cross between the resistant line P881072-75-1 and the susceptible lines P881072-75-2 and Monon, and 106 F_6 recombinant-inbred lines (mapping population II) were developed from a cross between the resistant line 72626E2-12-9-1 and the susceptible cultivar Arthur. Bulked-segregant analysis with random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellite or simple-sequence repeat (SSR) markers was conducted to identify those that were putatively linked to the *Stb1* gene. Segregation analyses confirmed that a single dominant gene controls the resistance to *M. graminicola* in each mapping population. Two RAPD markers, $G7_{1200}$ and $H19_{520}$, were tightly linked to *Stb1* in wheat line P881072-75-1 at distances of less than 0.68 cM and 1.4 cM, respectively. In mapping population II, the most closely linked marker was SSR *Xbarc74*, which was 2.8 cM proximal to *Stb1* on chromosome 5BL. Microsatellite loci *Xgwm335* and *Xgwm213* also were proximal to *Stb1* at distances of 7.4 cM and 8.3 cM, respectively. The flanking AFLP marker, *Eco*RI-AGC/*Mse*I-CTA-1, was 8.4 cM distal to *Stb1*. The two RAPD markers, $G7_{1200}$ and $H19_{520}$, and AFLP *Eco*RI-AGC/*Mse*I-CTA-1, were cloned and sequenced for conversion into sequence-characterized amplified region (SCAR) markers. Only RAPD allele $H19_{520}$ could be converted successfully, and none of the SCAR markers was diagnostic for the *Stb1* locus. Analysis of SSR and the original RAPD primers on several 5BL deletion stocks positioned the *Stb1* locus in the region delineated by chromosome breakpoints at fraction lengths 0.59 and 0.75. The molecular markers tightly linked to *Stb1* could be useful for marker-assisted selection and for pyramiding of *Stb1* with other genes for resistance to *M. graminicola* in wheat.

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Introduction

Septoria tritici blotch (STB) of wheat is caused by the fungal pathogen *Mycosphaerella graminicola* (anamorph *Septoria tritici*). The earliest known source of resistance to STB was the unimproved winter wheat cultivar Bulgaria 88 (Rillo and Caldwell 1966). This gene, designated *Stb1*, was transferred from Bulgaria 88 into the Indiana soft red winter cultivars Oasis and Sullivan (Patterson et al. 1975, 1979). These two wheat cultivars were first released during 1975 and 1979, respectively. The *Stb1* gene is dominant (Rillo and Caldwell 1966) and considered durable (Johnson 1984) because it remained effective for many years in widely grown cultivars across the STB-prone region of Indiana and adjacent states (Shaner and Finney 1982; Shaner and Buechley 1989).

Molecular markers such as random amplified polymorphic DNA [(RAPD); Williams et al. 1990], restriction fragment length polymorphisms [(RFLP); Nelson et al. 1995a, b, c], microsatellites or simple-sequence repeats [(SSR); Röder et al. 1998], amplified fragment length polymorphisms [(AFLP), Vos et al. 1995], and expressed sequence tags [(EST) Liu and Anderson 2003] have been used to tag fungal resistance genes in wheat. The abundance of these DNA markers in the wheat genome has facilitated mapping of other STB resistance genes. Using some of these molecular markers, genes *Stb2* and *Stb3* (Wilson 1985) were mapped to chromosomes 3BS and 6DS, respectively (Adhikari et al. 2004b); *Stb4* (Somasco et al. 1996) and *Stb5* were mapped near the centromere of chromosome 7D (Adhikari et al. 2004a; Arraiano et al. 2001), *Stb6* was mapped to chromosome 3AS (Brading et al. 2002), and *Stb7* was mapped on chromosome 4AL (McCartney et al. 2002). The *Stb8* gene was identified in the synthetic hexaploid wheat W7984 and mapped to the long arm of chromosome 7B with AFLP and SSR markers (Adhikari et al. 2003).

Gene *Stb4* was effective in California for more than 15 years but became ineffective recently (Jackson et al. 2000). Genes other than *Stb1* and *Stb4* have not been deployed extensively in commercial wheat cultivars, and their durability is not known. *Stb1* is the first named and, in the central United States, the most durable of the genes for resistance to *M. graminicola*, yet its chromosomal location has not been identified. The mapping and characterization of the *Stb1* locus may provide insight into strategies to extend the life of resistance genes in the field as well as to identify molecular markers for the use of breeding programs through marker-assisted selection (MAS). It may also provide a significant contribution towards positional cloning of this resistance locus. The objectives of this study were to (1) determine the inheritance of STB resistance in the two *Stb1*-containing wheat breeding lines SO852 and 72626E2-12-9-1; (2) explore the association of AFLP, RAPD, and SSR markers with the *Stb1* locus; (3) determine the chromosomal location of *Stb1*, using a set of deletion lines and previously mapped RFLP and SSR markers; and (4) develop a polymerase chain reaction (PCR)-based assay for MAS of the resistance gene.

Materials and methods

Mapping population I

The Purdue winter wheat cultivar Clark (Ohm et al. 1988), susceptible to *M. graminicola*, was crossed to a resistant spring wheat germplasm line, SO852 (parentage unknown), that was introduced from China. Two F_8-derived F_9 plants that differed for STB resistance in the field were selected and designated as P881072-75-1 (resistant) and P881072-75-2 (susceptible). Line P881072-75-1 was used as a resistant parent in subsequent crossing in place of the less-adapted SO852. To determine the inheritance of the resistance derived from SO852, a cross was made between P881072-75-1 and P881072-75-2 (Table 1). In addition to producing F_2 plants, the F_1 was crossed to the susceptible cultivar Monon (Patterson et al. 1978), which has no known resistance to *M. graminicola* (Ahmed et al. 1995; Shaner and Buechley 1989; Shaner and Finney 1982). In total, 148 F_1 plants from the three-way cross P881072-75-1/P881072-75-2//Monon were analyzed at the adult-plant stage for STB reaction and also for molecular mapping. The three-way cross F_2 families were tested at the seedling stage in a growth chamber to verify the F_1 adult-plant data. The backcross

Table 1 Segregation analysis of the septoria tritici blotch resistance gene *Stb1* in mapping population I and other wheat crosses derived from resistant line P881072-75-1

Cross	Generation	Testing stage	Resistant plants	Susceptible plants	Ratio	χ^2	*P*-value
P881072-75-1[a]/P881072-75-2	F_1	Adult	59	0	–[c]	–	–
	F_2	Seedling	215	63	3:1	0.81	0.37
P881072-75-1/P881072-75-2//Monon[b]	F_1	Adult	78	70	1:1	0.43	0.51
P881072-75-1/Monon	F_1	Adult	26	0	–	–	–
	F_2	Seedling	113	29	3:1	1.59	0.21
P881072-75-1/Monon//Monon	BC_1F_1	Adult	52	43	1:1	0.85	0.36
P881072-75-1/Sullivan//Monon	TCF_1	Adult	151	0	–	–	–
	TCF_2	Seedling	539	184	3:1	0.06	0.81

[a]The wheat line P881072-75-1 possesses the *Stb1* gene and was used as a resistant parent in place of the less adapted SO852
[b]A total of 148 F_1 plants (mapping population I) developed from the three-way cross (P881072-75-1/P881072-75-2//Monon) were analyzed by random amplified polymorphic DNA (RAPD) primers
[c]– Not applicable

P881072-75-1/Monon//Monon was made and the BC_1F_1 population was tested in the adult stage.

To determine the allelic relationship of the SO852 resistance locus with *Stb1*, the resistant line P881072-75-1 was crossed to Sullivan, a Purdue winter wheat cultivar that carries *Stb1* from Bulgaria 88 (Patterson et al. 1979). The F_1 plants were crossed to Monon to generate testcross F_1 and F_2 families. The testcross F_1 plants were analyzed for STB reaction in adult plants in the greenhouse, whereas the testcross F_2 families were inoculated at the seedling stage in the growth chamber (Table 1). In all experiments, P881072-75-1, P881072-75-2, Monon, Clark, and Sullivan were included as checks.

Mapping population II

The Purdue wheat breeding line 72626E2-12-9-1 was used as the donor of the *Stb1* gene for the second mapping population. The pedigree of this line is Arthur*2/2/dwarf Redcoat/Arthur sib/5/Sullivan/2/Arthur*2/Siete Cerros/3/Oasis/4/Mesa 5611 (Shaner and Buechley 1989). Sullivan or Oasis could have contributed the *Stb1* resistance in 72626E2-12-9-1. A mapping population of 106 recombinant-inbred lines (RILs) at the F_6 generation was developed by single-seed descent from a cross between the resistant line 72626E2-12-9-1 and the susceptible wheat cultivar Arthur. The sowing and growing of plant materials were as described previously (Adhikari et al. 2003).

Inoculation procedure

All experiments to test for resistance to *M. graminicola* were conducted in greenhouses and growth chambers at Purdue University, West Lafayette, Ind., USA. For inoculating mapping population I, infected leaves of the susceptible cultivar Monon were collected in the field from the Purdue Agronomy Research Center. Pycnidiospores were isolated and grown on V8 agar plates (200 ml V8 juice, 3 g $CaCO_3$, 15 g agar, and 800 ml distilled water). Fresh colonies of *M. graminicola* from these plates were used to inoculate 100 ml malt–sucrose broth (20 g malt extract, 20 g sucrose, and 5 g yeast extract per liter of distilled water). The samples were incubated at 20±2°C for 7 days. The resultant spore suspensions were adjusted to 10^7 spores/ml prior to inoculation. Adult plants were inoculated at the boot stage, and seedlings were inoculated at the two-leaf stage.

Mapping population II was tested under different conditions at two times. For the first test during 1997, cultures of *M. graminicola* obtained from eight individual pycnidia from flag leaves of the susceptible wheat cultivar Arthur from the Purdue Agronomy Research Center were used as a source of inoculum. These cultures were stored as fruiting lesions in leaves of cultivar Arthur in small vials at −80°C. When inoculum was needed, the 2 cm-square frozen leaf sections were placed in sterile petri dishes and flooded with 3 ml sterile water. The pycnidiospores were transferred aseptically to petri dishes that contained yeast–malt-extract agar (4 g yeast extract, 10 g malt extract, 4 g glucose, and 18 g agar per liter of distilled water). The plates were incubated for 4 days, and individual colonies were transferred to 250-ml Erlenmeyer flasks that contained 2% (w/v) malt-extract broth (Difco, Detroit, Mich., USA). These liquid cultures were placed on a shaker and maintained at 20°C with continuous low-intensity light provided by fluorescent tubes. After 3 days, 1–2 ml of broth was removed from the shake cultures and transferred to each of several petri dishes containing fresh yeast–malt-extract agar, and the fungus was allowed to grow for three more days at 22°C under continuous fluorescent light. Under these conditions, *M. graminicola* formed a dense carpet of spores morphologically identical to pycnidiospores. These spores were harvested by flooding the plates with sterile water, and the plants were inoculated with conidial suspensions (approximately 2.5 million spores per ml) after the flag leaves had emerged. For 72 h after inoculation, inoculated plants were kept moist by periodic misting in the greenhouse.

During the spring of 2003, inoculum was prepared from a tester isolate (IN95-Lafayette-1196-WW 1-4) of *M. graminicola* that was collected from Lafayette, Ind. during 1995 for a second inoculation of mapping population II. The pure culture was revived by placing a small piece (1–2 mm^2) of frozen (−80°C) filter paper containing mycelia and spores onto potato dextrose agar medium (Difco). Subsequent inoculum preparation and inoculation procedures were as described previously (Adhikari et al. 2003).

Disease assessment

Symptoms of STB were assessed on each plant between 21 and 27 days after inoculation by two methods (Gaunt et al. 1986; Kema et al. 1996; Rosielle 1972). These were (1) disease severity (DS) based on the visually estimated percentage leaf area with necrotic lesions containing pycnidia and (2) the level of sporulation estimated as pycnidial density within necrotic lesions ranging from 0 to 5 as described previously (Adhikari et al. 2003). Each pot contained a single plant, and each inoculated plant was treated as an experimental unit. Three to six replicates of each line were inoculated during each time. Means of DS and pycnidial density were calculated and averaged over all plants tested. A disease index was calculated for each parent and RIL by multiplying DS by pycnidial density.

Molecular analyses

Fourteen-day-old wheat leaves were harvested from all parents, the 148 F_1 plants from mapping population I, and the 106 F_6 RILs from mapping population II. Genomic DNA was extracted using either the methods described previously (Dweikat et al. 1997; Murray and Thompson 1980) or the Qiagen DNeasy Plant Mini Kit (QIAGEN, Valencia, Calif., USA). Each DNA sample was quantified with a fluorometer (Hoefer Scientific Instruments, San Francisco, Calif., USA) and adjusted to a final concentration of 25 ng/μl for AFLP, 10 ng/μl for RAPD, or 6 ng/μl for SSR analysis.

Molecular markers putatively linked to the resistance gene were identified by bulked-segregant analysis [(BSA) Michelmore et al. 1991]. Based on greenhouse phenotypic data, two bulk template DNA—a resistant bulk and a susceptible bulk—were made for AFLP, RAPD, and SSR analysis by pooling equal amounts of DNA from ten homozygous resistant and ten homozygous susceptible lines.

RAPD analysis was carried out as described previously (Hu et al. 1997). Oligonucleotide primers (10-mers) were purchased from the University of British Columbia (B.C., Canada) and Operon Technologies (Alameda, Calif., USA). A total of 148 F_1 plants (mapping population I) developed from the three-way cross (P881072-75-1/P881072-75-2//Monon) were analyzed with the RAPD primers.

Two RAPD bands produced with primers G7 (5′-GAACCTGCGG-3′) and H19 (5′-CTGACCAGCC-3′), fragments $G7_{1200}$ and $H19_{520}$, respectively, were cloned and sequenced from each end (Braden and Simon 1998). Four sequence-characterized amplified region [(SCAR) Paran and Michelmore 1993] markers were synthesized and used to determine polymorphism in mapping population II. All PCR conditions were the same as those described above.

AFLP analysis was performed using AFLP Analysis System II (Invitrogen Life Technologies, Carlsbad, Calif., USA) as described previously (Adhikari et al. 2003). AFLP loci were named based on the combination of nucleotides in each selective primer (*Eco*RI and *Mse*I) and relative size of the band. For example, AGC/CTA-1 denotes the first polymorphic band (numbered from high to low molecular weight) amplified with the primer combination *Eco*RI-AGC and *Mse*I-CTA. An AFLP amplicon, *Eco*RI-AGC/*Mse*I-CTA-1, closely linked to the *Stb1* locus, also was cloned (Braden and Simon 1998), sequenced, and SCAR primers were designed with

Primer Premier, version 4.0 (Premier Biosoft International, Palo Alto, Calif., USA), software.

For SSR analysis, 125 wheat markers designated as *Xgwm* [for Gatersleben (Germany) wheat microsatellite, Röder et al. 1998] covering all three wheat genomes (A, B, and D) were tested for mapping population II as described previously (Adhikari et al. 2003). To develop SCAR markers, a 598-bp fragment amplified with the primers for locus *Xgwm66* that was present in the resistant parent but absent in the susceptible parent was cloned and sequenced (Braden and Simon 1998). Four SCAR primers were designed and used in this experiment. Once the general chromosomal location of the *Stb1* gene was identified by linkage with previously mapped SSR markers, 18 BARC (Beltsville Agriculture Research Center) primers located on wheat chromosome 5B also were analyzed for associations with *Stb1* as described above. Primers for SCAR and both types of SSR markers were synthesized by MWG Biotech (Charlotte, N.C., USA). PCR reactions and band detection, using a modified DNA silver-staining system were as described previously (Adhikari et al. 2003).

RFLP analysis was performed as described previously (Francki et al. 1997). Eight enzymes, *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Sac*I, and *Xba*I, were used to digest DNA of the resistant parent P881072-75-1 and the susceptible parent P881072-75-2 according to the manufacturer's instructions (Promega, Madison, Wis., USA). Electrophoresis and Southern analysis were according to standard protocols. For physical mapping, RFLP probes associated with known deletion fraction lengths in chromosome group 5 were used (Gill et al. 1996).

Sequences of RFLP marker *Xpsr128* obtained from the GrainGenes database (http://wheat.pw.usda.gov/ggpages/ggdb.shtml) were used to design PCR primers and were analyzed by BSA of mapping population II.

Physical and genetic mapping of *Stb1*

Genomic DNA from Chinese Spring (CS) wheat and 42 nullisomic-tetrasomic (NT) lines of CS stocks (Sears 1966) plus chromosome 5BL ditelosomic and nine deletion lines for chromosome 5B (Endo and Gill 1996) was amplified with RAPD marker $H19_{520}$ as described above. To further confirm the *Stb1* locus on wheat chromosome 5B, genomic DNA of six NT lines of CS (N5AT5B, N5AT5D, N5BT5D, N5BT5A, N5DT5A, and N5DT5B), the resistant line 72626E2-12-9-1, and the susceptible cultivar Arthur was amplified by PCR, using the two specific SSR primers *Xgwm213* and *Xgwm335*, located on wheat chromosome 5B (Röder et al. 1998).

Chi-square analysis was used to test for deviation from the 1:1 or 3:1 single-gene segregation ratios expected in the various mapping populations as described by Adhikari et al. (2003). Linkage analyses of the STB resistance gene and molecular markers were with MAPMAKER (Lander et al. 1987), calculated using the same parameter values described previously (Adhikari et al. 2003).

Results

Stb1 segregation in mapping population I

All F_1 plants of the crosses P881072-75-1 × P881072-75-2 and P881072-75-1 × Monon were resistant. Segregation of all populations fit either a 1:1 or 3:1 ratio, supporting the hypothesis of a single dominant gene for resistance to STB (Table 1). All F_2 families of the 78 resistant (heterozygous) three-way F_1 plants were segregating, and all those of the 70 susceptible three-way F_1 plants were susceptible (data not shown).

By analysis of their pedigrees, Bulgaria 88 was the likely donor of STB resistance in both 72626E2-12-9-1 and Sullivan. However, the Chinese line S0852 is unrelated to the other Purdue University breeding materials, so it was thought to contain a different source of resistance. Identifying the allelic relationship between the resistance gene in SO852 and *Stb1* in Sullivan is important to test whether the genes are the same. If the two resistance genes were independent or loosely linked, then up to one-fourth of the plants in a testcross F_1 population would be susceptible. If the two genes were identical, the plants in the testcross F_1 population would all be resistant and plants in the testcross F_2 population would show a 3:1 segregation. To test this hypothesis, the resistant line P881072-75-1—derived from SO852—was crossed with the resistant cultivar Sullivan, and this F_1 was crossed with the susceptible cultivar Monon. The results showed that all 151 F_1 plants from the testcross P881072-75-1/Sullivan//Monon were resistant, indicating that the resistance gene in SO852 and the *Stb1* gene in Sullivan are allelic or very closely linked. In addition, when the segregation data in the testcross F_2 families were pooled, 539 seedlings were resistant and 184 were susceptible, which fit closely to a 3:1 ratio (Table 1).

Stb1 segregation in mapping population II

The 106 F_6 RILs were scored for resistance to *M. graminicola* in the greenhouse at two times by different individuals, with slightly different scoring systems and with different isolates of the pathogen. Both tests gave very similar results with a high correlation (r = 0.866, Fig. 1). Resistant lines had pycnidial density of 1.5 or less in both tests, while susceptible lines had scores of 2 or higher. Four lines had intermediate pycnidial density in both tests, and six lines were resistant in one test and susceptible in the other or vice versa (Fig. 1). These lines were tested again during the fall of 2003 to ascertain which response was correct.

The final data for both disease severity and pycnidial density were analyzed by a multiplicative index that revealed a 51:55 ratio of resistant:susceptible lines. This ratio did not differ from that expected for a single gene (χ^2 = 0.151, $P > 0.80$), confirming the presence of *Stb1* in wheat line 72626E2-12-9-1.

RAPD analysis of mapping populations I and II

Approximately 2% of the 620 random primers analyzed were polymorphic in BSA of mapping population I. The number of DNA fragments amplified per primer ranged from zero to ten. Two RAPD primers, G7 and H19, amplified DNA fragments of 1,200 bp and 520 bp from the resistant but not the susceptible parent, and these markers were designated as $G7_{1200}$ and $H19_{520}$, respectively. Among the 148 F_1 plants of the three-way cross (P881072-75-1/P881072-75-2//Monon) analyzed from

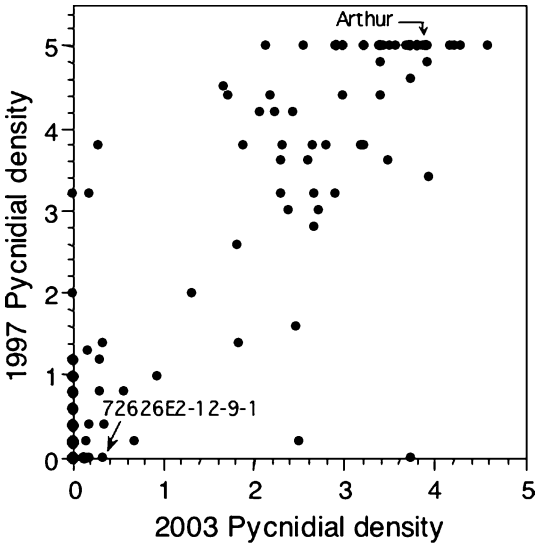

Fig. 1 Pycnidial density scores in response to *Mycosphaerella graminicola* among the 106 F_6 recombinant-inbred progeny lines from a cross between the resistant wheat line 72626E2-12-9-1 and the susceptible cultivar Arthur tested during 1997 versus 2003. The scores of the two parents are indicated by *arrows*

mapping population I (Table 1), the $G7_{1200}$ fragment was present in all resistant plants but absent in all susceptible plants. Similarly, the $H19_{520}$ band was present in 76 out of the 78 resistant F_1 plants but absent in all 70 susceptible plants.

The $H19_{520}$ and $G7_{1200}$ bands were converted to SCAR markers by designing primers specific for sequences obtained from the end of each fragment. A pair of specific SCAR primers (H19R: 5′-AAGCTCTCATCTGCCCTT-CAG-3′, H19F: 5′-GACATTCAAGGGGACTGGGTT-3′) for band $H19_{520}$ gave a plus or minus amplification pattern that duplicated the original RAPD polymorphism. However, the SCAR primers for band $G7_{1200}$ produced a similar-sized band with both parents so were not useful for characterizing mapping population I.

In mapping population II, the original two RAPD primers and the four SCAR markers derived from $G7_{1200}$ and $H19_{520}$ were monomorphic.

AFLP and SSR analyses of mapping population II

From 130 to 150 bands were obtained from each genotype with most of the 64 AFLP primer combinations analyzed. Almost all of the visible bands in the gels were reproduced consistently. Five primer combinations (*Eco*RI-AAG/*Mse*I-CTG, *Eco*RI-ACA/*Mse*I-CTA, *Eco*RI-ACT/*Mse*I-CTC, *Eco*RI-AGC/*Mse*I-CTA, and *Eco*RI-AGG/*Mse*I-CTC) each produced at least one polymorphic band present in the resistant parent and the resistant bulk but absent in the susceptible parent and the susceptible bulk. Three AFLP primer combinations (*Eco*RI-AAG/*Mse*I-CTG, *Eco*RI-ACT/*Mse*I-CTC, and *Eco*RI-AGG/*Mse*I-CTC) each amplified one polymorphic band in the susceptible parent and the susceptible bulk but absent in the resistant parent and the resistant bulk.

Among the 143 SSR loci (125 from the Gatersleben set and 18 BARC) tested in mapping population II, only four (nearly 3%) of the markers—*Xbarc74*, *Xgwm66*, *Xgwm213*, and *Xgwm335*—gave the expected patterns for linked markers in BSA and were used to genotype the 106 F_6 RILs. The SSR marker *Xbarc74* amplified an approximately 188-bp band specific to the resistant line 72626E2-12-9-1, and a 175-bp band from DNA of the susceptible parent Arthur (Fig. 2). The fragment sizes of the markers for loci *Xgwm213* and*Xgwm335* in the susceptible cultivar Arthur were 160 bp and 205 bp, respectively, compared with null alleles in the resistant parent. The SSR marker *Xgwm66* amplified a 598-bp fragment in the resistant parent that was absent in the susceptible parent. Because the 598-bp fragment was not expected with the SSR primers for locus *Xgwm66*, it was cloned and sequenced. Four SCAR primer pairs were designed from that clone, but the resulting banding patterns were monomorphic in the progeny (data not shown).

Linkage analysis and physical mapping of *Stb1*

Segregation analysis of the progeny from mapping population I indicated that RAPD markers $G7_{1200}$ and $H19_{520}$ were tightly linked to *Stb1*. No recombination was observed between $G7_{1200}$ and *Stb1*, so with a progeny size of 148, the RAPD markers must be within 0.68 cM of the resistance locus. The $H19_{520}$ marker was 1.4 cM from *Stb1*. To investigate possible linkages between AFLP markers and the *Stb1* gene, 33 markers generated by ten AFLP primer combinations were scored on the progeny from mapping population II. One AFLP marker, *Eco*R I-AGC/*Mse*I-CTA (374-bp allele), was linked to the resistance gene at a genetic distance of 8.4 cM (Fig. 3a). The other AFLP markers tested were false positives that were not linked to *Stb1*. The SSR marker *Xbarc74* was tightly

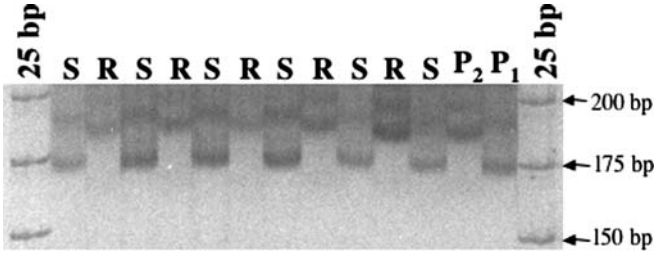

Fig. 2 DNA bands amplified from the parents and ten F_6 recombinant-inbred lines (RILs) derived from a cross between the resistant wheat line 7262E2-12-9-1 (*Stb1* gene for resistance to *M. graminicola*) and the susceptible parent Arthur, with microsatellite primer pair *Xbarc74* shown in a 3% agarose gel. A 25-bp DNA ladder was used as a standard size marker. P_1 Resistant parent wheat line 7262E2-12-9-1, P_2 susceptible parent Arthur. The resistant and susceptible progeny are indicated by *R* and *S*, respectively. The 188-bp DNA fragment was amplified from the resistant line 7262E2-12-9-1, and the resistant progeny and the 175-bp allele were amplified from the susceptible parent Arthur and the susceptible progeny

linked to the *Stb1* gene at a distance of 2.8 cM. However, *Xgwm66* was unlinked, and the other two marker loci, *Xgwm335* and *Xgwm213*, were linked to *Stb1* at distances of 7.4 cM and 8.3 cM, respectively. These marker loci exhibited segregation ratios ranging from 49:57 to 54:52, which were not significantly different from 1:1 by chi-square analysis.

Genomic DNA from CS and 42 NT lines was amplified with the markers $H19_{520}$, *Xgwm213*, and *Xgwm335* that were linked to *Stb1*. These markers were present in CS and all of the NT lines except for N5BT5D and N5BT5A (data not shown), confirming that these markers were located on chromosome 5B. The $H19_{520}$ allele was present in almost all lines tested, including the ditelosomic line for 5BL, and the two deletion lines L-14 and L-16 (Fig. 4) but was absent in lines L-6 and L-11. This indicated that marker $H19_{520}$ was located on the long arm of chromosome 5B.

The estimated fraction lengths of 5BL-11 and 5BL-14 were 0.59 and 0.75, respectively (Gill et al. 1996). Thus, marker $H19_{520}$ appeared to be in the region between fraction lengths of 0.59 and 0.75 (Fig. 3b). The RFLP probes *wg583* and *cdo400* were distal to the fraction breakpoint of 5BL-14 and showed no polymorphism between the two parents (data not shown). However, RFLP probe *bcd508*, which was distal to fraction length 5BL-11 but proximal to the fraction breakpoint of 5BL-14, i.e., between fraction lengths of 0.59 and 0.75, was polymorphic between the two parents (Fig. 3b). The PCR amplification products of a sequence-tagged site primer of probe *psr128*, located proximal to breakpoint 0.59, was not polymorphic (data not shown), suggesting that marker $H19_{520}$ was located between RFLP markers *pssr128* and *wg583*.

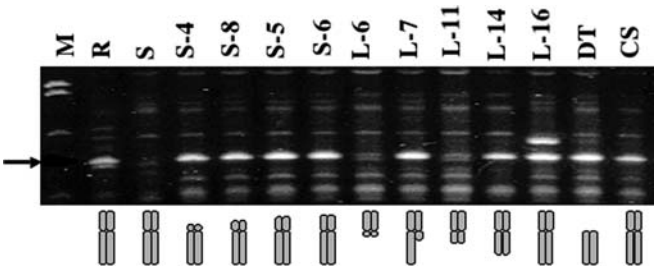

Fig. 4 RAPD marker $H19_{520}$ in wheat cultivar Chinese Spring chromosome 5B deletion lines. *M* Phage λ DNA digested with restriction enzyme *Pst*I, *R* resistant parent, *S* susceptible parent; *S-4 to L-16* deletion lines, *DT* ditelosomic line 5BL, *CS* Chinese Spring. The *arrow* indicates the $H19_{520}$ allele. Chromosomal configurations of normal, ditelosomic, and deletion lines are indicated at the *bottom of each lane*

Validation of the molecular markers

To examine the diagnostic value of the markers, 23 wheat accessions were analyzed with the two RAPD markers $G7_{1200}$ and $H19_{520}$ plus the SSR markers *Xbarc74* and *Xgwm335*. Bulgaria 88 and its derivatives Oasis and Sullivan all had RAPD markers $G7_{1200}$ and $H19_{520}$. Among the other STB-resistant cultivars, some had neither, both or one of the markers (Table 2). Israel 493 (*Stb3* gene) is resistant to STB, but had neither of the two RAPD markers. Veranopolis (*Stb2* gene), although it is resistant to STB, had $H19_{520}$ but not $G7_{1200}$. The susceptible cultivar CS had $H19_{520}$ but not $G7_{1200}$, which allowed us to use the special genetic stocks in the CS background, such as NT and deletion lines, to map $H19_{520}$.

The two SSR markers *Xbarc74* and *Xgwm335* showed differences in their patterns of amplification when tested on a range of unrelated wheat cultivars that differed for the

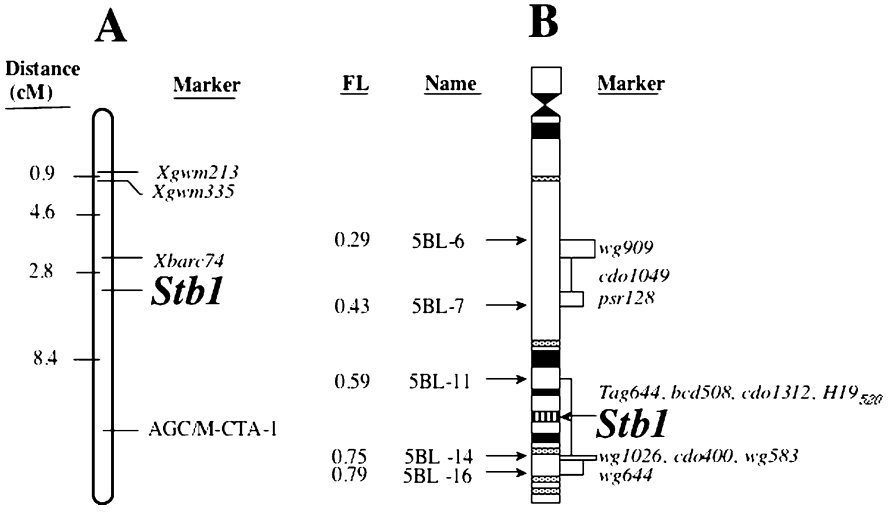

Fig. 3a, b Genetic and physical locations of the *Stb1* gene for resistance to *M. graminicola* on the long arm of wheat chromosome 5B. **a** Linkage map of amplified fragment length polymorphism and microsatellite markers constructed from 106 F_6 RILs derived from a cross between the resistant wheat line 72626E2-12-9-1 and the susceptible cultivar Arthur. Distances in centiMorgans (*cM*) from *Stb1*, are indicated on the *left*; marker type and resistance locus are shown on the *right*. **b** Cytologically based physical map on the right reproduced from Gill et al. (1996) consists of fraction breakpoints indicated by *arrows* on the left of the chromosome and restriction fragment length polymorphism markers, random amplified polymorphic DNA (RAPD) marker $H19_{520}$, and the resistance gene *Stb1* indicated on the *right*

Table 2 Validation of random amplified polymorphic DNA (*RAPD*) and microsatellite or simple-sequence repeat (*SSR*) markers in resistant and susceptible wheat cultivars

Line/cultivar	PI/CI number[b]	Reaction to STB[c]	RAPD marker		SSR marker[a]	
			$H19_{520}$ allele	$G7_{1200}$ allele	*Xbarc74* allele	*Xgwm335* allele
Anza	CI 15284	R	–[d]	+	188	212
Arminda	PI 428503	R	+	+	175	205
Bobwhite	PI 590172	R	–	+	170	205
Bulgaria 88	PI 94407	R	+	+	188	215
Iassul 20	CI 15396	R	–	+	188	212
Israel 493	N/A[e]	R	–	–	160	212
Kavkaz/K4500	N/A	R	+	+	170	205
Lakhish	PI 384031	R	+	–	160	212
Oasis	CI 15929	R	+	+	188	215
Olaf	CI 15930	R	+	+	160	212
Pilcraw	CI 5540	R	+	–	188	215
SO852	N/A	R	+	+	170	212
Sullivan	CI 17684	R	+	+	188	215
Toropi	PI 344200	R	–	+	160	212
Veranopolis	PI 297008	R	+	–	188	215
72626E2-12-9-1	N/A	R	–	–	188	215
Arthur	CI 14425	S	–	–	175	205
Chinese Spring	CI 14108	S	+	–	160	225
Clark	PI 512337	S	–	–	188	215
Gerek 79	PI 559560	S	–	–	175	205
Kavkaz	PI 361879	S	–	–	160	205
Monon	CI 13278	S	–	–	188	215
Morocco	PI 431591	S	+	–	188	212

[a]Microsatellite loci with approximate sizes of bands (in base pairs) amplified by each primer. *Xbarc74* amplified four alleles (188, 175, 170, and 160 bp) from the wheat cultivars tested. Similarly, *Xgwm335* also amplified four DNA bands (225, 215, 212, and 205 bp)
[b]*PI* Plant introduction, *CI* cereal inventory
[c]Resistance to septoria tritici blotch caused by *Mycosphaerella graminicola* as determined in previous analysis (Yang 2000). *R* Resistant, *S* susceptible
[d]Presence of allele (+) and absence of allele (−)
[e]*N/A* Not applicable

presence or absence of *Stb1*. SSR marker *Xbarc74* amplified PCR products of approximately 188, 175, 170, and 160 bp from both the resistant and the susceptible cultivars (Table 2). The 188-, 175-, and 160-bp alleles were amplified from both resistant and susceptible wheat cultivars. The 170-bp allele amplified only from the three resistant cultivars Bobwhite, Kavkaz/K4500 and SO852 (Table 2). However, that was not the allele linked to *Stb1* in Bulgaria 88.

The SSR marker *Xgwm335* also amplified four DNA bands (225, 215, 212, and 205 bp) when tested on the 23 wheat cultivars (Table 2). The largest band (225 bp) was present only in CS. The remaining three alleles were amplified from both resistant and susceptible cultivars (Table 2).

Discussion

Stb1 was identified originally in the winter wheat cultivar Bulgaria 88 (Rillo and Caldwell 1966). The original tests were done with combined inoculum from six single-spore cultures from Indiana, none of which survived. Although we cannot be certain that the gene identified with contemporary isolates from Indiana is the same as that identified almost 40 years ago, it seems highly likely. The *Stb1* gene was transferred into US wheat cultivars such as Oasis and Sullivan during the 1970s (Patterson et al. 1975, 1979). These and other Bulgaria 88 derivatives were grown widely for many years with no loss of resistance (Patterson et al. 1975, 1979; Rillo and Caldwell 1966; Shaner and Buechley 1989; Shaner and Finney 1982). Due to the long-term effectiveness of *Stb1* against *M. graminicola* in Indiana, we assume that the resistance identified with our isolates is the same as that identified originally with mixed-isolate inoculum in Bulgaria 88.

The resistant RIL P881072-75-1 used to develop mapping population I in this study was indistinguishable in terms of its STB phenotype from that of cultivar SO852. Moreover, segregation analysis fit either the 1:1 or 3:1 ratio for resistant to susceptible that is expected for single-gene resistance to *M. graminicola* in testcross and F_2 progeny of SO852. Line SO852 came originally from China and was expected to contain different genes from those already available in the US wheat collection. The allelism tests, however, indicated that the dominant STB resistance gene of SO852 is allelic, or very closely linked, to the *Stb1* gene of Bulgaria 88. Similarly, a Chinese line with resistance to *Erysiphe graminis* f. sp. *tritici* was shown to have the resistance gene *Pm1* (Hu et al. 1997), a gene that has been used extensively in North America.

The two RAPD markers that were linked to the *Stb1* gene in P881072-75-1 may be useful for introgressing the resistance into additional cultivars. No recombination was observed between $G7_{1200}$ and the resistance gene among 148 progeny tested. If the next individual tested were a recombinant, then the recombination value would be 1/149 or 0.00671. Therefore, $G7_{1200}$ must be less than 0.68 cM from *Stb1*. $H19_{520}$ also was tightly linked to *Stb1* at a genetic distance of 1.4 cM. However, whether these two RAPD markers flank the resistance gene and their position relative to the centromere are not known.

Attempts to convert the RAPD polymorphisms into SCAR markers were only partially successful, indicating that developing sequence-specific markers from RAPD primers is not an efficient process in wheat. Specific primers at each end of the $H19_{520}$ sequence produced a plus or minus segregation that duplicated the original RAPD polymorphism. Several pairs of specific primers for the $G7_{1200}$ band either did not work or amplified the same-sized band from resistant and susceptible individuals. Our inability to successfully convert the $G7_{1200}$ allele into a SCAR marker presumably was because the original RAPD polymorphism resulted from differences in nucleotide sequence at the priming sites, and all of our primers were internal. Similar results have been reported in other host–pathogen systems. For example, Paran and Michelmore (1993) found that six of nine RAPD polymorphisms for downy mildew resistance genes in lettuce were derived from mismatches in one or a few nucleotides in the priming sites. Extended SCAR primers produced undifferentiated amplification products from both parents, most likely because they were not affected by these mismatches (Paran and Michelmore 1993). Similar phenomena probably are occurring with the $G7_{1200}$ marker in wheat.

The resistant wheat line 72626E2-12-9-1 used to develop mapping population II in this study contained the Bulgaria 88 derivatives Sullivan and Oasis in its pedigree as well as a dwarf derivative of Redcoat, all of which could have contributed resistance to STB (Shaner and Buechley 1989). Although allelism tests among these cultivars were not performed, the resistant line 72626E2-12-9-1, Oasis (Patterson et al. 1975), and Sullivan (Patterson et al. 1979) most likely all inherited the *Stb1* gene for resistance to *M. graminicola* from Bulgaria 88.

Analysis of mapping population II identified one AFLP and three SSR markers linked to *Stb1*, but more markers are needed for maximum effectiveness of MAS. This is because the only marker distal to *Stb1*, AFLP locus *Eco*RI-AGC/*Mse*I-CTA-1, was relatively distant from the resistance gene and could not be converted successfully into a SCAR marker. Both regular and inverse PCR (Braden and Simon 1998) were tried in attempts to convert the 374-bp AFLP marker into an allele-specific SCAR. Each specific primer pair amplified a single product of the same size as the progenitor AFLP marker, but did not differentiate the two parental genotypes under a variety of PCR conditions, presumably because the polymorphism was near the 3′ end of the original primer sequence or was within one of the restriction-enzyme recognition sites.

Cytogenetic stocks allowed localization of the *Stb1* gene to the physical map of chromosome 5BL. Analysis of the nine deletion lines suggested that $H19_{520}$ was located in the region between the fraction lengths of 5BL-11 and 5BL-14 (Fig. 3b). Since RAPD marker $H19_{520}$ is approximately 1.4 cM away from the *Stb1* locus, it is likely that resistance gene *Stb1* is located between fraction lengths 0.59–0.75, i.e., between the two RFLP markers *Tag644* and *wg1026* (Gill et al. 1996; Nelson et al. 1995a).

The lack of complete correspondence between the *Stb1* resistance gene and the four most closely linked molecular markers is not surprising, given the distance of the markers from the gene. The only cultivars with all four markers were Bulgaria 88 and its descendents Oasis and Sullivan, indicating that a linkage block of at least 7.5 cM was maintained intact during the backcrossing process. Susceptible cultivars never had more than two of the four markers. The closely linked marker $G7_{1200}$ was absent from all of the susceptible cultivars but also was missing from resistant line 72626E2-12-9-1. Therefore, none of the markers was diagnostic for the *Stb1* gene.

The high level of polymorphism of the SSR markers may increase their effectiveness for marker-assisted selection. Four alleles were identified at each SSR locus when they were tested on a range of resistant and susceptible cultivars. The specific alleles linked to resistance usually occurred together; 73% of the accessions with one of the resistance-associated alleles also had the other. This association may mean that both alleles have been in coupling with the resistance allele since it first originated. However, SO852 has neither of the specific resistance-related SSR alleles, possibly indicating a separate origin for the resistance in this line. In contrast, the two susceptible cultivars Clark and Monon do have the resistance-related alleles, possibly reflecting a crossover between locus *Xbarc74* and the resistance gene during the breeding process.

In addition to the *Stb1* gene, the long arm of chromosome 5B carries *Ph1* (Gill et al. 1993), which is considered to be the genetic system that governs exclusive homologous pairing in polyploid wheat (Riley and Chapman 1958), and the *tsn1* gene for resistance to a necrosis-inducing isolate of *Pyrenophora tritici-repentis*, the cause of tan spot (Stock et al. 1996). Unlike some other STB

resistance genes (Adhikari et al. 2004a), *Stb1* does not appear to be located in a cluster of genes for resistance to diverse pests and pathogens.

Genetic characterization of new germplasm is the first step towards its utilization, particularly for a potential source of resistance to *M. graminicola*, of which relatively little is known. Although we found no evidence that line SO852 contains any other gene for resistance to STB, it still may be useful because of its other traits. In addition to *Stb1*, SO852 has resistance to *Blumeria graminis* f. sp. *tritici* and *Puccinia triticina* (H.W. Ohm, unpublished data). Of particular utility to wheat improvement in the Midwestern states is that SO852 is an early maturing and short cultivar, whereas STB-resistant lines derived from Bulgaria 88 were commonly late and tall (Patterson et al. 1975, 1979; Rosielle and Brown 1979).

Earliness is a highly desirable trait in Indiana because it facilitates double cropping of wheat with early-maturing soybeans. However, in some cases, early maturity was highly associated with susceptibility to *M. graminicola* (Baltazar et al. 1990; Rosielle and Boyd 1985; Shaner et al. 1975). In regions where STB is a major problem, disease-favorable conditions with cool temperatures and rain are more probable early in the spring. Thus, flag leaves often will emerge on early-maturing cultivars when weather is conducive for infection (Shaner et al. 1975). Genetic linkages between earliness and susceptibility to *M. graminicola* also have been reported as a possible explanation for this association (Baltazar et al. 1990; Rosielle and Boyd 1985). Many semidwarf wheat cultivars possess one or both of the Norin 10 height-reducing genes (Rht_1 or Rht_2) in their parentage (Gale et al. 1981). Differences in levels of resistance to *M. graminicola* were observed in wheat, depending on which dwarfing gene they possess (Baltazar et al. 1990). Use of SO852 in a breeding program could help mitigate these negative associations between earliness and susceptibility to STB.

Conventional breeding for resistance against STB is complicated by the long latent period of the disease and lack of defined tester isolates of *M. graminicola*. Tightly linked molecular markers could help to overcome these problems and are now available for eight major genes for resistance to *M. graminicola* (Goodwin and Adhikari 2003). *Stb1* was effective in Indiana for many years with no loss of resistance (Shaner and Finney 1982; Shaner and Buechley 1989). The *Stb4* gene was effective in California for about 15 years but became ineffective recently (Jackson et al. 2000). The long-term effectiveness of these genes was not expected for a pathogen with high genetic diversity and frequent sexual recombination (Ahmed et al. 1995; McDonald and Linde 2002). Although unexplained, the apparent durability of *Stb1* indicates that a single major gene can confer resistance for many years against *M. graminicola*, at least within a limited geographical area. Combining *Stb1* with other genes for resistance to STB may help extend the useful life of all of these genes to provide more durable resistance to *M. graminicola*.

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