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RESEARCH

A New Map Location of Gene *Stb3* for Resistance to Septoria Tritici Blotch in Wheat

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ABSTRACT

Septoria tritici blotch (STB), caused by *Mycosphaerella graminicola* (synonym: *Zymoseptoria tritici*; asexual stage: *Septoria tritici*), is an important disease of wheat (*Triticum aestivum* L.) worldwide. Management of the disease usually is by host resistance or fungicides. However, *M. graminicola* has developed insensitivity to most commonly applied fungicides, so there is a continuing need for well-characterized sources of host resistance to accelerate the development of improved wheat cultivars. Gene *Stb3* has been a useful source of major resistance, but its mapping location has not been well characterized. On the basis of linkage to a single marker, a previous study assigned *Stb3* to a location on the short arm of chromosome 6D. However, the results from the present study show that this reported location is incorrect. Instead, linkage analysis revealed that *Stb3* is located on the short arm of wheat chromosome 7A, completely linked to microsatellite (simple-sequence repeat) locus *Xwmc83* and flanked by loci *Xcfa2028* (12.4 cM distal) and *Xbarc222* (2.1 cM proximal). Linkage between *Stb3* and *Xwmc83* was validated in BC_1F_3 progeny of other crosses, and analyses of the flanking markers with deletion stocks showed that the gene is located on 7AS between fraction lengths 0.73 and 0.83. This revised location of *Stb3* is different from those for other STB resistance genes previously mapped in hexaploid wheat but is approximately 20 cM proximal to an STB resistance gene mapped on the short arm of chromosome $7A^m$ in *Triticum monococcum*. The markers described in this study are useful for accelerating the deployment of *Stb3* in wheat breeding programs.

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Abbreviations: DH, doubled haploid; FL, fraction length; LOD, logarithm of odds; PCR, polymerase chain reaction; SSR, simple-sequence repeat; STB, Septoria tritici blotch; TRAP, target region amplification polymorphism.

Septoria tritici blotch (STB), caused by the ascomycete *Mycosphaerella graminicola* (synonym: *Zymoseptoria tritici*; asexual stage: *Septoria tritici*), is one of the most common and important diseases of wheat (*Triticum aestivum* L.) worldwide (Eyal et al., 1987). Septoria tritici blotch often is the predominant disease problem of wheat in areas with cool, wet spring weather, such as northern Europe and the United Kingdom, and is usually one of the top three or four diseases in other parts of the world, depending on the year. Even when other diseases such as leaf and stem

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rust, Fusarium head blight, or Stagonospora nodorum leaf and glume blotch are more prominent, STB still occurs and can contribute to losses, particularly when it spreads to flag leaves late in the season.

Fortunately, resistance is available and can mitigate losses caused by STB. Whether resistance was inherited quantitatively or qualitatively was debated for many years and was only settled less than a decade ago when wheat and *M. graminicola* were shown to interact in a gene-for-gene manner (Brading et al., 2002). To date, 18 major genes for resistance to STB have been identified and 16 have been mapped (Chartrain et al., 2009; Goodwin, 2012; Tabib Ghaffary et al., 2011; Tabib Ghaffary et al., 2012), along with many quantitative genes with minor effects (Jlibene et al., 1994; Simón et al., 2004; Simón et al., 2012). For some of the mapped STB resistance genes, multiple molecular markers have been identified that flank the resistance gene and have been verified in two or more populations (e.g., Chartrain et al., 2009). For others, the precise chromosome location is not known or is based only on linkage in one population to a single molecular marker, so a need remains for additional linked markers to accelerate the deployment of these genes in plant improvement programs.

The major resistance gene *Stb3* was assigned to the short arm of chromosome 6D on the basis of linkage to the single microsatellite or simple-sequence repeat (SSR) locus *Xgdm132* (Adhikari et al., 2004). The original donor of *Stb3* was the Australian red wheat cultivar Israel 493 (Wilson, 1985). This cultivar has a very strong resistance phenotype but has not been used extensively in breeding programs, in part because of the lack of a set of linked markers for marker-assisted selection. While the SSR locus *Xgdm132* was reported to be 3 cM from *Stb3*, this location on chromosome 6DS had not been verified in independent segregating populations. If *Stb3* is to be used effectively in future wheat improvement programs, it is necessary to identify additional markers that are closely linked to and that flank the resistance gene. The goals of this research were to address this need while validating the map location of *Stb3* in one or more independent populations.

MATERIALS AND METHODS

Mapping Populations

The original mapping population for *Stb3* consisted of 97 doubled-haploid (DH) lines derived from a cross between the susceptible wheat line RAC875-2 and the resistant cultivar Israel 493 (Adhikari et al., 2004). Developed at the University of Adelaide, Australia, RAC875-2 is a hard white spring wheat that is susceptible to Australian isolates of *M. graminicola*. Israel 493 is a red spring wheat cultivar selected in Australia from a cross between Miriam 4 and Lakhish 1552-3 (Wilson, 1979) and carries the *Stb3* gene for resistance to *M. graminicola* (Wilson, 1985).

Validation of the revised map location for *Stb3* was done with two BC_1F_3 populations developed at the University of California, Davis, by crossing Israel 493 to the STB-susceptible wheat lines UC1110 and Clear White. From each pairing, BC_1 plants were allowed to self-pollinate for two generations to generate BC_1F_3 populations. Individual plants were tested during the BC_1F_2 generation to identify those that were homozygous at *Xwmc83*, the SSR locus identified as linked to the STB resistance locus in Israel 493. Eight seeds from BC_1F_2 individuals homozygous for the *Xwmc83* allele from Israel 493 and eight seeds from BC_1F_2 individuals homozygous for the *Xwmc83* allele from the susceptible parents UC1110 and Clear White were planted to test for susceptibility to STB. To ensure an unbiased test, plants were scored for resistance phenotype without prior knowledge of the marker genotype.

Plant Inoculation and Disease Scoring

Testing of the mapping population for susceptibility to the Paskeville isolate of *M. graminicola* was described previously (Adhikari et al., 2004). Briefly, plants were grown in individual 4-inch pots in a greenhouse and spray-inoculated with a spore suspension after the flag leaves had emerged fully. Plants were scored for percentage of leaf area covered with lesions and for density of pycnidia (0 to 5 scale) after lesions developed. Doubled-haploid lines with disease severity less than 5% and with pycnidial density of 0 to 1 averaged over 6 to 8 replicate plants were considered resistant (Adhikari et al., 2004). These criteria gave a good separation between resistant and susceptible plants and showed a bimodal distribution when plotted as a histogram (Adhikari et al., 2004).

Plants of the BC_1F_3 populations were tested by a sheath-inoculation method. Spores of *M. graminicola* were produced by growing isolate T48 (IN95-Lafayette-1196-WW 1–4) in 100 mL of yeast-sucrose broth medium (10 g of yeast extract, 10 g of sucrose, and 25 mg of kanamycin sulfate in 1 L of distilled water) for 3 to 5 d at room temperature on an orbital shaker at 120 rpm. Once the cultures became opaque, they were passed through a single layer of cheesecloth to remove mycelia and other large material. Spore suspensions were then adjusted to 1.0×10^6 spores/mL with a haemacytometer.

Wheat plants at the five-leaf stage were inoculated using a 3-mL Leur-Lok syringe fitted with a 20G needle. The needle was inserted into the sheath at the whorl of developing leaves of each plant at an oblique angle to prevent emergence from the other side. Spore suspension was injected into each sheath until it emerged from the top of the leaf whorl, ensuring thorough contact with the developing leaf blade. Following inoculation, the plants remained on the greenhouse bench with no other treatment. Within 2 d, the inoculation point emerged from each sheath and could be identified by a puncture wound along the leaf blade.

Sheath-inoculated plants were evaluated for resistance 16 d after inoculation. Each plant was rated on a 0 to 5 scale for lesion size and pycnidial density. Average lesion and pycnidial density scores were added together to yield a composite score of 0 to 10 describing the overall resistance and susceptibility of each wheat line. Lines with combined scores of 4 or lower were considered resistant, while those with combined scores greater than 4 were considered susceptible. Plants for which the inoculation point could not be identified clearly were not scored.

Molecular Markers, Linkage Analysis, and Mapping

Twelve SSR loci previously mapped near locus *Xgdm132* on the short arm of chromosome 6D (6DS) were tested for polymorphism between the parents of the original *Stb3* mapping population by Marla Hall at the USDA Small Grain Genotyping Center (Manhattan, KS).

Bulked segregant analysis (Michelmore et al., 1991) with SSR markers was performed on DNA from the parents and two bulks comprised of 10 resistant and 10 susceptible progeny that showed clear phenotypes. Markers that were polymorphic between the resistant parent and resistant bulk versus the susceptible parent and susceptible bulk were then tested on the 10 individual progeny comprising each bulk. Markers that still appeared to be linked to the *Stb3* resistance locus (>70% of the progeny with the expected alleles) were tested subsequently on the complete set of 97 DH lines.

Each polymerase chain reaction (PCR) for SSR loci contained 13 μL of water, 2.5 μL of 10X PCR buffer, 2.5 μL of dNTPs, 1.5 μL of 25 mM $MgCl_2$, 4 μL of 4 μM primers, 0.5 μL of MangoTaq DNA polymerase, and 1 μL of 12.5 ng/μL DNA. Cycling parameters were: 95°C for 5 min; 35 cycles of 94°C for 30 sec, 55 or 60°C (on the basis of primer annealing temperature) for 30 sec, 72°C for 1 min; and a final extension at 72°C for 7 min before cooling to 4°C. Polymerase chain reaction products were separated by electrophoresis on 3% (w/v) agarose gels in 0.5X TAE buffer (0.04 M tris-acetate, 0.001 M EDTA) and stained with ethidium bromide. Products were further analyzed when necessary by separating on 9% nondenaturing polyacrylamide gels and were detected by silver staining, as described by Adhikari et al. (2003).

For locus *Xcfa2028*, bands also were separated on a sequencing gel after amplification with a fluorescent primer. Amplification was done as described above with the original Xcfa2028-R primer used in combination with the Xcfa2028-F primer with a 6FAM fluorescent dye attached to the 5′ end. Following amplification, 1 μL of the PCR product was mixed with 8.9 μL of Hi-Di Formamide and 0.1 μL of Genescan-500 Liz size standard, both from Applied Biosystems, Inc. Separation of the amplification products was completed at the Purdue Genomics Facility in West Lafayette, IN. The results were analyzed with GeneMarker software from Softgenetics.

Target region amplification polymorphism (TRAP) marker analysis (Hu and Vick, 2003) was used to identify candidate chromosomes containing the *Stb3* gene. For this approach, DNA samples from the resistant and susceptible parents plus resistant and susceptible bulks were first screened for polymorphisms with TRAP markers amplified by the two random primers T03 (5′-CGTAGCGCGTCAATTATG-3′) and T13 (5′-GCGCGATGATAAATTATC-3′) in combination with the nine fixed primers described by Li et al. (2006). The fixed primers were designed on the basis of deletion-mapped wheat EST sequences (www.ncbi.nlm.nih.gov, accessed 9 Sept. 2014) and provide good genome coverage of all wheat chromosomes except for 4A and 5A (Li et al., 2006). To visualize bands, the two random primers were labeled at their 5′ ends with two dyes (IR dye 700 or IR dye 800) for detection with a LI-COR Global Genotyper system (LI-COR Biosciences). Polymerase chain reaction was performed in final reaction volumes of 15 μL in 96-well microtiter plates containing the following reagents: 2 μL of genomic DNA, 1.5 μL of 10X PCR buffer, 5 mmol dNTPs, 1 μL of 25 mM $MgCl_2$, 0.3 pmol each of IR-700 and IR-800 dye-labeled primers, 10 pmol of the fixed primer, and 1.5 units of *Taq* DNA polymerase (Clonetech). All PCR conditions and cycles were performed as described previously (Li et al., 2006). Each PCR product was mixed with loading dye, and a 1-μL aliquot was loaded onto a 6.5% (w/v) polyacrylamide sequencing gel in a LI-COR Global DNA Sequencer using instructions provided by the manufacturer (LI-COR Biosciences). Electrophoresis was conducted at 1500 V for 3.5 h. The amplified polymorphic fragments from 100 to 1000 bp were scored visually on the basis of the printed images collected by the SAGA^{GT} software (Li-Cor Biosciences).

To identify the chromosome containing a polymorphic TRAP marker amplified with fixed primer W33 (5′-ACTGCTCTAACGGGAAAC-3′) and random primer T03, additional TRAP amplifications were performed on DNA from wheat cultivar Chinese Spring and its 42 nullisomic-tetrasomic lines as described by Röder et al. (1995).

Linkage analyses were done with Mapmaker version 2.0 (Lander et al., 1987). The Kosambi mapping function was used with a logarithm of odds (LOD) score of 3.0 and maximum recombination fraction of 0.4 to determine locus order. Alternative orders were tested by trying the markers in sliding windows of five at a time with the RIPPLE command.

Physical Mapping of Loci Linked to *Stb3*

For physical mapping of the loci that were linked to *Stb3*, deletion stocks 4546 L1 (7AS-1, FL 0.89), 4546 L2 (7AS-2, FL 0.73), 4546 L5 (7AS-5, FL 0.59), 4546 L8 (7AS-8, FL 0.45 and 7AL-17, FL 0.71), 4546 L12 (7AS-12, FL 0.83), 4547 L1 (7AL-1, FL 0.39), 4547 L5 (7AL-5, FL 0.63), and 4547 L21 (7AL-21, FL 0.74) were ordered from the Wheat Genetics Resource Center (Kansas State University, Manhattan). Each of these stocks has a single deletion from the short or long arms of chromosome 7A, except for 4546 L8, which has a deletion on each arm (Qi et al., 2009). To determine the physical location of each marker, PCR amplification was performed as described above with each deletion stock, the three nulli-tetrasomic stocks, and the two ditelosomic lines for chromosome 7A.

Cloning, Sequencing, and Specific Polymerase Chain Reaction Amplification

Attempts to improve the specificity of the primers for loci *Xwmc83*, *Xwmc603*, and *Xcfa2028* were made by cloning and sequencing the polymorphic bands produced by the two parents. Cloning was done with the TOPO TA Cloning kit (Invitrogen Corporation), according to the manufacturer's instructions. Positive clones were identified as those with inserts following digestion of plasmid DNA with the restriction enzyme *Eco*RI and electrophoresis on agarose gels. Sequencing with M13 forward and reverse primers was done by standard methods at the Low-throughput Sequencing Laboratory at the Purdue Genomics Core Facility (www.genomics.purdue.edu/~ltl, accessed 9 Sept. 2014). The sequences were aligned with each other and, when available, with the sequences for the original loci downloaded from GrainGenes (http://wheat.pw.usda.gov,

accessed 9 Sept. 2014) to identify the cause of the observed polymorphisms. Alignments were made with ClustalX (Larkin et al., 2007) and prepared for publication with Jalview (Clamp et al., 2004). The original primers were extended by 1 to 5 bp and all combinations were tried on the parents of the mapping population to test for improved resolution.

RESULTS

Molecular Mapping of *Stb3*

Among the 12 microsatellite loci previously mapped to the short arm of chromosome 6D, five were polymorphic between the parents of the *Stb3* mapping population. Testing the five polymorphic markers (*Xbarc54*, *Xbarc173*, *Xcfd1*, *Xcfd49*, and *Xcfd75*) on the complete progeny set revealed that none were linked to *Stb3* (data not shown). These results suggested that *Stb3* is not located on the short arm of chromosome 6D and prompted a whole-genome scan for linkage with markers located on other chromosomes.

Testing of primer pairs for 251 SSR loci covering all 21 wheat chromosomes identified only 27 that were polymorphic between the resistant parent and resistant bulk versus the susceptible parent and susceptible bulk. However, linkage analysis revealed that none of these loci were linked to *Stb3* (data not shown).

The TRAP marker analysis on parents and bulks with nine fixed primers in combination with the two random primers detected one polymorphic band of approximately 468 bp amplified by fixed primer W33 with random primer T03 (*TRAP-W33T03*). This marker (Fig. 1) was located approximately 15.7 cM from the resistance gene when data from all 97 progeny lines were included, and at a distance of 11 cM from *Stb3* when all potentially ambiguous data were removed from the linkage analysis (75 lines). An additional polymorphic TRAP marker was identified but was not linked to *Stb3* (data not shown). Analysis of Chinese Spring and its 42 nullisomic-tetrasomic derivatives showed that the 468-bp TRAP band was missing from lines nullisomic for chromosomes 3A and 7A, indicating that the resistance locus could be on either of those chromosomes.

Following the TRAP analysis, 81 SSR loci on chromosomes 3A and 7A were tested for polymorphism on the parents and progeny of the mapping population. Twenty-six loci on chromosome 3A were tested; three were polymorphic but none were linked to *Stb3* (data not shown). Among the 55 additional loci tested on chromosome 7A, 15 were polymorphic and formed a linkage block with *Stb3* and *TRAP-W33T03* (Fig. 2A). Linkage analysis of these 15 microsatellite loci with Mapmaker showed that *Stb3* is located on the short arm of wheat chromosome 7A, completely linked to SSR locus *Xwmc83* and flanked by SSR markers *Xcfa2028* (12.4 cM, distal) and *Xbarc222* (2.1 cM, proximal).

Ten loci on 7A had been screened during the previous bulked segregant analysis, so 65 loci on this chromosome were tested in total. Loci that were tested but were not polymorphic between the parents of the *Stb3* mapping population included *Xbarc64*, *Xbarc70*, *Xbarc105*, *Xbarc112*, *Xbarc127*, *Xbarc151*, *Xbarc154*, *Xbarc157*, *Xbarc1005*, *Xbarc1025*, *Xbarc1034*, *Xbarc1088*, *Xcfa2028*, *Xcfa2040*, *Xcfa2049*, *Xcfd193*, *Xcfd242*, *Xgpw2103*, *Xgwm60*, *Xgwm63*, *Xgwm130*, *Xgwm233*, *Xgwm260*, *Xgwm276*, *Xgwm282*, *Xgwm332*, *Xgwm350*, *Xgwm471*, *Xgwm554*, *Xgwm573*, *Xgwm635*, *Xgwm666*, *Xpsp3050*, *Xpsp3094*, *Xpsp3114*, *Xwmc9*, *Xwmc17*, *Xwmc65*, *Xwmc158*, *Xwmc168*, *Xwmc179*, *Xwmc182*, *Xwmc388*, *Xwmc405*, *Xwmc422*, *Xwmc479*, *Xwmc497*, *Xwmc593*, *Xwmc596*, *Xwmc695*, *Xwmc809*, and *Xwmc826*. Loci that were polymorphic but did not form part of the linkage group with *Stb3* included *Xcfd62*, *Xwmc338*, and *Xwmc646*.

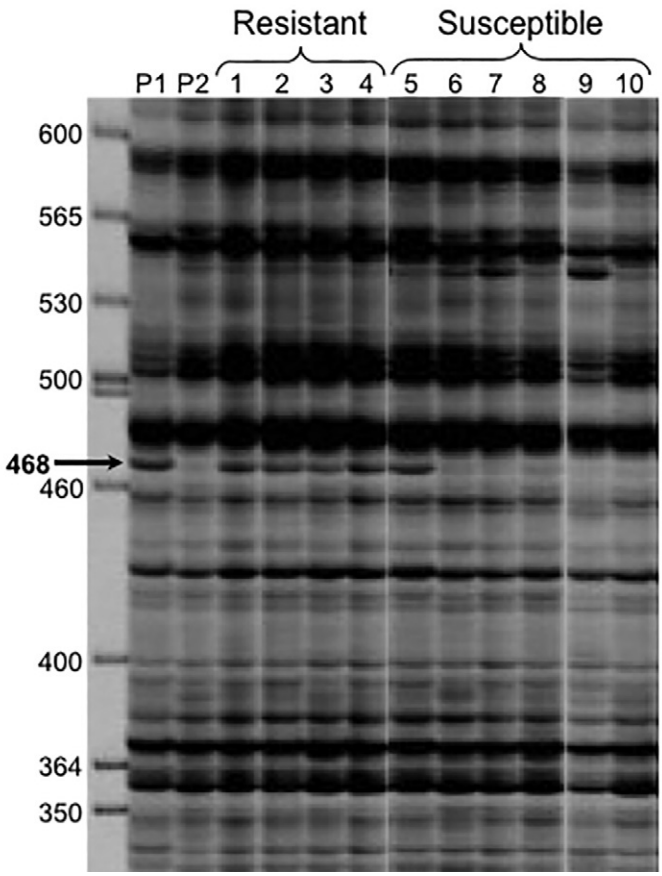

Figure 1. Portion of a target region amplification polymorphism gel of amplification products generated with fixed primer W33 and random primer T03 showing segregation of a locus linked to the *Stb3* gene for resistance to *Mycosphaerella graminicola* in wheat (*Triticum aestivum* L.). P1, resistant parent Israel 493; P2, susceptible parent RAC875-2; lanes 1 to 4, resistant doubled-haploid progeny lines 5, 6, 7, and 8, respectively; lanes 5 to 10, susceptible doubled-haploid progeny lines 1, 3, 4, 9, 11, and 14, respectively. Sizes in base pairs of the bands for the molecular marker standard in lane M are indicated to the left of each band. The arrow on the left indicates the approximately 468-bp allele at locus *TRAP-W33T03* that is linked to the *Stb3* gene for resistance to *M. graminicola*. All progeny lines have the expected marker phenotype except for line 1 (lane 5), which was susceptible but had the marker allele from the resistant parent.

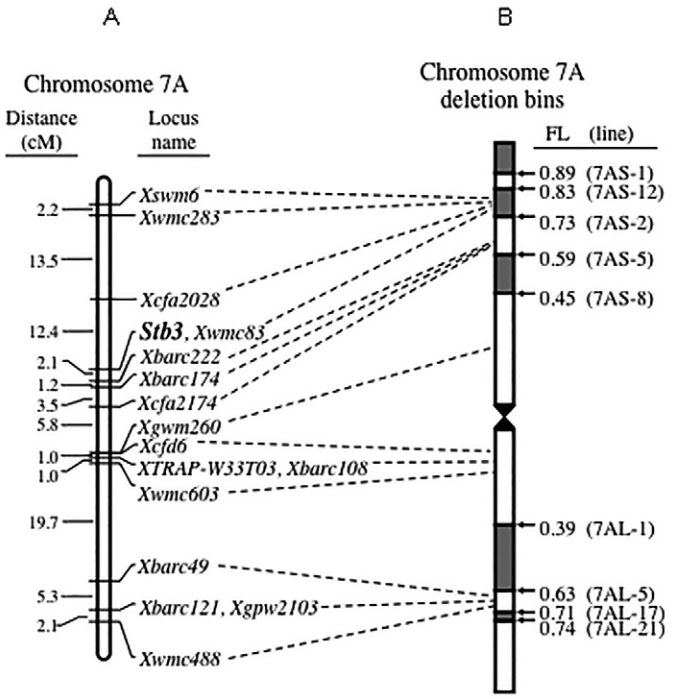

Figure 2. (A) linkage and (B) physical mapping of the region containing *Stb3* on wheat (*Triticum aestivum* L.) chromosome 7A. *Stb3* is indicated in bold. *XTRAP-W33T03* is a target region amplification polymorphism locus identified with fixed primer W33 and random primer T03. Other names beginning with *X* indicate simple-sequence repeat loci. Dashed lines connect the locations of the markers on the linkage map (A) to deletion bins on the physical map (B). The genetic distance between loci is indicated on the left. FL, fraction length of the bins defined by the breakpoints of the chromosomes in the deletion lines indicated in parentheses.

Physical Mapping of Loci Linked to *Stb3*

Analysis of the chromosome 7A cytogenetic stocks confirmed that all the mapped SSR markers were located on chromosome 7A, with eight on the short arm and seven on the long arm. The three loci most closely linked to *Stb3* were on 7AS, between fraction lengths (FL) 0.59 and 0.83 (Fig. 2B). Since locus *Xwmc83*, which co-segregated with *Stb3*, lies distal to the breakpoint of deletion stock 7AS-2 (Fig. 3A), the most likely location of the resistance gene is within FL 0.73 to 0.83.

Use of the RIPPLE command in Mapmaker revealed that the closely linked markers *Xwmc603*, *Xbarc108*, *TRAP-W33T03*, *Xcfd6*, and *Xgwm260* were ordered with a LOD score <2. Analysis of the cytogenetic stocks showed that *Xgwm260* was on the short arm (Fig. 3B), but the other markers in this group such as *Xbarc108* were on the long arm (Fig. 3C). A map with the order of these markers inverted had a LOD score 1.02 lower but was not significantly different from the first order at LOD 2. Therefore, the alternative order (Fig. 2B) was chosen because it was consistent with the results of the physical mapping.

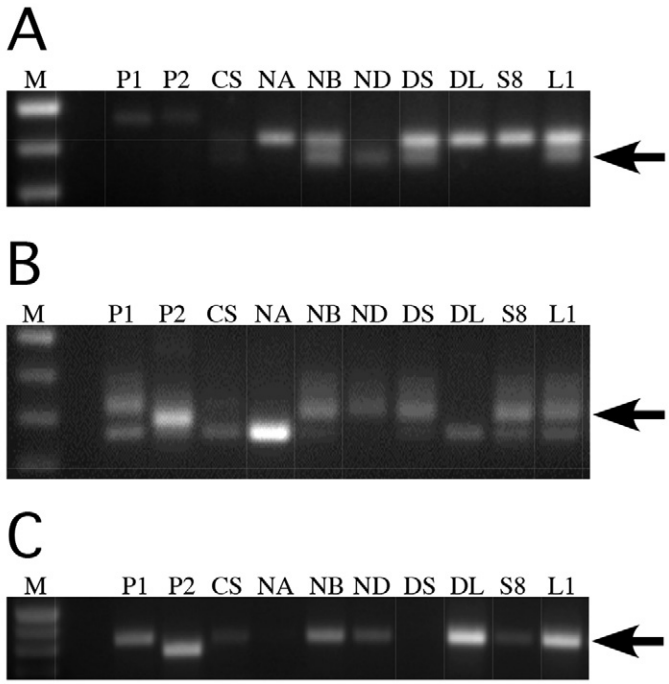

Figure 3. Physical mapping of markers linked to *Stb3* with cytogenetic stocks of wheat (*Triticum aestivum* L.). (A) Primers for locus *Xwmc83* gave an amplification product with all lines except for N7AT7D, DT7AL, and 7AS-8, indicating that it (and presumably *Stb3* as well) is on the short arm of chromosome 7A, distal to the deletion breakpoint at fraction length (FL) 0.45. Amplification products from both parents and 'Chinese Spring' had different sizes at this locus; DNA from Chinese Spring gave amplification from two loci, one on chromosome 7A and the other on 7D. (B) Primers for locus *Xgwm260* gave an amplification product with all lines except for N7AT7D and DT7AL, indicating that it is on the short arm of chromosome 7A, proximal to the deletion breakpoint at FL 0.45. (C) Primers for locus *Xbarc108* gave an amplification product with all lines except for N7AT7D and DT7AS, indicating that it is on the long arm of chromosome 7A, proximal to the deletion breakpoint at FL 0.39. Bands scored are indicated by arrows to the right. The middle band in panel A was on chromosome 7B, and the upper bands in panels B and D were on chromosome 7D. M, HyperLadder V size marker from Bioline; P1, resistant parent Israel 493; P2, susceptible parent RAC875-2; CS, wheat cultivar Chinese Spring; NA, cytogenetic stock N7AT7D (nullisomic for chromosome 7A, tetrasomic for 7D); NB, cytogenetic stock N7BT7A (nullisomic for 7B, tetrasomic for 7A); ND, cytogenetic stock N7DT7B (nullisomic for 7D, tetrasomic for 7B); DS, ditelosomic stock DT7AS (missing the long arm of chromosome 7A); DL, ditelosomic stock DT7AL (missing the short arm of 7A); S8, deletion stock 7AS-8 (missing part of the short arm of chromosome 7A); and L1, deletion stock 7AL-1 (missing part of the long arm of 7A).

Attempts to Develop Improved Primers for Simple-Sequence Repeat Loci

Sequencing of the polymorphic bands produced by the susceptible (RAC875-2) and resistant (Israel 493) parents of the *Stb3* mapping population at locus *Xwmc83* showed that the alleles were very different except for five copies of a TG microsatellite near the first primer region (Fig. 4A). The sequence from which the original primers were

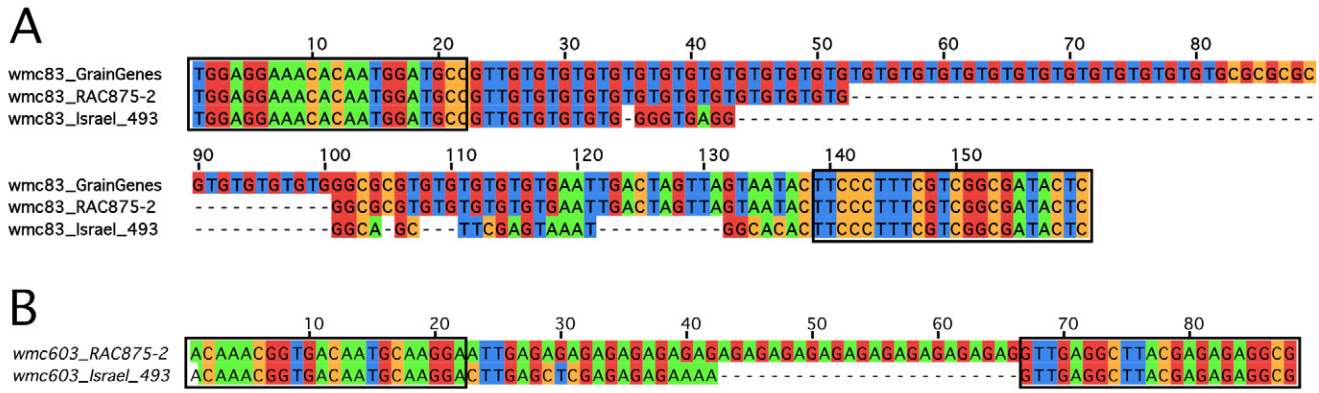

Figure 4. Alignment of DNA sequences of the alleles from the susceptible (RAC875-2) and resistant (Israel 493) parents of the *Stb3* mapping population at simple-sequence repeat loci (A) *Xwmc83* and (B) *Xwmc603*. Gaps are indicated by dashes. Black boxes at the beginning and end of each alignment indicate the primer sequences used to generate the amplification products that were sequenced.

made (downloaded from GrainGenes) contained a complex microsatellite at this locus, with 29 copies of a TG repeat, followed by four copies of CG, five more of TG, 6 bp of GGCGCG, followed by six more copies of TG. In contrast, the allele at this locus in the susceptible parent RAC875-2 had a 48-bp deletion that reduced the first TG microsatellite to only 14 repeats and eliminated the internal CG and TG repeats (Fig. 4A). The corresponding allele in Israel 493 was very different from those in the other two cultivars with the first TG microsatellite truncated to only five copies and the other repeats eliminated (Fig. 4A). Sizes of the alleles also varied dramatically, from 160 bp in the sequence from GrainGenes to 112 bp for that from RAC875-2 and only 88 bp for the allele from Israel 493. The parental bands for locus *Xwmc603* were cloned and sequenced as well and were also very different except for the primer sequences (Fig. 4B). Extension of the primer sequences for both loci by 1 to 5 bp reduced the number of bands present, but the results were not always better than using the original primers (results not shown).

Validation of the Map Location

Marker analysis identified nine BC_1F_2 plants that were homozygous for the *Xwmc83* allele linked to the resistance gene in Israel 493 and six that were homozygous for the alleles from the susceptible parents Clear White and UC1110. Between six and eight BC_1F_3 progeny from each BC_1F_2 plant were successfully scored for disease phenotype. Mean disease severities of progeny from plants homozygous for the Israel 493 (resistant) allele at locus *Xwmc83* ranged from 0.5 to 1.4, while those for progeny from plants homozygous for the alleles from the susceptible parents ranged from 1.4 to 3.6. The corresponding values for the resistant (Israel 493) and susceptible (UC1110 and Clear White) parents were 0.25, 3.4, and 3.2, respectively. Mean pycnidial density values ranged from 0.0 to 0.88 for progeny from plants that were homozygous for the Israel 493 allele and from 0.9 to 3.7 for progeny from plants that were homozygous for the *Xwmc83* allele from the susceptible parents. Corresponding scores for the parents were 0.0, 3.1, and 2.2 for Israel 493, UC1110, and Clear White, respectively. A plot of disease severity versus pycnidial density values gave good separation between resistant and susceptible progeny sets and confirmed that *Xwmc83* genotype gives excellent predictions of disease phenotypes (Fig. 5A), as expected by its close linkage with *Stb3*. With the exception of the BC_1F_3 progeny line B1, all lines with pycnidial density greater than 0.5 were homozygous for the susceptible-linked allele at locus *Xwmc83*; those with pycnidial density lower than 0.5 were always homozygous for the Israel 493 allele.

Analysis of the combined scores revealed a similar pattern. All lines with combined scores of 2.25 or lower were homozygous for the Israel 493 allele at *Xwmc83*; those with combined scores of 2.63 or higher were homozygous for the susceptible allele (Fig. 5B).

DISCUSSION

Analysis of additional molecular markers on wheat chromosome 6D revealed none that were linked to resistance locus *Stb3*. While a lack of detectable linkage with markers on the same chromosome is possible if the loci are far enough apart to appear unlinked, all the tested loci were chosen because they were known to be near the previously published location for *Stb3*. Lack of linkage with so many markers from the same location is highly unlikely. Therefore, we conclude that the previously reported location for *Stb3*, on the short arm of wheat chromosome 6D, was not correct. Assignment of *Stb3* to the 6DS location was based on a single SSR locus (Adhikari et al., 2004) in one mapping population and therefore may have been the result of spurious linkage.

Finding the correct location for *Stb3* required multiple approaches owing to the relatively low level of polymorphism between the parents of the mapping population. Scanning the genome with 251 additional SSR markers identified 27 polymorphic loci, but analysis of the

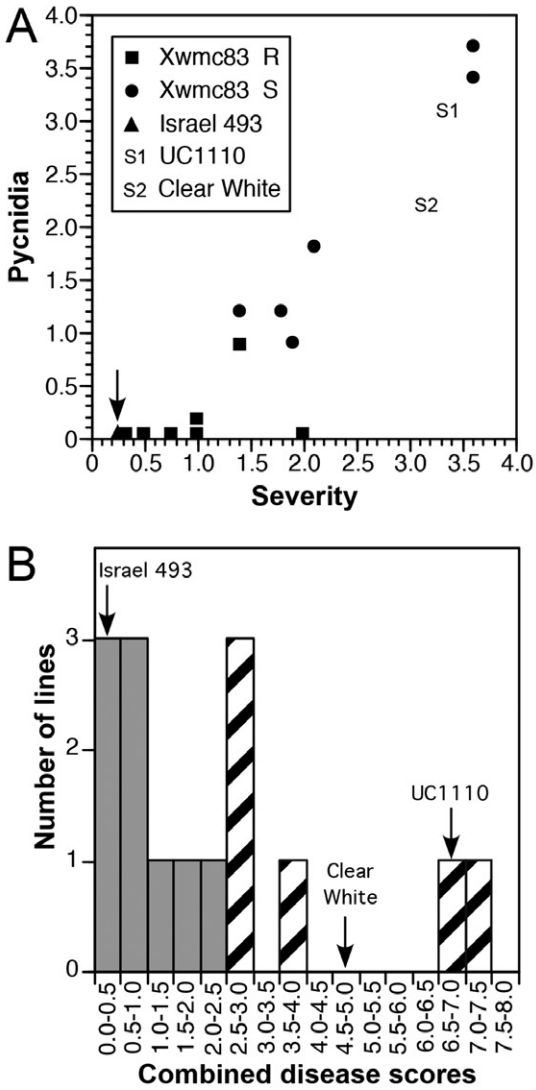

Figure 5. Validation of the *Xwmc83* marker for linkage to *Stb3* in BC_1F_3 progeny sets. (A) A plot of disease severity versus pycnidial density averaged over 6 to 8 plants of each BC_1F_3 progeny set showed a separation between lines that were homozygous for the Israel 493 (resistant) allele at locus *Xwmc83* compared with those that were homozygous for the alleles from the susceptible parents. The vertical arrow points to the solid triangle for Israel 493. (B) A histogram of the combined disease severity and pycnidial density scores also showed a separation between lines with and without the resistance and susceptibility markers. Solid bars: BC_1F_3 progeny lines homozygous for the *Xwmc83* allele from Israel 493; hatched bars: BC_1F_3 progeny lines homozygous for the allele from the susceptible parents. Vertical arrows point to the scores for the resistant parent Israel 493 and the susceptible parents UC1110 and Clear White.

individuals comprising each bulk in the bulked segregant analysis revealed no linkage between these markers and the resistance gene. Fortunately, the TRAP analysis (Hu and Vick, 2003; Li et al., 2006) identified two possible genomic locations (3A and 7A), one of which was confirmed as the chromosome containing *Stb3*. An advantage of TRAP analysis is that it scans multiple regions of a genome and is particularly useful when polymorphism is low (Li et al., 2006). Once the location was narrowed down, testing of additional microsatellite loci identified linkages only with SSR markers on chromosome 7A. The missing 468-bp TRAP band in N3AT3B and N3AT3D may be the result of residual variation in the 7A region of this line, as reported previously for other nullisomic-tetrasomic lines (Devos et al., 1999).

The 7AS location of *Stb3* was further confirmed by linkage to the closest marker *Xwmc83* in blind tests in two independent BC_1F_3 segregating populations. *Stb3* and *Xwmc83* cosegregated in the original mapping population of 97 DH progeny lines so must be closely linked. On the basis of the size of the DH population it can be estimated with 95% confidence (Hanson 1959) that the completely linked loci *Stb3* and *Xwmc83* are 3 cM or less apart. We currently do not know whether *Xwmc83* is distal or proximal to *Stb3*. On the proximal side of *Stb3*, two additional loci, *Xbarc222* and *Xbarc174*, were within 3.3 cM, providing good alternative markers for populations where *Xwmc83* is not polymorphic. On the distal side, *Xcfa2028* is the closest marker, but since it is 12.4 cM away from *Stb3*, there is a need to develop additional markers in this region.

Xwmc83 has the potential to serve as a good selectable marker for *Stb3*, but attempts to improve the primer sequences for this locus were not successful. Amplification at this locus often was faint, and in Chinese Spring the primers amplified two bands, the correct one on chromosome 7A and a larger one located on 7D, on the basis of analysis of nullisomic-tetrasomic lines (Fig. 3A). Therefore, some care is needed when assaying this locus with the current primers to be certain that the amplification is from the correct location. Because of the complex sequence of this locus (Fig. 4A), improving the primers will require extending the sequence outward from the existing primers rather than inward.

The location of *Stb3* on the short arm of chromosome 7A indicates that it is different from all other genes for STB resistance that have been identified to date, because so far no others have been mapped to this chromosome in bread wheat. Interestingly, very few genes for resistance to any pests or pathogens have been mapped to wheat chromosome 7AS, with the exceptions of a gene for greenbug resistance (Boyko et al., 2004) and several quantitative trait loci for resistance to Fusarium head blight (Jayatilake et al., 2011). Most of the resistance genes now on 7A were transferred to bread wheat from other species. For example, leaf rust resistance genes *Lr19* and *Lr47* were transferred from *Thinopyrum ponticum* (Zhang et al., 2005) and *Aegilops speltoides* (Dubcovsky et al., 1998) to the short arms of chromosome 7A in durum and bread wheat, respectively. The reason for this apparent paucity of resistance genes on wheat chromosome 7A is not known.

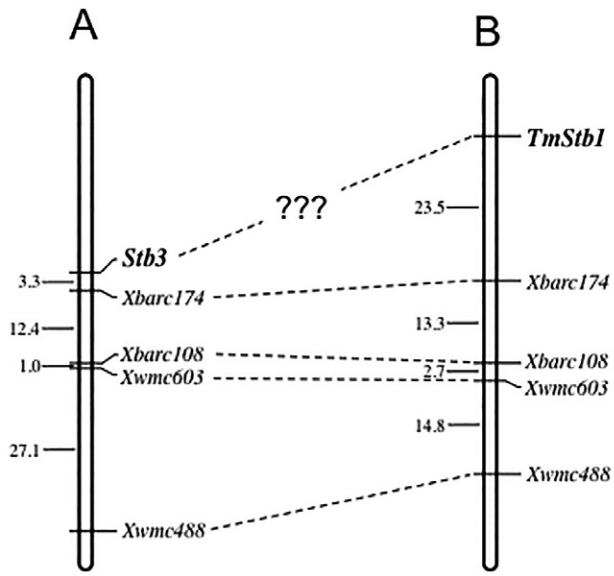

Figure 6. Comparative map locations for (A) *Stb3* from Israel 493 and (B) *TmStb1* from *Triticum monococcum* on wheat chromosome 7A. Both resistance genes are indicated in bold. Names of simple-sequence repeat loci begin with *X*. Distances in centiMorgans and genetic loci are indicated to the left and right of each map, respectively. Dashed lines connect the locations of each marker from one map to the other. Map A is a simplified form of the *Stb3* map in Fig. 2. Map B is adapted from Fig. 3c of Jing et al. (2008).

Another gene for resistance to STB, *TmStb1*, was mapped to the short arm of chromosome 7A in the diploid wheat relative *T. monococcum* (Jing et al., 2008), and the linkage map containing *TmStb1* has five SSR loci in common with that for *Stb3* (Fig. 6). On the basis of their relative distances to common marker *Xbarc174*, it appears that *TmStb1* is approximately 20 cM closer to the telomere than is *Stb3*. The distance between *Xbarc174* and *Stb3* is only 3.3 cM, compared with a distance of 23.5 cM between this SSR locus and *TmStb1* (Fig. 6). Other distances on the two maps are similar, although that between loci *Xwmc488* and *Xwmc603* is about twice as large in the *Stb3* map (27.1 cM), compared with that in the *TmStb1* map (14.8 cM). Because they originated from different species and map to apparently distinct locations, both genes appear to be unique. However, since genetic distances can vary in different populations, an allelism test or a more precise mapping of both genes is required to demonstrate that they are different. An allelism test will be complicated in this particular case because A-genome chromosomes from hexaploid wheat recombine poorly with the homoeologous *T. monococcum* chromosomes in the presence of the *Ph1* gene (Dubcovsky et al., 1995). Therefore, a more detailed comparative map of these two regions may be the best strategy for answering this question.

Wheat cultivar Israel 493, the original source of *Stb3* (Wilson, 1979), has been reported to contain three isolate-specific resistances to different isolates of *M. graminicola* (Chartrain et al., 2004). Unfortunately, it is not possible to know which of these genes, if any, corresponds to the original *Stb3*, because different isolates were used during phenotypic testing in each analysis. *Stb3* was identified originally from phenotypes scored in the field in Australia, but since no isolates were obtained or saved from that study, a subsequent definitive identification of the original gene is not possible. Because the mapping population for the current analysis was phenotyped using an isolate from Australia (Adhikari et al., 2004) and it is the only STB gene from Israel 493 to be mapped so far, it can be considered as a new standard for *Stb3*. In future analyses, it would be interesting to test whether the Israel 493 × RAC875-2 DH population segregates for additional STB resistance genes when screened with different isolates of *M. graminicola*.

Cytogenetic stocks were instrumental in determining the physical location of *Stb3* and for clarifying the results of the linkage analysis. Two alternative orders were detected for the markers from *Xwmc603* to *Xgwm260* that differed less than LOD 2. The selected order (Fig. 2) was the one that assigned the markers to the short and long arms correctly. The alternative order had all of the loci from *Xwmc603* to *Xgwm260* on the long arm of chromosome 7A. However, analyses of the cytogenetic stocks showed that locus *Xgwm260* clearly was on the short arm, in agreement with the order with the higher LOD score.

Use of the sheath-inoculation method simplified the phenotyping of the BC_1F_3 progeny plants. One of the difficulties with the usual spray-inoculation method is the need to maintain high humidity and moderate temperatures during the first 2 to 3 d after inoculation to allow for good infection. Injection of spores into the leaf sheath of the developing whorl allows the plant itself to provide protection for the growing fungal spores and provides conditions that are conducive for infection. The physical damage caused by the needle did not increase susceptibility of resistant lines, as they remained immune. Although this technique requires more time for a large number of plants compared with spray inoculation, the advantage of more uniform environmental conditions for infection makes it ideally suited for testing small numbers of plants.

The revised and validated map location for *Stb3* and linked molecular markers provide the tools to deploy this resistance gene in breeding programs. Since this is a wheat resistance gene, there are no expected restrictions on recombination that would hinder the separation of *Stb3* from any putative deleterious linkages identified in the future. If *Stb3* and *TmStb1* are demonstrated to be different genes, it could prove valuable to combine them on a single chromosome. This would require crosses using the *ph1b* mutation to enable recombination between the common wheat and *T. monococcum* chromosomes (Dubcovsky et al., 1995; Luo et al., 2000). Such a combination could increase the strength and durability of the resistance compared with what either gene provides in isolation and could help to reduce the damage caused by the devastating STB disease.

Acknowledgments

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