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ORIGINAL PAPER

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Genomic analysis and marker development for the *Tsn1* locus in wheat using bin-mapped ESTs and flanking BAC contigs

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Abstract The wheat *Tsn1* gene confers sensitivity to the host-selective toxin Ptr ToxA produced by the tan spot fungus (*Pyrenophora tritici-repentis*). The long-term goal of this research is to isolate *Tsn1* using a positional cloning approach. Here, we evaluated 54 ESTs (expressed sequence tags) physically mapped to deletion bin 5BL 0.75–0.76, which is a gene-rich region containing *Tsn1*. Twenty-three EST loci were mapped as either PCR-based single-stranded conformational polymorphism or RFLP markers in a low-resolution wheat population. The genetic map corresponding to the 5BL 0.75–0.76 deletion bin spans 18.5 cM and contains 37 markers for a density of 2 markers/cM. The EST-based genetic map will be useful for tagging other genes, establishing colinearity with rice, and anchoring sequence ready BAC contigs of the 5BL 0.75–0.76 deletion bin. High-resolution mapping showed that EST-derived markers together with previously developed AFLP-derived markers delineated *Tsn1* to a 0.8 cM interval. Flanking markers were used to screen the Langdon durum BAC library and contigs of 205 and 228 kb flanking *Tsn1* were assembled, sequenced, and anchored to the genetic map. Recombination frequency averaged 760 kb/cM across the 228 kb contig, but no recombination was observed across the 205 kb contig resulting in an expected recombination frequency of more than 10 Mb/cM. Therefore, chromosome walking within the *Tsn1* region may be difficult. However, the sequenced BACs allowed the identification of one microsatellite in each contig for which markers were developed and shown to be highly suitable for marker-assisted selection of *Tsn1*.

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Introduction

Tan spot, caused by fungus *Pyrenophora tritici-repentis* (Died.) Drechs, is an economically important disease of common wheat (*Triticum aestivum* L., $2n=6x=42$, AABBDD genomes) and durum (*T. turgidum* L., $2n=4x=28$, AABB genomes) in many wheat-growing areas throughout the world. Characteristic symptoms of this disease include tan necrosis and extensive chlorosis, which are genetically independent (Lamari and Bernier 1991). Severe disease during grain filling can result in significant yield losses because severe spotting reduces the photosynthetic area of the upper leaves.

P. tritici-repentis races are known to produce host selective toxins (HSTs) that induce necrosis or chlorosis in sensitive wheat genotypes (DeWolfe et al. 1998). Ptr ToxA was the first HST produced by *P. tritici-repentis* to be described and well characterized (Tomás and Bockus 1987; Ballance et al. 1989; Touri et al. 1995; Zhang et al. 1997). This toxin causes necrosis when infiltrated into leaves of sensitive wheat genotypes, while insensitive wheat genotypes have no reaction to the toxin. Insensitivity to Ptr ToxA is conditioned by a single recessive gene in wheat (Lamari and Bernier 1989), which has been designated *tsn1* (Faris et al. 1996). Anderson et al. (1999) indicated that chromosome deletion lines without the *tsn1* locus were also insensitive to Ptr ToxA. The *Tsn1* allele was a major factor responsible for the symptom of tan necrosis in disease development, while

wheat genotypes possessing the *tsn1* allele were resistant to necrosis of tan spot (Lamari and Bernier 1989). Therefore, the *Tsn1* gene plays an important role in host–pathogen interaction and the product of *Tsn1* is actively involved in the susceptibility of the disease.

Because of the importance of *Tsn1* in the host–pathogen interaction and disease susceptibility, it is desirable to isolate the gene by positional cloning. Saturation mapping and molecular tagging of *Tsn1* is an essential prerequisite to map-based cloning. Faris et al. (1996) mapped *Tsn1* on the long arm of chromosome 5B and identified RFLP markers flanking it at 5.7 and 16.5 cM. Later, Faris et al. (2000) placed the gene on the wheat physical map in the deletion bin 5BL 0.75–0.76 and delineated *Tsn1* to a 4.0 cM interval using RFLP markers. More recently, Haen et al. (2004) delineated *Tsn1* to a 0.8 cM interval using amplified fragment length polymorphism (AFLP) markers in combination with bulked segregant analysis (AFLP-BSA). The AFLP markers were cloned and converted to RFLP markers.

The size of the haploid wheat genome is about 17,300 Mb (Bennett and Leitch 1995) and known to contain an abundance (80%) of repetitive sequences (SanMiguel et al. 2002; Wicker et al. 2001), which makes chromosome walking difficult. Therefore, it is highly desirable to identify more markers closely linked to *Tsn1* using additional resources. Expressed sequence tags (ESTs) are short sequences derived from expressed genes. Over 600,000 ESTs have been generated from wheat and closely related species (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html), and more than 16,000 EST loci have been physically mapped to specific regions of wheat chromosomes using deletion lines (Qi et al. 2004). Therefore, the EST sequence and bin-mapping data provide a powerful tool for direct selection of markers for saturation mapping of target genes.

The objectives of this study were to estimate the genetic order and recombination frequencies among EST loci within the deletion bin 5BL 0.75–0.76, to further saturate the *Tsn1* region with markers, and to determine the feasibility of isolating *Tsn1* using positional cloning methods by evaluating recombination frequencies across flanking BAC contigs.

Materials and methods

Plant materials

A common wheat population consisting of 117 recombinant substitution lines (RSLs) derived from a cross of Chinese Spring (CS) with a CS-*T. dicoccoides* chromosome 5B disomic substitution line (CS-DIC 5B, i.e., the CS 5B chromosome pair was replaced by a pair of *T. dicoccoides* 5B chromosomes) was used to determine the genetic order of ESTs within deletion bin 5BL 0.75–0.76. The procedure of developing the population was described in Gill et al. (1996). Both CS and CS-DIC 5B are insensitive to Ptr ToxA so the population does not segregate for *Tsn1*. This population was used for genetic mapping here because a high-density map between breakpoints 0.75 and 0.79 has been constructed with RFLP markers, and the RFLP markers flanking *Tsn1* have been identified (Faris et al. 2000).

A durum wheat F_2 population derived from Langdon (LDN)×LDN-DIC 5B was used for high-resolution mapping of markers found to be tightly linked to *Tsn1* in the CS×CS-DIC 5B population. Nearly 11,000 F_2 plants derived from LDN×LDN-DIC 5B were screened for reaction to Ptr ToxA as described in Haen et al. (2004), and 2,719 Ptr ToxA-insensitive F_2 plants were selected and used for mapping because they are homozygous for the *tsn1* allele.

The utility of SSR markers developed in this research was evaluated using *T. aestivum* cultivars CS, Alsen, BR34, Grandin, Atlas 66, Erik, Bobwhite, Kulm, and Opata 85. The CS aneuploid nullisomic for chromosome 5B and tetrasomic for chromosome 5D (N5BT5D) was used as a negative control.

EST marker analysis

Sequences of ESTs that were physically mapped within deletion bin 5BL 0.75–0.76 by the wheat NSF-EST project were downloaded from http://www.wheat.pw.usda.gov/index.shtml. ESTs were mapped as either single-stranded conformational polymorphism (SSCP) or RFLP markers. For SSCP, which relies on conformational intrastrand differences in DNA to detect polymorphism between PCR-amplified fragments, PCR primers were designed on the basis of the EST sequences using the software Primer 3 (Rozen and Skaletsky 2000) and validated using the software Amplify 1.2 (University of Wisconsin-Madison, Genetics, WI). Expected sizes of PCR products ranged from 300 to 700 bp; however, the sizes could be larger than expected as an amplified region may contain intron(s). For PCR amplification, a 20 µl reaction mixture contained 2 µl of 10× buffer, 2 mM of $MgCl_2$, 0.25 mM each of dATP, dCTP, dGTP and dTTP, 400 nM of each primer, 1 unit of Biolase DNA polymerase (Bioline Inc., London) and 200 ng of genomic DNA as template. PCR conditions were 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 55, 53, or 60°C (depending on annealing temperature of the primer pair) for 1 min, and 72°C for 2 min, followed by 72°C for 7 min. The PCR product was mixed with 25 µl loading dye (95% formamide, 0.02 M EDTA, 9.9 mM NaOH, 0.25% w/v bromophenol blue, and 0.025% w/v xylene cyanol) and 5.5 µl was loaded onto a gel consisting of 20 ml MDE® gel solution (Cambrex Bio Science Rockland, Inc. Rockland, ME), 4.8 ml 10× TBE, 55.2 ml ddH_2O, 320 µl 10% APS (ammonium persulfate) and 32 µl Temed. Gels were run at 4 W for 12 h and stained with SYBR Green II (Sigma, St. Louis) for 30 min. The image was then scanned into a

computer using a Typhoon 9410 variable mode imager (GE Healthcare, Waukesha, WI).

For those EST markers that were monomorphic between the parents in SSCP analyses or whose primers amplified no products in PCR, RFLP analyses were employed. The plasmids having EST inserts were kindly provided by B.S. Gill (Kansas State University, Manhattan, KS). The inserts were PCR-amplified using standard M13 primers, purified, and used as probes for hybridization. Probes were tested for polymorphism between parents digested with the restriction enzymes *Apa*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I, *Sca*I, and *Xba*I. Those probes revealing polymorphism were used for mapping. The procedures for DNA extraction, Southern blotting, labeling, hybridization, and membrane washing were previously described (Faris et al. 2000). After washing, membranes were exposed to phosphorimaging screens for 3–12 h and scanned using a Typhoon 9410 variable mode imager (GE Healthcare, Waukesha, WI).

Construction of genetic maps

The computer program Mapmaker (Lander et al. 1987) version 2.0 for Macintosh was used to calculate linkage distances using the Kosambi mapping function (Kosambi 1944) and a LOD score of 3.0. Those markers whose LOD scores were below 3.0 were placed in the most plausible locations on the map.

Identification, sequencing and analysis of BAC clones

The LDN durum BAC library (Cenci et al. 2003) was screened using RFLP probes FCG17 and FCG9, which were described in Haen et al. (2004). Plasmid DNA was isolated from positive BAC clones using standard protocols. BACs were fingerprinted by digesting 20 μg of each plasmid with *Hin*dIII followed by separation of the fragments on 0.9% agarose gels. The gels were then stained with ethidium bromide, photographed, and visualized for overlapping fragments. The same gels were Southern blotted and hybridized with the probe used to identify the clone initially. The resulting autoradiograph images were used to identify BACs belonging to chromosome 5B by comparing the size of the hybridizing fragment with polymorphic *Hin*dIII fragments generated by the same probe and mapped in the LDN×LDN-DIC 5B population.

Two minimally overlapping BACs detected by each flanking probe were selected for sequencing. BACs 378P21 (AY914085), 1154L7 (AY914086), and 404J6 (DQ157837) were sequenced by Myriad Genetics, Inc. (Salt Lake City, UT), and BAC 533E21 was sequenced as described in Faris et al. (2003). BAC sequences were submitted to the Rice Genome Automated Annotation System (RiceGAAS; http://www.ricegaas.dna.affrc.go.jp/) and surveyed for putative low-copy sequences near the ends of the BACs. Primers for low-copy sequences were designed using MacVector 7.2 (Accelrys, San Diego, CA) and used to PCR amplify low-copy fragments from BAC clones. The PCR-amplified fragments were separated on 2% agarose gels, purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and used as RFLP probes for mapping.

BAC sequences were scanned for SSRs using MICAS-Microsatellite Analysis Server (http://www.210.212.212.7/MIC/). Primers flanking SSRs were designed using MacVector 7.2 (Accelrys, San Diego, CA). SSRs were PCR-amplified following the protocol of Röder et al. (1998) except that the annealing temperatures varied according to the TM of the primers. Amplified fragments were separated on 2% agarose gels, stained, and photographed, or separated on 6% nondenaturing polyacrylamide gels, stained with Sybr Green II (Sigma, St. Louis, MO), and visualized with a Typhoon 9410 variable mode imager (GE Healthcare, Waukesha, WI).

Results

EST mapping

A total of 54 unique ESTs were physically mapped in the deletion interval 5BL 0.75–0.76 (http://www.wheat.pw.usda.gov/index.shtml) by the wheat NSF-EST project. PCR primers developed from the 54 ESTs (Table 1) were tested for their ability to amplify fragments from CS and CS-DIC 5B. Fifty-one primer pairs (94%) successfully amplified products. Primers for ESTs BM138094, BM138151, and BM140357 failed to amplify fragments. None of the PCR products revealed polymorphisms when separated on 2.0% agarose gels. However, 16 (30%) of 51 markers showed polymorphism between the parents in the SSCP analysis and 12 of these behaved as codominant markers (Fig. 1). The 16 EST-SSCPs were subsequently evaluated on the entire CS×CS-DIC 5B RSL population and used for mapping (Fig. 2). The remaining 38 ESTs (including BM138094, BM138151, and BM140357) were then used to develop RFLP probes. Seven of the EST-derived probes detected RFLPs between the parents and were subsequently mapped. Thus, a total of 23 ESTs (43% of 54 ESTs) were mapped in the CS×CS-DIC 5B RSL population.

The genetic map of the CS×CS-DIC 5B RSL population constructed here is 24.6 cM (Fig. 2). Of the 23 ESTs that were polymorphic between the parents, 19 were mapped in the population at a LOD > 3.00. The remaining four ESTs were placed at the most likely intervals. With one exception, each of 23 ESTs detected a single locus. EST BM138151 detected two loci, *XBM138151.1* and *XBM138151.2*, which we confirmed to map in deletion bins 5BL 0.75–0.76 and 5BL 0.76–0.79, respectively, using deletion lines 5BL-16, 5BL-9, and 5BL-14. These findings agree with those found by the wheat NSF-EST group in detecting two loci for

Table 1 ESTs mapped to deletion bin 5BL 0.75–0.76 by the wheat NSF-EST project, the primers used to evaluate them as SSCP markers, and the marker types used to map them in the CS×CS-DIC 5B population

Accession number	PCR primers	Annealing temperature	Marker type for mapping (enzyme)
BE398803	CTCTCCATCGCGCACCAGACCCGCT GAGTAGTAACTTCAGCGGCCTTAGT	55	Monomorphic
BE399111	GCACAACGATGATCGATCTCCAA CTGAGGAAGCTGAGGAAGGAGGT	55	Monomorphic
BE399939	TGCTTGTGCAGTCTGATCTCTATAC GGGCGAAAGAAGAAACAAATTTAGG	55	Monomorphic
BE403217	GTACAATGCTTATAATCGCTCACCG TCTACACCTAAATTTGATCCCAGTC	55	RFLP (*Eco*RI)
BE403702	TATAATCAGAACAGAGCCATCAACC CCTGACATATCAGTAGGCTGTGTAT	55	SSCP
BE403968	CGAGGCTTGGTTTTGGTTTTCTT GAGCTTTGAAGTGATTATCGAAATC	53	SSCP
BE404740	CTCCTCTCCAGATCCCGACCAT AACCTGCTCCAGAATGAATGCAA	55	Monomorphic
BE406507	GATGTACTGAACGAGGACACTGATT GGGTCACACCATGAGACGCAGA	55	Monomorphic
BE423505	GCAACAAGCAAGTCATTATTAAAAGGC GATATCATTTGGAATTGAAGCATCTG	53	SSCP
BE425878	CTCCTCCACGAGGCTTCCTGCGCGTG CGGCAGCGACCTAGAACTCATTT	60	SSCP
BE426161	ACCGAAAACTCATGTTACGTACTG GTGGATAGCTGGTTGACGTTCCCG	55	SSCP
BE426183	GTCGACGACGGAGAAACGGCCGC ATAGAACTAACACCACCCTCTACCA	55	Monomorphic
BE442858	GCAGGACGCCTTCTTCAGGGCGTT CTGAATGTGGCTAATATGGTCTTGC	55	Monomorphic
BE442978	GCCAGCTGATGATTTACATCTGCTT TACTGGAAACTGGATTTTGTTGGAG	55	SSCP
BE445619	GTGTGTGTCAGCTAATGGTATTACG GTGCTCTGACTTGTGTCACTGTATC	55	SSCP
BE445997	ACCCTAACCCTAACAACCAGTAGAT CTGCAGTTTCCTTTTGATTCTGTAT	55	Monomorphic
BE446301	GTAAGTGCATGCTTCGCTAAATTGT TTCAGTCACCACATACATTACAAGG	55	Monomorphic
BE446352	AAGCAGACCTGACTTCATCTGTAAA TCCTGTCCATATATGAAAGTAGTCG	55	Monomorphic
BE471131	CGGCTTCTTCTCTGCCTGCTGGTC GTCACTTGATCTATTCGATTTTGAG	53	SSCP
BE471214	GTTGACTCACGCCACTGTGCCTTC GCTCAGTCAACTCCTTATTCAGTTC	55	Monomorphic
BE488792	GGCCAGGTATATGGGTATAGGAG ACAACGCACCGTGAACAGCCCGT	55	RFLP (*Bgl*II)
BE490237	AGACCAAAACTAGGGGCTTTAATTC GGAGCAAATGTTTTAGCACAGAC	55	Monomorphic
BE497490	AGTTCGACGACGAGAAGATCCTGG CAGTGGTGTTAACAGGAAACAAAG	55	Monomorphic
BE499738	GCGTCGAAGATGAGGGAGTGCAT AGCCCTGCAGTCCAGTGCAGTTG	60	Monomorphic
BE500596	GACAATCAACACCAACACCATAG TCAATTAAAGTTGTCATCAAGCCC	55	Monomorphic
BE500658	GATGAGGTATGAAGAATCCATCTTG GTTGTACAAGGTGAAGAAGACACTG	53	SSCP
BE585769	CTTCTATTGAAGATGCTCGAAGTGT AAGTTTGAGCTCGCACATATTCTC	55	Monomorphic
BE590499	AGGCGGTAGTAACAAAGCCAGTA CGGAACATGAACCGTACGTTTTTAT	55	SSCP
BE591416	GTCAGTGTTAAGGAAGAGGTTGATG GCAAAAAGACTACCCACAGCTATTA	55	RFLP (*Xba*I)
BE591798	CGCAATGACATCTGTTGATCAGTTC CAGGCTATTATTATTGTCTTTCCG	53	SSCP
BF202652	CGTGATCGATTTAGGAAAGTATCTC GACTATATAAACTGCAAACCGTTCGCC	53	Monomorphic
BF203136	AGGAGCACAACTAAAGCACTACTTC GTAAGTGGATCAACTAATTGCAGTG	55	RFLP (*Bam*HI)

Table 1 (Contd.)

Accession number	PCR primers	Annealing temperature	Marker type for mapping (enzyme)
BF292996	CAAGTTTAGGACCAACCAAGGAT TTTTTAAACTAATGCCCGTGTTTAT	53	Monomorphic
BF293157	TCTCTCTTCCTCTCAAGTCTAACCA GCATTGGCAAGAACAAGTTTAGTG	55	SSCP
BF428724	AATCATCTGTCTTCAACACCAGTC AGTCGGATGCAGATGATGGATAG	55	Monomorphic
BF482522	CGAAAATGATTTCCATAACCCAACA CGCCAGCTATAATCTTGGTTGCAAT	55	Monomorphic
BF483248	GCTGTCATATGCTCCAGATTTTAT GGTCCATGCTTTATCATGTCAAA	53	Monomorphic
BF483357	GCCTTTCCGCTGCTAGTCTAGTCC AGAAACCACGCTCGTCACTTCAC	60	Monomorphic
BF483506	GTTCAAAAGAGCGAGTAAGCTAGT GATATCAGTGGTCCATGTCACAATA	55	SSCP
BF483510	TAACTGGAATGATGGGGGTCTTAAT GGTTGAACATATGGCACAAAAGGT	55	SSCP
BF484437	AGTGTCTCCGATTTCCTGAATGT ACTGTTAGAGACCGAACACGAGATA	55	RFLP (*Eco*RI)
BF484835	CTGGATTCTGCAGAGGTCCTTCCG AACATATATCCCAGGATCCTCAATC	55	SSCP
BF604547	GAGAGGGCTAGAGAAGGGAGAAG ATTTCTCCACGAAAAACATGCTTCG	55	Monomorphic
BG263083	GTATACACTTTCGAGTTTCGACACA GCTGACCATCAGGGACGTCAAGCC	55	Monomorphic
BG274124	GTAAATGTCCCAATTGATAACCAAG GTGATTACGAAAAGAGGAAACAGGT	53	Monomorphic
BG606115	AGTCTAAGTTGAGTCTTGCCCCTGT CCCTGAACGAAAAAGGAAAACACT	55	Monomorphic
BG608197	CTTCTCCCTCTTCGACAAGAAC CTTGTTTGACACAACAGGTCTCTG	55	SSCP
BI479122	AGAGAGAGGGAGGGAGAGAGAGAT GAGCTGAAATTTATTCGGTTGTATG	53	Monomorphic
BM134343	TTACGGGAGGCTCATTGCCTTAT TTTCCCTCTGCAAGAATCCAGTG	55	Monomorphic
BM138094[a]	GAATTTTGAAGCTCGATTAAAAAGG GACAATATGGTAGTCCAATGCTTTT	55	Monomorphic
BM138151[a]	GTTGTGCTCAAATTTATAGTTCCTGG CGCATCTTACAAGCTGCAAGTTATT	55	RFLP (*Eco*RI, *Xba*I)
BM140357[a]	GTTGAAATGATTGTCACACAAAATC TAAAATTGTGGAAAACAGCAAGGG	53	RFLP (*Eco*RV)
BM140591	ATCTCTCCTTCCTCCTCGCTCCT GAACGACGATCAGCAGCAAGTTT	55	Monomorphic
BQ283386	GTCCTTAACTACCTGCCACCAAT AGATTAGGAACAAAGTGCCCATC	55	Monomorphic

[a]No amplification products were obtained using the primers indicated for these ESTs

BM138151; one within the 5BL 0.75–0.76 bin and one in the 0.76–0.79 bin.

Markers *XBM138151.1*, *XBE425878*, *Xfcg9*, *Xfcg10*, and *Xfcc1* cosegregated in the RSL population. *Xfcg9* was previously shown to map proximal to the 5BL-9 breakpoint, whereas *Xfcg10* and *Xfcc1* mapped distal to 5BL-9 (Haen et al. 2004). The wheat NSF-EST group indicated that BE425878 detected a locus proximal to the 5BL-9 breakpoint, but upon viewing the autoradiograph image, it was apparent that the data for 5BL-9 was somewhat ambiguous. Therefore, we tested BE425878 as an SSCP marker on the deletion lines and found that *XBE425878* was located in the deletion interval 5BL 0.76–0.79.

Similarly, marker locus *XBG608197* mapped distal to the presumed 5BL-9 breakpoint on the genetic map, but the wheat NSF-EST group indicated that BG608197 detected a locus proximal to 5BL-9. We evaluated this marker on the deletion lines and found that it amplified fragments in 5BL-16, but not in 5BL-9 and 5BL-14. Therefore, the *XBG608197* marker identified in this study maps to the 5BL 0.76–0.79 deletion bin.

ESTs BE591416, BF484835, and BE471131 detected loci more than 16 cM proximal to *XBF483510* (data not shown). We tested these EST markers on deletion lines 5BL-16, 5BL-9, and 5BL-14, and found that the polymorphic fragments detected in the CS×CS-DIC 5B RSL population physically mapped proximal to the 5BL-14 deletion breakpoint (data not shown). Although the wheat NSF-EST project did not report loci for these ESTs proximal to 5BL-14, it is possible that they went undetected due to lack of intergenomic

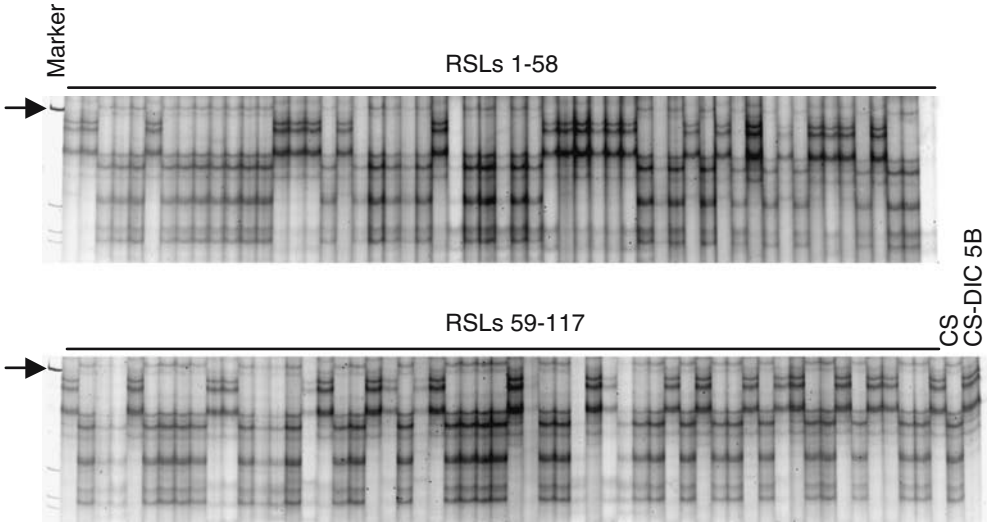

Fig. 1 Genotyping of the CS×CS-DIC 5B RSL population with EST marker *XBE403968* using SSCP analysis. *Arrows* indicate a 500 bp size marker

polymorphism and that the 5BL 0.75–0.76 deletion bin loci detected by these ESTs were monomorphic in our population.

Faris et al. (2000) placed 11 RFLP markers on the region of the CS×CS-DIC 5B RSL genetic map corresponding to the 5BL 0.75–0.76 deletion bin, and Haen et al. (2004) placed eight AFLP-derived markers. With the addition of the 18 EST markers mapped in this work, the linkage map corresponding to the 5BL 0.75–0.76 deletion bin now accounts for 18.5 cM and contains 37 markers for an average density of one marker per 0.5 cM. Based on comparative analysis of previously published maps (Faris et al. 1996, 2000; Haen et al. 2004), the *Tsn1* gene was located to a 2.6 cM interval near the distal end of the deletion bin.

High-resolution mapping

Two AFLP-BSA-derived markers, *Xfcg17* and *Xfcg9*, were previously shown by Haen et al. (2004) to flank *Tsn1* at 0.2 and 0.6 cM, respectively, in the high-resolution LDN×LDN-DIC 5B F_2 population, which consisted of 930 F_2s at the time of publication. Here, these markers were re-mapped in the now expanded LDN×LDN-DIC 5B population consisting of 2,719 F_2s, and flanked *Tsn1* at distances of 0.3 and 0.5 cM (Fig. 2).

XBF483506 and *XBM138151.1*, the EST markers identified in the present study, flanked *Tsn1* at 0.4 and 0.5 cM in the LDN×LDN-DIC 5B F_2 population. The EST marker *XBF483506* was 0.1 cM further from *Tsn1* than *Xfcg17* (Fig. 2). *XBM138151.1* and *Xfcg9* cosegregated in the high-resolution mapping population. As with the CS×CS-DIC 5B RSL population, the EST BM138151 detected a second locus in the LDN×LDN-DIC 5B population. However, high-resolution mapping was conducted using only plants with recombination events between markers *Xfcg6* and *Xfcc1*. Therefore, *XBM138151.2* cosegregated with *Xfcc1* among the recombinants of the LDN×LDN-DIC 5B population because it likely maps distal to *Xfcc1*.

BAC contig analysis

Probe FCG17 detected BACs 378P21 and 1154L7, which were 126 and 139 kb in size, respectively. Alignments of the two BACs indicated they shared about 60 kb of overlapping sequence. Therefore, these two BACs formed a contig spanning about 205 kb (Fig. 3). Sequence analysis of 378P21 indicated the presence of a putative subtilisin-like serine protease gene approximately 6 kb from the nonoverlapping end of the BAC. We amplified a 518 bp fragment of the putative gene from 378P21 and used the amplified product as a probe for mapping in the high-resolution LDN×LDN-DIC 5B population where the marker, designated as *Xfcg21*, cosegregated with *Xfcg17*.

The first 8 kb of the nonoverlapping end of BAC 1154L7 had similarity to the wheat retrotransposon Tar1 (data not shown), but the adjacent 2 kb of sequence had no similarities to anything. Therefore, we amplified a 510 bp segment of this putative low-copy region and developed the RFLP marker *Xfcg22*. Upon mapping of *Xfcg22* in the LDN×LDN-DIC 5B population we found that it, too, cosegregated with *Xfcg17* (Fig. 3). Therefore, no recombination events were observed across 191 kb in 5,438 gametes. This indicates that recombination is highly suppressed in this region and results in a physical to genetic distance ratio of greater than 10 Mb/cM.

Probe FCG9 hybridized to overlapping BACs 533E21 and 404J6, which were about 93 and 153 kb in size, respectively, and had about 18 kb of overlapping sequence. Therefore, these two BACs formed a contig of about 228 kb. Sequence analysis of these BACs revealed putative low-copy sequences near both ends of the

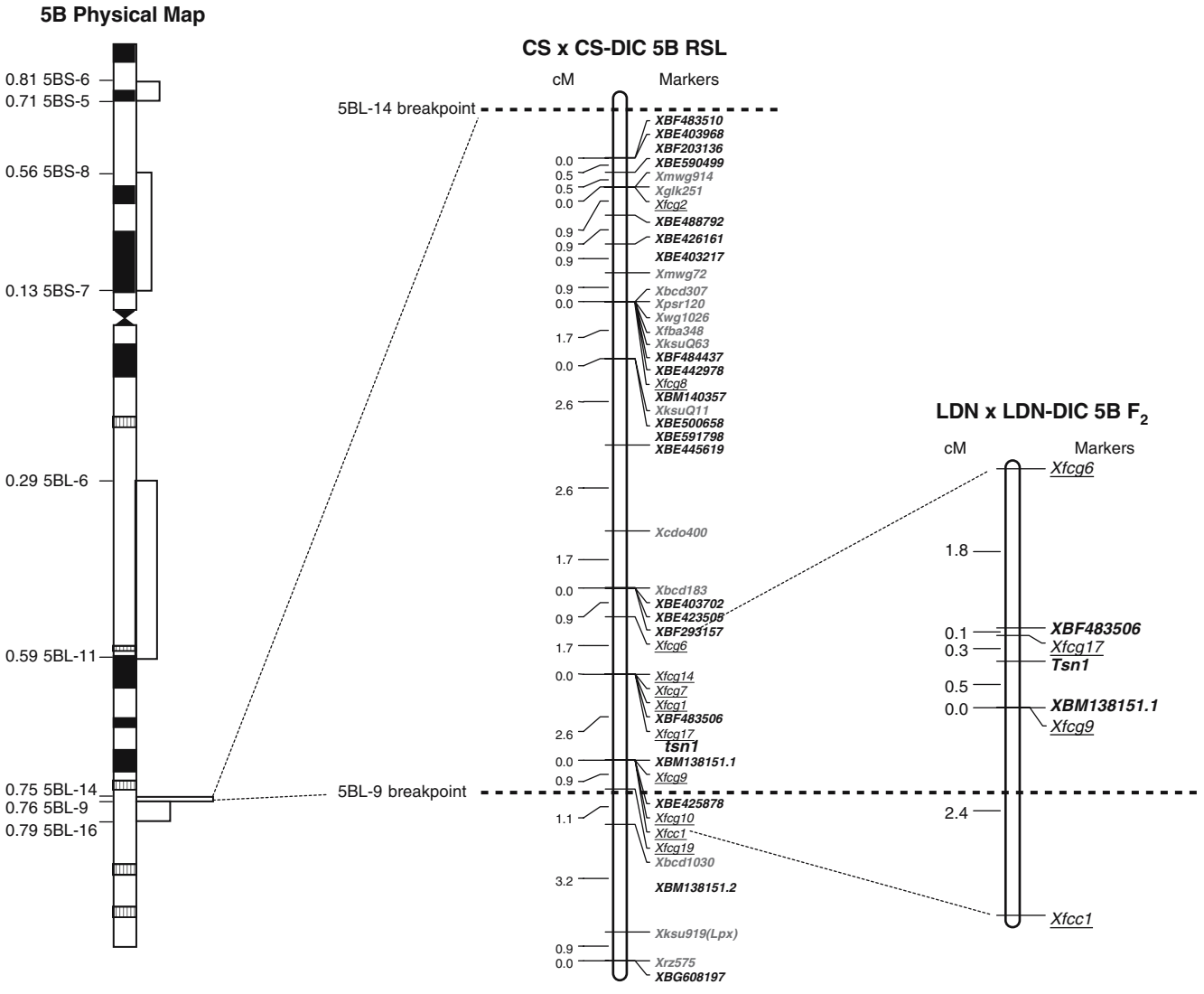

Fig. 2 Genetic linkage maps constructed from the low-resolution CS×CS-DIC 5B RSL population (*middle*) and from the high-resolution LDN×LDN-DIC 5B F_2 population at the *Tsn1* locus (*right*). A physical map showing fraction breakpoints for chromosome deletion lines and the location of the 5BL 0.75–0.76 deletion bin is shown for reference (*left*) (Gill et al. 1996). RFLP markers (Faris et al. 2000) are shown in *gray*, AFLP-BSA derived markers (Haen et al. 2004) are *underlined*, and EST-derived markers (this research) are in *bold*

contig. Fragments of 589 and 558 bp were each amplified approximately 2 kb from the nonoverlapping ends of 404J6 and 533E21 and used to develop the markers *Xfcg23* and *Xfcg24*, respectively. *Xfcg24* mapped 0.02 cM closer to *Tsn1* than *Xfcg9* in the LDN×LDN-DIC 5B population, and *Xfcg23* mapped 0.28 cM distal to *Xfcg9* (Fig. 3). Therefore, the physical to genetic distance ratio across this contig is approximately 760 kb/cM, which is about a 13-fold increase in recombination frequency compared to the proximal contig.

The sequence for EST marker *XBM138151*, which cosegregated with *Xfcg9* in the high-resolution mapping population, was aligned with sequences for BACs 533E21 and 404J6. The EST aligned with 95% identity to a sequence about 9 kb from the nonoverlapping end of 533E21, which is about 70 kb closer to *Tsn1* than *Xfcg9*. Therefore, the single recombination event that occurred between *XBM138151* and *Xfcg24* occurred within a 7 kb segment while no recombination event occurred along the 70 kb between *XBM138151* and *Xfcg9*.

Development of SSR markers

The entire 433 kb of BAC sequence generated in this work was scanned for SSRs. Two SSRs were identified and used to develop markers *Xfcp1* and *Xfcp2* (Table 2). The SSR marker *Xfcp1* is located on BAC 1154L7 approximately 11 kb from *Xfcg17*, and *Xfcp2* is about 57 kb distal to *Xfcg9* on BAC 404J6 (Fig. 3). Therefore, *Xfcp1* and *Xfcp2* delineate *Tsn1* to an interval of approximately 1 cM.

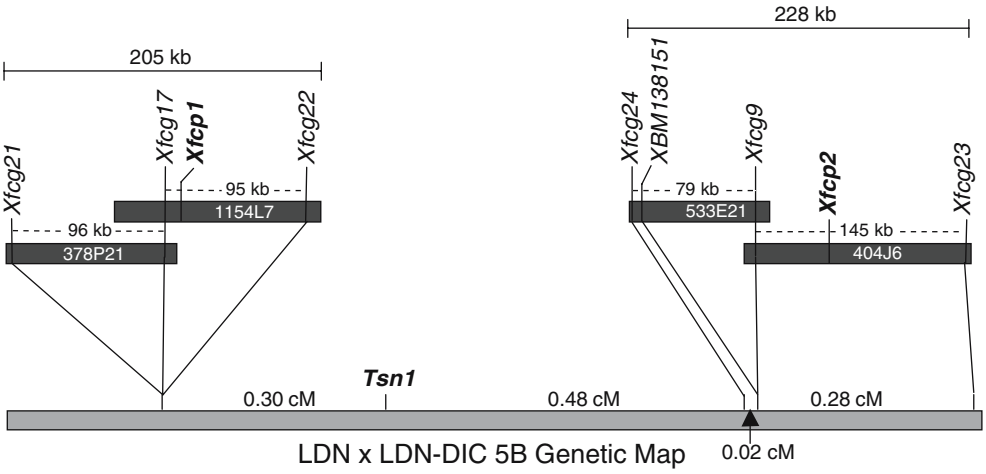

Fig. 3 Physical map of the BAC contigs flanking the *Tsn1* locus anchored to the high-resolution genetic map constructed in the LDN×LDN-DIC 5B population of 2,719 F_2 plants. Names of the SSR markers are indicated in *bold*, and their approximate physical locations on the BAC contigs are given. Note that the orientation of the 205 kb contig is unknown because no recombinants between *Xfcg21* and *Xfcg22* were identified

Table 2 SSRs developed from sequenced BAC contigs for genotyping and marker-assisted selection of *Tsn1*

Locus	PCR primers	Repeat	Annealing temperature	LDN (bp)
Xfcp1	ATAACTCCGTCACGACCACCTCCTCTCAAG CAGTCTGAAAACGCCATACCCG	(GT)44	65	402
Xfcp2	GTGAGCCCTGGCTGCCTACTTATCTCACTCT GTAGGCATTTGAAGATGAGGTAGCAC	(GC)9 (AC)16	67	498

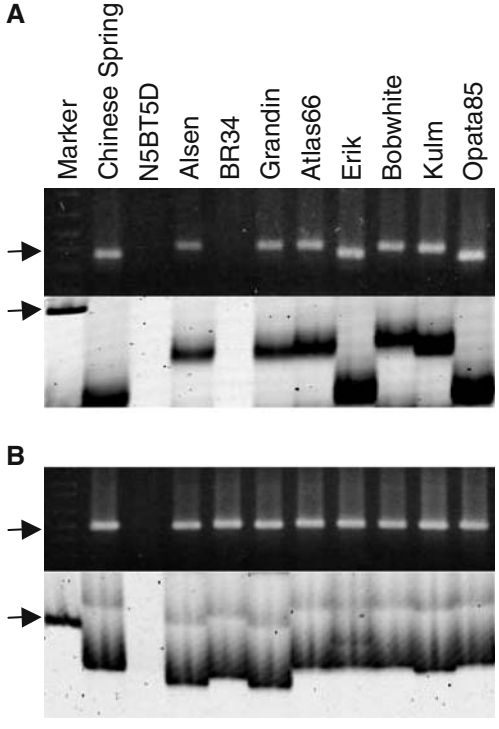

Fig. 4 PCR amplifications of SSR loci *Xfcp1* (**a**) and *Xfcp2* (**b**) in ten wheat genotypes. PCR reactions were electrophoresed on standard 2% agarose gels (*top panels*) and 6% nondenaturing polyacrylamide gels (*bottom panels*). *Arrows* indicate location of 500 bp marker in each gel

Evaluation of *Xfcp1* and *Xfcp2* on ten wheat genotypes including N5BT5D indicated that they are highly specific to chromosome 5B. Variation at *Xfcp1* was sufficient to detect polymorphism among the ten genotypes by standard agarose gel electrophoresis (Fig. 4). In comparison, the variation at *Xfcp2* was much less and polymorphism could be detected only by polyacrylamide gel electrophoresis.

Discussion

The abundance of wheat EST sequence and bin-mapping data provide a valuable resource for genome analysis, comparative mapping studies, evolutionary studies, and saturation mapping and tagging of target genes. Every EST has the potential to be developed as a PCR-based or RFLP marker, and here we exploited the abundance of EST sequence and mapping data for saturation mapping and tagging of the *Tsn1* gene.

Our results show that EST-derived PCR markers are user-friendly and highly effective when used to detect SSCPs, and the primer sequences presented here will be useful to other researchers for mapping 5BL 0.75–0.76 bin-mapped ESTs in other populations. The SSCP gel system was originally developed for rapid analysis of mutations (Orita et al. 1989). This gel system could separate two single-stranded DNA fragments in which the nucleotide sequences differed at

only one position (Orita et al. 1989), maximizing the ability of a gel system to detect polymorphism. In our study, at least 53 of 54 genes evaluated existed in both CS and CS-DIC 5B. Thirty percent of the corresponding EST sequences were variable between the parents, and the variation was detectable by SSCP analysis but not by standard agarose gel electrophoresis. Therefore, the SSCP analysis provides a means of rapidly mapping EST sequences in wheat, and they should be effective for marker-assisted selection schemes as well. However, it should also be noted that the populations used in this research are chromosome substitution lines and are therefore recombinant for a single pair of chromosomes in a homogeneous background. SSCP banding patterns would likely increase in complexity in conventional populations making it somewhat more difficult to establish allelic loci and score for mapping purposes.

The EST-based maps of the 5BL 0.75–0.76 deletion bin constructed in this research will be useful for tagging additional genes within this gene-rich region. In addition, the resolution of EST loci within the bin is extremely valuable for establishing the level of colinearity with rice (H.J. Lu, J.D. Faris, submitted), especially considering that wheat homoeologous group 5 chromosomes appear to be highly rearranged compared to rice chromosomes (Sorrells et al. 2003; La Rota and Sorrells 2004). Furthermore, the EST-based linkage maps will be useful for the assembly of sequence-ready BAC contigs for which this gene-rich deletion bin is a prime target (Qi et al. 2003).

When embarking on a map-based cloning project, it is important to first conduct saturation and high-resolution mapping of the target gene. Selection of RFLPs and SSRs from previously published maps is useful to establish a basic map structure in the population being used, which can then provide information regarding which deletion bin harbors the gene of interest. Once the chromosome bin location for a gene of interest is known, only ESTs located within that bin need to be tested for polymorphism, which provides a means of direct selection of markers for saturation mapping. The level of colinearity of the target region with rice can be established by scanning the rice genome for sequences homologous to the mapped ESTs. If a suitable level of colinearity exists, additional markers can be generated based on rice sequences and mapped in the reference wheat population. AFLP-BSA can be used if additional markers are needed, but this technique is by far the more laborious especially if AFLP fragments are to be cloned and converted to RFLP markers for library screening (Faris and Gill 2002; Haen et al. 2004).

Akhunov et al. (2003) showed that in wheat, relative gene density and recombination rate increased with the relative distance of a deletion bin from the centromere. The genomic region defined by the 5BL 0.75–0.76 is located in the distal 25% of the long arm and constitutes a gene-rich recombination hot spot (Faris et al. 2000). Cytological experiments indicated that this deletion bin accounts for approximately 1% of the long arm of chromosome 5B (Gill et al., 1991; Endo and Gill, 1996) and is estimated to be about 5 Mb in size (Faris et al. 2000). The wheat NSF-EST project mapped 16,000 EST loci (Qi et al. 2004), of which 2,338 exist on homoeologous group 5 chromosomes (Linkiewicz et al. 2004). The homoeologous group 5 physical maps now consist of 2,555 loci for a mean density of 1.1 loci/Mb (Linkiewicz et al. 2004). If genes were distributed at random, we would expect there to be only five genes located in this deletion bin. The 5BL 0.75–0.76 bin is now known to contain at least 70 genes (Gill et al. 1996; Faris et al. 2000; Erayman et al. 2004; Haen et al. 2004; Linkiewicz et al. 2004; http://www.wheat.pw.usda.gov/wEST/binmaps/wheat5_rice.html). Thus, gene density in this region is approximately 14 times greater than the genomic average.

The genetic size of the 5BL 0.75–0.76 deletion bin was 18.5 cM. Therefore, the physical to genetic distance ratio for the interval is 270 kb/cM. This estimate is in close agreement for that reported for the 5AL-0.87 deletion interval on chromosome 5A (Faris and Gill 2002; Faris et al. 2003). However, combining the average recombination frequencies across the two BAC contigs gives an average physical to genetic distance ratio of 1.4 Mb/cM near the *Tsn1* locus. Therefore, recombination frequencies are likely to be highly variable across the 5BL 0.75–0.76 deletion interval.

Variable recombination frequencies within small physical segments have previously been reported in wheat. For example, over a physical distance of about 350 kb at the *Lr10* locus in wheat, Stein et al. (2000) reported an average physical to genetic distance ratio of 1.4 Mb/cM, but ratios varied from 400 kb/cM to 12 Mb/cM. These ratios are similar to what we observed across the two contigs flanking *Tsn1*.

Recombination frequencies have also been shown to be highly variable among different genomic regions. Spielmeyer et al. (2000) showed that recombination frequencies were 20–50 kb/cM at the seed storage protein loci on chromosome 1D of *Aegilops tauschii*, while Yan et al. (2003) reported a ratio of 6.25 Mb/cM at the *Vrn1* locus. We observed no recombination event in 5,438 gametes across the 205 kb contig proximal to *Tsn1*. This result is reminiscent of that reported by Ling et al. (2003) where they observed no recombination event in more than 4,000 gametes across 105 kb at the *Lr1* locus on chromosome 5D. Our results indicate it may be difficult to conduct chromosome walking at the *Tsn1* locus due to the apparent suppression of recombination. However, we will continue to extend the contigs and evaluate recombination frequencies in an effort to span the *Tsn1* gene.

SSRs are well suited for marker-assisted selection of target genes because they are user-friendly, amenable to high-throughput protocols, usually locus-specific, and

display higher levels of polymorphism, even between wheat varieties (Plaschke et al. 1995; Röder et al. 1995; Liu et al. 2005). In this research, the sequencing of BAC contigs led to the discovery of SSRs flanking the *Tsn1* locus. We are currently using these markers to introgress the *tsn1* allele into Ptr ToxA-sensitive wheat germplasm. Closely flanking codominant markers are particularly useful for marker-assisted selection of *tsn1*. Selection based on phenotypes alone will not allow one to distinguish between sensitive homozygotes and heterozygotes in progeny derived from backcrosses to sensitive recurrent parents. The codominant SSRs allow the heterozygotes to be identified and selected for in subsequent rounds of backcrossing while retaining the desired insensitivity allele.

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