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Genetic Maps of Stem Rust Resistance Gene *Sr35* in Diploid and Hexaploid Wheat

Wenjun Zhang, Eric Olson, Cyrille Saintenac, Matt Rouse, Zewdie Abate, Yue Jin, Eduard Akhunov, Mike Pumphrey, and Jorge Dubcovsky*

ABSTRACT

Puccinia graminis f. sp. *tritici* is the causal agent of stem rust of wheat. A new race designated TTKSK (also known as Ug99) and its variants (TTKST and TTTSK) are virulent to most of the stem rust resistance genes currently deployed in wheat cultivars worldwide. Therefore, identification, mapping, and deployment of effective resistance genes are critical components of global efforts to mitigate this threat. Multipathotype seedling tests demonstrated that resistance gene *Sr35* is effective against the three TTKS variants and another broadly virulent race from Yemen, TRTTF. Two genetic maps of *Sr35* are presented in diploid (*Triticum monococcum*) and two in hexaploid wheat (*T. aestivum*). The *Sr35* resistance to TRTTF and RKQQC races was mapped in diploid wheat within a 2.2 to 3.1 cM interval on the long arm of chromosome $3A^m$ between markers *XBF483299* and *XCJ656351*. This interval corresponds to a 174-kb region in *Brachypodium* that includes 16 annotated genes. The *Sr35* map location was confirmed in two backcross-derived hexaploid populations segregating for *Sr35*. Recombination between diploid and hexaploid chromosomes was 10-fold lower than between homologous chromosomes, but was sufficient to reduce the introgressed diploid segment. These maps provide markers closely linked to *Sr35* that will be useful to accelerate its deployment and pyramiding with other stem rust resistance genes.

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Abbreviations: EST, expressed sequence tag; RIL, recombinant inbreed lines; SSR, simple sequence repeat; TTKS-complex: TTKSK, TTKST, and TTTSK stem rust races.

Resistance genes derived from wild relatives have played a major role in the fight against the stem rust of wheat (caused by *Puccinia graminis* f. sp. *tritici*), and have provided adequate resistance for the last several decades. A potentially devastating new race of *P. graminis* with an unusually broad virulence spectrum was identified in Uganda in 1999 and is commonly known as Ug99. This race is identified as TTKSK based on the North American stem rust nomenclature (Jin et al., 2008; Pretorius et al., 2000; Wanyera et al., 2006). TTKSK was the first stem rust race reported to be virulent on *Sr31*, a gene present in the short arm of chromosome 1R from 'Petkus' rye and introgressed into hexaploid wheat as a 1RS·1BL translocation. This translocation continues to play a major role in wheat improvement and has been deployed worldwide in spring, facultative, and winter wheat for more than 30 yr (Bartos et al., 1973; Jin and Singh, 2006; Zeller and Hsam, 1983).

The initial TTKSK race was not virulent on *Sr24* and *Sr36,* two additional wild-relative-derived stem rust resistance genes frequently used by wheat breeders (Olson et al., 2010a). *Sr24* was originally

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transferred from *Thinopyrum ponticum* to bread wheat and is effective against most stem rust races worldwide (Smith et al., 1968; Yu et al., 2010). *Sr36* was transferred from *Triticum timopheevii* (Allard and Shands, 1954) and is present in several commercial wheat varieties (Olson et al., 2010a; Yu et al., 2010). Unfortunately, two new variants of TTKS with virulence on *Sr24* (TTKST) and *Sr36* (TTTSK) were identified in Kenya (Jin et al., 2008; Jin et al., 2009). These two new races have further broadened the virulence spectrum of this race complex (henceforth, TTKS-complex) and elevated the threat to wheat production worldwide. TTKS variants are currently affecting areas in Ethiopia and other East African countries (Wanyera et al., 2006), and have recently moved to Yemen and Iran (Nazari et al., 2009).

The advance of TTKSK toward the main wheat-growing regions of the world has triggered a coordinated global response (Stokstad, 2007). As part of this global response, major efforts have been initiated to precisely map and eventually clone resistance genes that are effective against these highly virulent races of stem rust. Molecular markers tightly linked to different TTKS resistance genes can be used to accelerate their deployment using marker-assisted selection (MAS) and also to combine multiple resistance genes in the same genetic background ("gene pyramiding"). The presence of multiple resistance genes is expected to extend the durability of resistance, since the probability of simultaneous mutations in the pathogen to overcome multiple resistance mechanisms is much lower than the probability to overcome individual mutations.

Sr35, originally transferred from *Triticum monococcum* to hexaploid wheat (McIntosh et al., 1984), is effective against TTKSK (Jin et al., 2007). Monogenic lines carrying *Sr35* exhibited resistant to moderately resistant infection responses with relatively low disease severity in field nurseries in Kenya in 2005 and 2006 (Jin et al., 2007). *Sr35* was first assigned to the long arm of chromosome 3A (McIntosh et al., 1984) and later mapped 41.5 cM from the centromere and 1cM from the red grain color gene *R2*. Babiker et al. (2009) recently mapped a gene conferring resistance to stem rust race QTH relative to four simple sequence repeat (SSR) markers on the long arm of chromosome 3A and suggested that this gene was *Sr35*. However, their *Sr35* map showed inconsistencies with previously published maps and therefore, we remapped *Sr35* in two diploid wheat (*T. monococcum*) and two hexaploid wheat (*T. aestivum*) populations, and confirmed a different map location. The identity of *Sr35* was further validated by the molecular characterization of the *Sr35* genetic stock Marquis*5/G2919.

The chromosomes of *T. monococcum* are known to recombine poorly with the wheat chromosomes in the presence of the *Ph1* gene (Dubcovsky et al., 1995; Luo et al., 1996; Luo et al., 2000) which may interfere with the precise mapping of *Sr35* in hexaploid wheat. To avoid this problem we employed two mapping populations in diploid wheat *T. monococcum*, where reduced recombination is not expected. An additional advantage of using *T. monococcum* is that a bacterial artificial chromosome (BAC) library from the TTKSK-resistant parent DV92 is already available (Lijavetzky et al., 1999) and that genetic mapping in a diploid species is easier and faster than in polyploid wheat. Two additional hexaploid wheat mapping populations were developed to validate the diploid results and to reduce the length of the *T. monococcum* segment introgressed into hexaploid wheat. The lines with smaller *T. monococcum* introgressions and the polymerase chain reaction (PCR) markers tightly linked to *Sr35* identified in this study will provide useful tools to accelerate the deployment of *Sr35* in the wheat breeding programs. In addition, the precise map of *Sr35* in diploid wheat provides the initial step for the positional cloning of this resistance gene.

MATERIALS AND METHODS

Plant Materials

The first diploid wheat mapping population used in this study was derived from the cross between cultivated *T. monococcum* ssp. *monococcum* accession DV92 and wild *T. monococcum* ssp. *aegilopoides* accession G3116 (Dubcovsky et al., 1996). This mapping population included the original F_2 population and 142 $F_{6:8}$ recombinant inbred lines (RIL) from the same cross. To validate the location of *Sr35,* a second population was generated from the cross between *T. monococcum* ssp. *monococcum* accession G2919 (= PI428170, donor of *Sr35* into hexaploid wheat, McIntosh et al., 1995) and the susceptible *T. monococcum* ssp. *aegilopides* accession TA189 (= PI427796). The susceptible accession TA189 was backcrossed (BC) as female to the F_1 hybrid and 269 BC_1F_1 lines were generated and screened for resistance to stem rust and for markers flanking *Sr35*.

We also compared the hexaploid genetic stock Marquis*5/G2919 with the diploid *T. monococcum* ssp. *monococcum* accession G2919. The Marquis*5/G2919 stock was developed by crossing the resistance from G2919 into the susceptible cultivar Marquis for five generations. *Triticum monococcum* accessions G2919 and C69.69 are the two sources used to transfer *Sr35* into the tetraploid and hexaploid wheat varieties (McIntosh et al., 1995).

Marquis*5/G2919 was also used as the *Sr35* donor in the two hexaploid wheat segregating populations. These two populations were generated by backcrossing to eliminate a second stem rust seedling gene present in the Marquis*5/G2919 genetic stock (likely *Sr19*) that is effective against some North American races. The first population of 176 F_3 families was generated from the cross Fuller*2///2174*2/Marquis*5/G2919 and will be referred as U5932 hereafter. The second population of 91 F_3 families, henceforth U5931, was generated from the cross Postrock*2///2174*2/Marquis*5/G2919.

Markers were assigned to physical chromosome bins using deletion lines C-3AL3–0.42, 3AL3–0.42–0.78, 3AL5–0.78–0.85, and 3AL8–0.85–1.00 (Endo and Gill, 1996).

Stem Rust Assays

Seedling resistance tests for DV92 and G3116 were performed at the USDA-ARS Cereal Disease Laboratory with TTKSK,

Table 1. Reactions to stem rust races from North American, East African, and Yemen in *T. monococcum* parental lines G3116 and DV92, diploid genetic stocks for *Sr21,* and *Sr21* + *Sr35* and the hexaploid genetic stock for *Sr22.* G2919 is the diploid genetic stock for *Sr35* + *Sr21*, PI10474 is the diploid genetic stock for *Sr21,* and Sr22TB is the monogenic hexaploid genetic stock for *Sr22*.

Race†	G3116	DV92‡	G2919 *Sr21* + *Sr35*	PI10474 *Sr21*	Sr22TB *Sr22*
MCCFC	1	1	;,1	;1	1
TTTTF	3	X LIF	X- LIF	3	1+2-
TPMKC	4	;,3,4 LIF	–	3	2
RKQQC	4	0	0	3+	;1
QFCSC	3	3+	4	3+	1+
TTKSK	2,2+	0;	0	2,2+	2-;
TTKST	2,2+	0;	–	–	2-
TTTSK	1,2	0;	–	2	2-
TRTTF	3+,4	0	0	4	2-;

†TTKSK, Ug99; TTKST, Ug99 + *Sr24* virulence; TTTSK, Ug99 + *Sr36* virulence; TRTTF, race from Yemen

‡X, mesothetic reaction, also described by '4,3,;' or ';1,2,3,' LIF, low infection frequency with most leaves with infection type (IT) 0 with rare pustules.

its variants TTKST (*Sr24* virulence), TTTSK (*Sr36* virulence), TRTTF (Yemen race) and five other races of stem rust (MCCFC, QFCSC, RKQQC, TPMKC, and TTTTF). Inoculation, incubation, and scoring disease reactions were performed as described previously (Jin et al., 2007). Since DV92 and G3116 were both resistant to the three races of the TTKS-complex, the 142 recombinant inbred lines were tested with races TRTTF and RKQQC that differentiated these two accessions. Plants were evaluated for their reaction to specific race isolates. From each genotype, 12 seedlings were screened. Infection types (ITs) 0,;, 1, 2, or combinations thereof were considered low ITs, indicating a resistant wheat line; whereas ITs 3 to 4 were considered high ITs, indicating a susceptible wheat line. After the initial mapping, 21 RILs showing critical recombination events between the *Sr35* flanking markers *Xcfa2193* and *Xwmc169* were re-sent to the Cereal Disease Laboratory for a blind validation of the mapping location. The 269 BC_1F_1 lines from the cross TA189//G2919/TA189 were screened with stem rust race RKQQC in Kansas State University.

The hexaploid mapping populations were assayed by seedling phenotyping using stem rust race RKQQC which is avirulent to *Sr35*. For each BC_1F_3 family, 16 seedlings were grown in 10 by 10 cm pots in Metro-Mix 200 medium (Hummert, Inc., Earth City, MO) in a greenhouse. Urediniospores were removed from liquid nitrogen storage and heat-shocked in a 42°C water bath for 5 min. Spores were suspended in Soltrol 170 isoparaffin oil (Chevron Phillips Chemical Company LP, The Woodlands, TX) and sprayed onto two to three leaf-stage seedlings. Inoculated plants were incubated in a dew chamber at 24 ± 1°C, 100% relative humidity for 16 h and then grown in a greenhouse at 21 ± 4°C with 16 h light/8 h dark cycle. Infection types were assessed 14 d after inoculation as described before (Stakman et al., 1962) and lines were classified as resistant or susceptible using the same criteria described above for diploid wheat.

PCR Marker Development and Detection

Primer sequences for the SSR markers from chromosome 3AL were obtained from GrainGenes (http://wheat.pw.usda.gov [verified 5 Aug. 2010). To develop additional markers in the *Sr35* region, *Brachypodium* and rice orthologous regions were initially identified using the sequence of the *Sr35*-linked restriction fragment length polymorphism (RFLP) marker *Xpsr1205*. *Brachypodium* and rice genes from this region were used to screen the GenBank wheat expressed sequence tag (EST) database using BLASTN and BLASTX programs. The annotated gene structure in *Brachypodium* and rice was used to predict the putative exon structure of the wheat ESTs and to design PCR primers in the exons that would amplify one or more introns. The PCR amplification products from both parental lines were treated with shrimp alkaline phosphatase and exonuclease I Mix (USB) at 37°C for 30 min, followed by inactivation at 80°C for 15 min, and were then sequenced directly using an ABI3730 sequencing equipment. Single nucleotide polymorphisms (SNPs) between the parental lines were used to develop cleavage amplification polymorphic sequences (CAPS) or degenerate CAPS (dCAPS) markers (Michaels and Amasino, 1998) or were mapped directly using the KASPar SNP Genotyping System (KBioscience, http://www.kbioscience.co.uk/ [verified 5 Aug. 2010]). The SSR- and EST-derived PCR markers were separated in 6% nondenaturing acrylamide gel (29:1) (http://maswheat.ucdavis.edu/PDF/SSR_Protocol.pdf [verified 5 Aug. 2010]) and stained directly with ethidium bromide.

Genetic Map

Linkage analysis was performed using MapMaker version 3.0b (Lander et al., 1987). Map distances were computed with the Kosambi mapping function. The map was initially constructed at a LOD of 3.0. Additional markers were added using the TRY command and their order was refined using the RIPPLE command.

RESULTS

Resistance Gene Postulation for the Diploid Wheat Parental Lines

We screened the parental *T. monococcum* lines DV92 and G3116 with nine different stem rust races including races in the TTKS lineage (Table 1). Line G3116 was susceptible to most races but was resistant to all three variants of TTKS-complex and race MCCFC (Table 1). Based on this race-specificity, we postulated G3116 to contain *Sr21.*

The cultivated *T. monococcum* ssp. *monococcum* accessions DV92 and G2919 had broader resistance than the *T. monococcum* ssp. *aegilopoides* accession G3116 (Table 1). The infection types to MCCFC, TTTTF, QFCSC, TTKSK, and TRTTF in cultivated line DV92 are very similar to those described for G2919, the donor of the *Sr35* and *Sr21* genes.

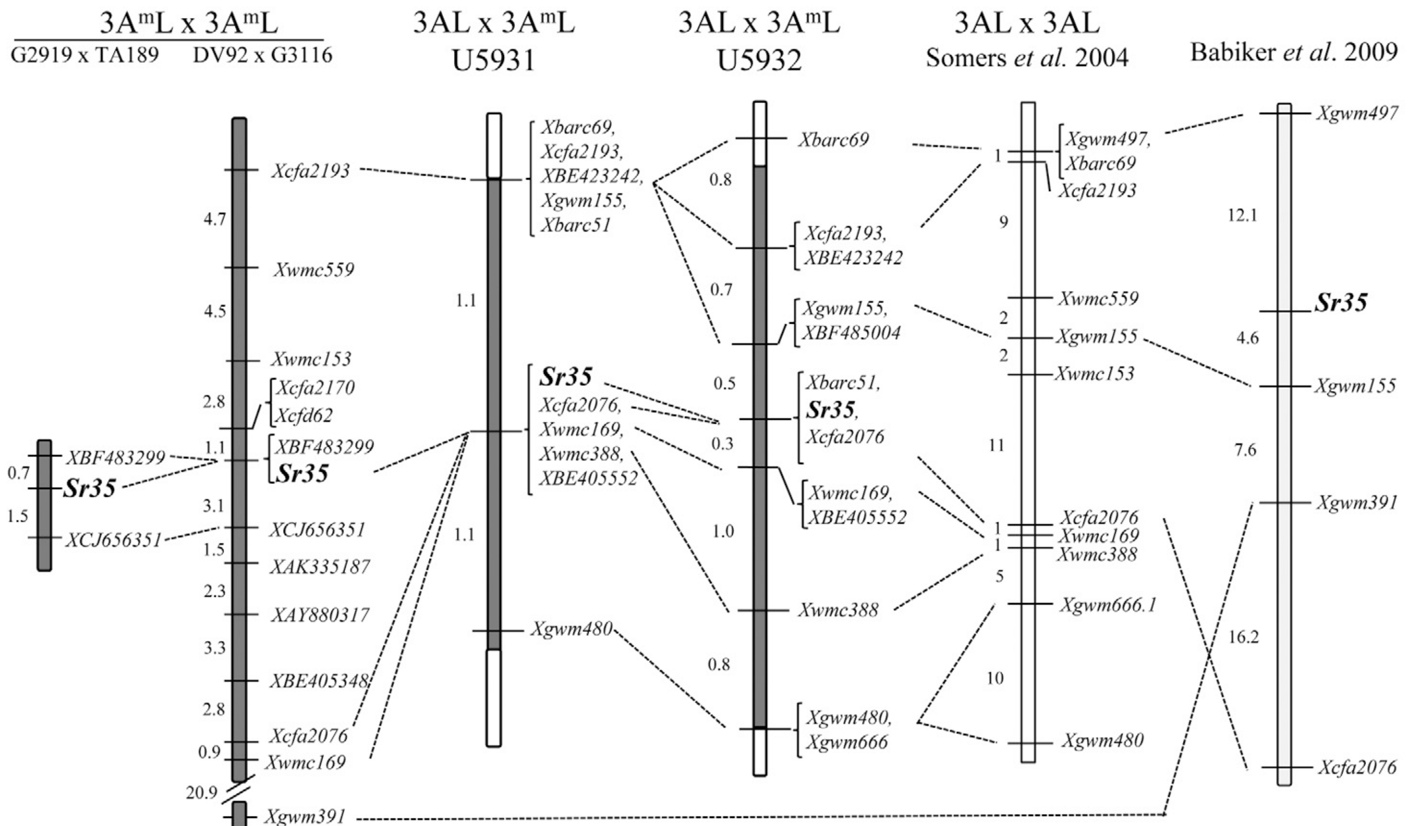

Figure 1. Genetic maps of stem rust resistance gene *Sr35* in diploid wheat *T. monococcum* ($3A^mL \times 3A^mL$, BC_1F_1 TA189//G2919/TA189 and RIL DV92 × *G*3116) and in *T. monococcum* introgression lines in hexaploid wheat ($3AL \times 3A^mL$, populations U5931 and U5932). Shaded areas indicate *T. monococcum* introgressed chromosome segments, their comparison with a microsatellite consensus map for hexaploid wheat (Somers et al., 2004), and a previous map of *Sr35* (Babiker et al., 2009).

We postulated the presence of *Sr35* in DV92 based on the immune ("0") or very resistant (";") infection types to races TTTTF, TPMKC, RKQQC, TTKSK, TTKST, TTTSK, and TRTTF. These races are avirulent on lines with *Sr35.*

We postulated the presence of *Sr21* in DV92 based on the "1" infection type to race MCCFC which is avirulent on lines with *Sr21,* but virulent on lines with *Sr35* alone. We confirmed that both G3116 and DV92 have the same gene-conferring resistance to MCCFC since all 142 RILs were resistant and showed a similar infection type to MCCFC. Race QFCSC is virulent on both *Sr21* and *Sr35* and produced high infection types on all four *T. monococcum* accessions. The '*Sr22*TB' stock, a hexaploid line with the *Sr22* resistance gene derived from *T. monococcum*, was included as a control and showed the expected resistance to QFCSC. Since both parental lines were resistant to all three variants of the TTKS-complex, we used races RKQQC and TRTTF to screen the RILs and map the resistance gene.

Sr35 Mapping in Diploid Wheat

The resistance to the RKQQC and TRTTF races was initially mapped in the 142 *T. monococcum* RILs to a single locus on the long arm of chromosome $3A^m$ linked to SSR marker *Xcfa2170* (Fig. 1). Additional SSR markers were then selected from the distal part of the long arm of chromosome 3A or $3A^m$ using the GrainGenes database, and the seven polymorphic ones were mapped on the 30 cM region between *Sr35* flanking markers *Xcfa2193* and *Xwmc169* (Fig. 1). Some of these markers were also added to the original *T. monococcum* F_2 population (Dubcovsky et al., 1996) to integrate the SSR and RFLP markers (Fig. 2).

To generate additional markers in the *Sr35* region, we identified the colinear regions in the sequenced genomes of *Brachypodium*, rice, and sorghum using the sequence of the RFLP probe used to map the *Xpsr1205* locus, which was mapped 9 cM distal to *Sr35.* We selected 12 genes from this region, identified wheat EST sequences in GenBank, designed primers, and sequenced the PCR products from both parents. Six genes showed SNPs and one an indel (insertion/deletion polymorphism). We developed CAP or dCAP markers for these polymorphisms (Table 2) and mapped five of them on the *Sr35* region (Fig. 1). The resistance to the RKQQC and TRTTF races was mapped linked to *XBF483299* and 3.1 cM proximal to *XCJ656351* in the DV92 × G3116 *T. monococcum* RIL population (Fig. 1). This chromosome location was further confirmed by an independent determination of infection types in 21 RILs showing recombination between *Sr35* flanking markers with race RKQQC.

To further validate the location of *Sr35,* we developed a second *T. monococcum* population of 269 BC_1F_1 lines from

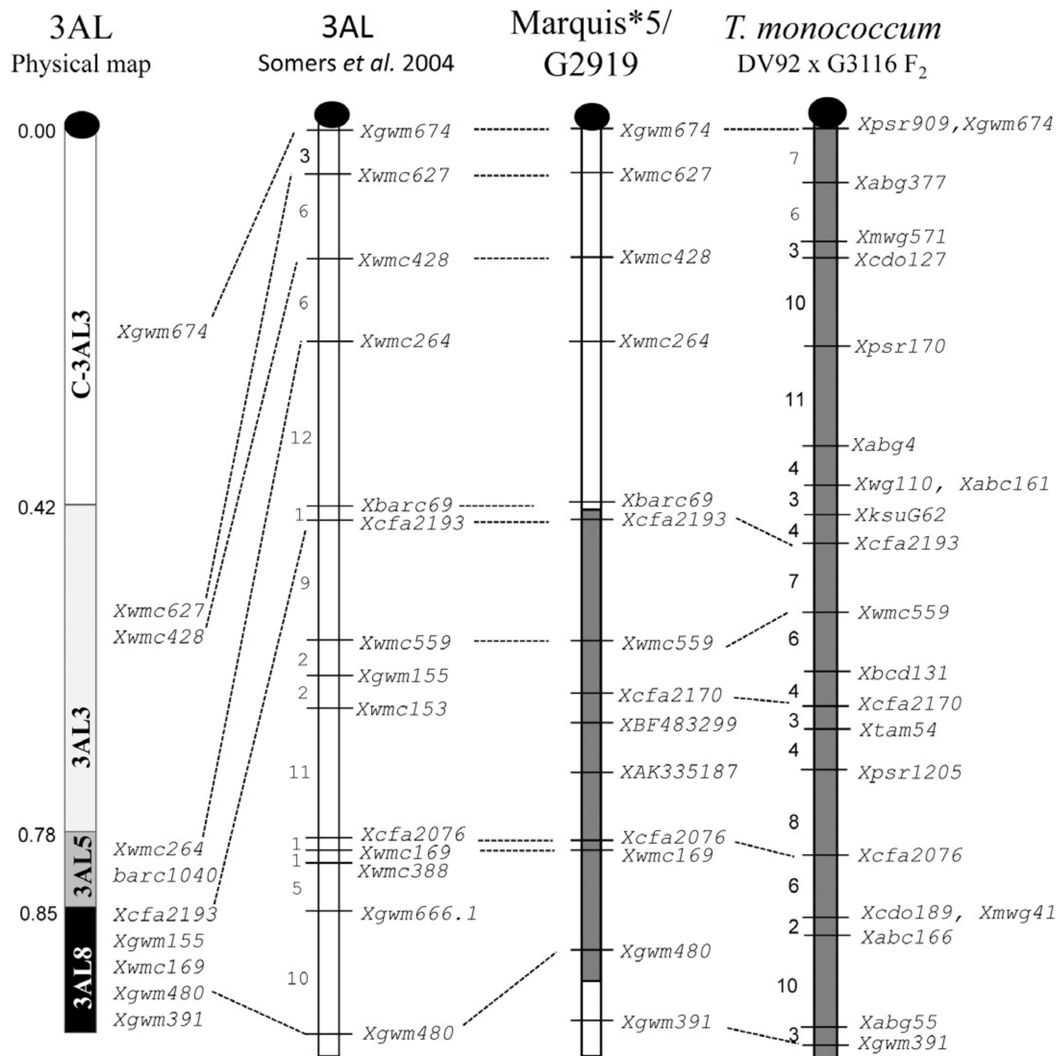

Figure 2. Comparison of physical and genetic maps of the long arm of chromosome 3A in hexaploid wheat with the introgression of a $3A^mL$ chromosome segment from *T. monococcum* (shaded gray area) in hexaploid wheat Marquis*5/G2919. The last *T. monococcum* map integrates the microsatellite markers used in this study into the same F_2 population used to construct the RFLP map of *T. monococcum* (Dubcovsky et al., 1996).

the cross TA189//G2919/TA189, evaluated their resistance to RKQQC, and genotyped them using KASPar assays for *XBF483299* and *XCJ656351*. In this population, we found two recombination events between *XBF483299* and *Sr35* (0.74 cM), and four recombination events between *Sr35* and *XCJ656351* (1.49 cM, Fig. 1). These results validated the location of *Sr35* in the DV92 × G3116 populations and showed that *XBF483299* is proximal to *Sr35*.

Sr35 Gene Region in Hexaploid Wheat

Markers mapped in the *T. monococcum* segregating population and in a *T. aestivum* consensus map (Somers et al., 2004) were used to determine the region of *T. monococcum* accession G2919 transferred to the *Sr35* hexaploid genetic stock Marquis*5/G2919. Markers showing the same allele as G2919 in Marquis*5/G2919 were considered to be within the *T. monococcum* introgression whereas those showing the same allele as the recurrent hexaploid parent Marquis were considered to be outside of the introgression (Table 3). The markers included in the 30-cM region between *Xcfa2193* and *Xgwm480* all showed the same allele as the diploid *Sr35* gene donor G2919, suggesting that this region was transferred from the diploid into the hexaploid wheat (Fig. 2).

The proximal marker *Xbarc69* did not show the G2919 allele in Marquis*5/G2919, indicating that the initial transfer of the distal $3A^mL$ *T. monococcum* segment occurred through a recombination event between markers *Xbarc69* and *Xcfa2193* (Fig. 2). The most distal marker *Xgwm391* also showed the absence of the G2919 allele in Marquis*5/G2919, indicating that a second recombination event between *Xgwm480* and *Xgwm391* restored the distal region of chromosome arm 3AL to the recombined chromosome (Fig. 2). Markers used in this study were also mapped in the 3AL deletion bin to validate the genetic mapping results and to provide an estimate of the physical location of the

Table 2. The EST-derived PCR markers in the *Sr35* region. Band sizes correspond to the *T. monococcum* DV92 resistant allele followed by the G3116 susceptible allele (between brackets) in the diploid population and to the G2919 resistant allele followed by the susceptible Marquis allele (between brackets) in the hexaploid populations.

Wheat EST	Primer sequence	Restriction enzyme	Marker type†	Map	Band size (bp) *Sr35* donor/(susceptible)
BF483299	GATATGATTTCCTCATCCAGTGGTAC GCCAGAAAAGGGATGCTACACT	*Kpn*I	dCAP	Diploid	122/(144)
CJ656351	AAATGTTTTTGTATATTCTTGAGCAG AACTGTGGAAGCCATTCTTAAA	*Pvu*II	CAP	Diploid	76/(101)
AK335187	GGTTCAACATCGTCGGACAG CCAGCACGACGTACTTGGAG	–	indel	Diploid	~205,290/(205)
AY880317	AATTTGAACTTGAAACATGCAACACA TTAGTAATTCCACCGGCAACAAGAT	*Eco*RV	dCAP	Diploid	122/(100)
BE405348	ATTCCAGGTCCAGGAACTCC TTGTAAACCACCTATTGAGTTTGTTT	*Nco*I	dCAP	Diploid	142/(123)
BE423242	TCTGACCAATGCAAAATGGA GCTGATTGGCTTGGAAGGTA	–	indel	Hexaploid	430/(426)
BE405552	CACCATCTTCGTCACCATCA CACAGTGCAGCGAACAGATT	–	indel	Hexaploid	377/(371)
BF485004	TGCAGAATGCGTTCCTTCTA GGCCAGAGAATTTCTTGAGG	–	indel	Hexaploid	Null/(597)

†CAP, cleavage amplification polymorphism; dCAP, degenerate cleavage amplification polymorphism; indel, insertion/deletion.

markers (Fig. 2, Table 3). With the exception of the markers located in the first 15 cM from the centromere, all other markers were mapped in the distal bin 3AL8–0.85–1.00. Since the *Sr35* flanking markers were all mapped within this distal bin, we conclude that *Sr35* is physically located within the distal 15% of the long arm of chromosome 3A.

Sr35 Mapping in Hexaploid Wheat

In both hexaploid populations the segregation between susceptible, heterozygous, and resistant F_3 families showed a 1:2:1 ratio, which is consistent with segregation for a single resistance gene (U5931 χ^2 $P = 0.51$, U5932 χ^2 $P = 0.06$,). In the U5931 population, recombination events were detected only between three groups of markers. The proximal group included markers *Xcfa2193*, also mapped in *T. monococcum,* and marker *Xgwm155,* which is an important reference marker to compare with the map published by Babiker et al. (2009). This group of markers is 1.1 cM distal to the second group that includes the *Sr35* resistance gene and microsatellite markers *Xcfa2076, Xwmc169*, and *Xwmc388*. Finally, the most distal group includes only *Xgwm480*. The distance between the most proximal (*Xbarc69*) and most distal marker (*Xgwm480*) in the U5932 population is 2.2 cM, compared with 42 cM in the *T. aestivum* consensus map (Fig. 1). The hexaploid wheat consensus map and *T. monococcum* map distances are very similar (Fig. 2), suggesting that the genetic distances are reduced in the U5931 population.

The U5932 population showed a slightly higher recombination between the most proximal and distal markers (4.1 cM) than the U5931 population, but this distance was still 10-fold lower than the 42 cM observed in the *T. aestivum* consensus map. In the U5932 population the *Sr35* gene was mapped distal to *Xgwm155* and proximal to *Xwmc169*.

Table 3. Assignment of loci to physical bins by comparisons of allele size in the recurrent hexaploid wheat variety Marquis (accessions PI 351208 and CItr 3641), the introgression line Marquis*5/G2919, and the *T. monococcum* donor of *Sr35* (G2919). The *T. monococcum* alleles are indicated in bold.

Bin	Locus	Marquis	Marquis*5/G2919	G2919
C-3AL3–0.42	*Xgwm32*	169	169	168
3AL3–0.42–0.78	*Xcfa2134*	308	308	234
3AL5–0.78–0.85	*Xbarc1060*	260	258	249
3AL5–0.78–0.85	*Xbarc1040*	195	195	null
3AL8–0.85–1.00	*Xbarc69*	155	155	139
3AL8–0.85–1.00	***Xcfa2193***	230	**243**	**243**
3AL8–0.85–1.00	***XBE423242***	426	**430**	**430**
3AL8–0.85–1.00	***Xgwm155***	162	**160**	**null**
3AL8–0.85–1.00	***XBE485004***	597	**null**	**na†**
3AL8–0.85–1.00	***Xbarc51***	249	**237**	**237**
3AL8–0.85–1.00	***XBE405552***	371	**377**	**377**
3AL8–0.85–1.00	***Xwmc169***	153	**143**	**143**
3AL8–0.85–1.00	***Xgwm480***	188	**null**	**null**
3AL8–0.85–1.00	*Xgwm666*	122	122	107
3AL8–0.85–1.00	*Xbarc1099*	127	127	125
3AL8–0.85–1.00	*Xgwm162*	null	null	225
3AL8–0.85–1.00	*Xgwm391*	94	94	262
3AL8–0.85–1.00	*Xcfd2*	338	338	340

†na, not analyzed.

Sr35 Colinear Regions in Other Cereal Genomes

The order of all five wheat EST-derived markers mapped in the *T. monococcum Sr35* region was colinear with the order of the orthologous genes in *Brachypodium* chromosome 2 (Bd2) (Fig. 3). The order of these genes was also conserved in rice chromosome 1 (R1) and sorghum chromosome 3 (Sb3).

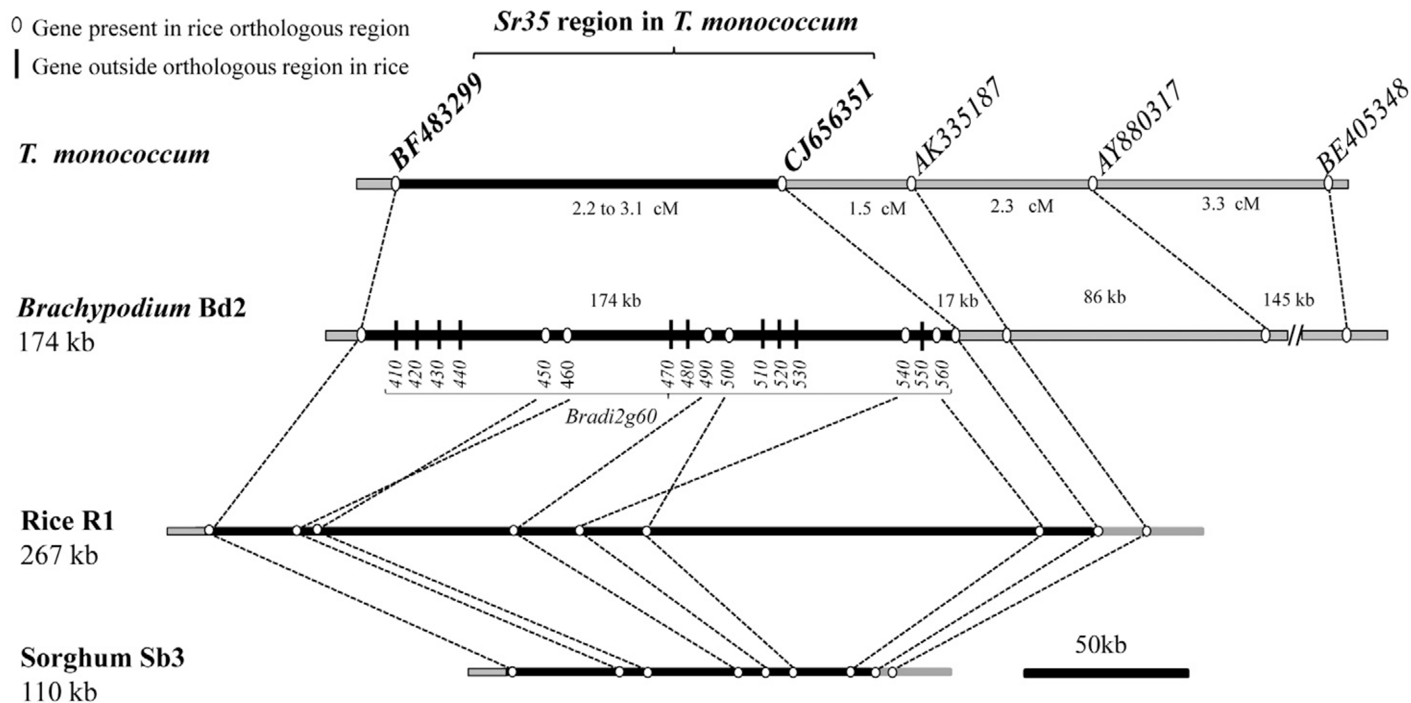

Figure 3. Comparison of the *T. monococcum* map in the *Sr35* (black line) and flanking regions (gray line) and its comparison with the annotated sequences of the *Brachypodium*, rice, and sorghum genomes. The bar represents 50 kb. The numbers in the *Brachypodium* map (black line) are the annotated genes (*Bradi2g60410–Bradi2g60560*) in the region between the *Brachypodium* orthologs to wheat genes flanking *Sr35* (*BF483299–CJ656351*). Orthologous genes found in *Brachypodium*, rice, and sorghum are indicated with white circles and the *Brachypodium* non-colinear genes with black vertical lines.

Six of the *Brachypodium* genes (*Bradi2 g640450*, *Bradi2 g640460*, *Bradi2g60490*, *Bradi2g60500*, *Bradi2g60540,* and *Bradi2g60560*) located between the *BF483299* and *CJ656351* orthologs were also present in the colinear regions in rice and sorghum (Fig. 3). The order of these genes was relatively well conserved with the exception of two small inversions including *Brachypodium* genes *Bradi2g60450* and *Bradi2g60460* and *Bradi2g60500* to *Bradi2g60540* (Fig. 3). In addition to the six colinear genes, 10 additional *Brachypodium* genes located within this region were not colinear with rice (Fig. 3).

The annotation of the *Brachypodium* genes and their closest rice homologs is summarized in Table 4. Wheat ESTs with significant similarity to the *Brachypodium* genes in this region were identified and are also listed in Table 4. Polymorphisms between the *T. monococcum* parental lines DV92 and G3116 were identified only for two of the wheat ESTs (*AK331221* and *AF445790*), but they were not linked to the markers in the *Sr35* region.

DISCUSSION

Identification of the Resistance Gene as *Sr35*

The stem rust resistance gene identified in the two *T. monococcum* mapping populations is postulated to be *Sr35* based on its diploid origin, infection types with multiple races, map location, and presence of the same *T. monococcum*-derived region in the *Sr35* hexaploid genetic stock Marquis*5/G2919. Previous studies have identified three stem rust resistance genes from *T. monococcum* (*Sr21*, *Sr22*, and *Sr35*) that are still effective against the races within the TTKS-complex (Jin et al., 2007; Singh et al., 2006). These three genes are described below.

The *Sr22* resistance gene was identified in *T. monococcum* ssp. *aegilopoides* accession G-21 (Gerechter-Amitai et al., 1971) and in *T. monococcum* ssp. *monococcum* accession RL5244 (Kerber and Dyck, 1973). *Sr22* was mapped on the long arm of chromosome 7A within a relatively large *T. monococcum* chromosome segment (The et al., 1973), which was recently shortened in new secondary recombinant hexaploid lines (Olson et al., 2010b). *Sr22* is effective against all stem rust races listed in Table 1, including QFCSC. Since both G3116 and DV92 are susceptible to QFCSC, the presence of *Sr22* in these two lines can be ruled out.

The *Sr21* resistance gene was identified in *T. monococcum* ssp. *monococcum* accession C.I.2433 (= PI 10474, which is used as a pathotype differential) and mapped on chromosome 2AL, 2 cM from the centromere (The et al., 1979). *Sr21* confers resistance in diploid wheat to TTKSK (Pretorius et al., 2000), but there is a progressive dilution of resistance when this gene is transferred from diploid to tetraploid and hexaploid wheat, suggesting an effect of polyploidy or genetic background on the expression of the *Sr21* resistance (Jin et al., 2007; McIntosh et al., 1984). In diploid wheat, the *Sr21* gene is effective against race MCCFC (C17 = race 56) which has known virulence on *Sr35*, but is not effective against TPMKC, RKQQC, and TRTTF. Since G3116 is

Table 4. *Brachypodium* genes identified in the region between *Brachypodium* orthologues to wheat markers *BF483299* and *CJ656351* (flanking markers for *Sr35*).

Brachypodium	Wheat EST	Annotation (putative function)†	Rice gene
Bradi2g60400	BF483299	Proximal flanking marker	*Os01g71300*
Bradi2g60410	AK331487	NB/LRR domains (disease resistance)	*Os11g43700*§ (*Os10g03570, Os01g41890*)§
Bradi2g60420			
Bradi2g60430			
Bradi2g60440			
Bradi2g60450	AK332451	Hexokinase (carbohydrate transport and metabolism)	*Os01g71320*
Bradi2g60460	BM148354	FAD/FMN-containing dehydrogenase (catalytic activity)	*Os01g71310*
Bradi2g60470		DUF1719 domain protein (unknown function)	*Os04g01560*§
Bradi2g60480			
Bradi2g60490	AK331482	β-glucanase (hydrolysis)	*Os01g71380*
Bradi2g60500	DR435176	β-glucanase/lichenase (hydrolysis)	*Os01g71474*
Bradi2g60510	AK334855‡	DUF3615 domain protein (hypothetical protein)	*Os08g38620*§
Bradi2g60520		No similarity to any known protein (hypothetical protein)	*No homolog*
Bradi2g60530	AK445790‡	Serine/Threonine kinase (phosphotransferase)	*Os02g42150*§
Bradi2g60540	AK331221‡	Metallophosphatase MPP (Ser/Thr phosphatase)	*Os01g71420*
Bradi2g60550		B3 DNA binding domain protein (plant transcription factor)	*Os03g42240*§
Bradi2g60560		β-1,3-endo-glucanase (hydrolysis)	*Os01g71670*
Bradi2g60570	CJ656351	Distal flanking marker	*Os01g71690*

†LRR, leucine rich repeat; NB, nucleotide binding.
‡Mapped outside *Sr35* region.
§Most similar rice gene outside *Sr35* region.

susceptible to the last three races (Table 1) and resistant to MCCFC and the three races in the TTKS-complex, we postulate that this accession has *Sr21*.

DV92 and the complete RIL population showed resistance to MCCFC suggesting that both G3116 and DV92 carry *Sr21*. However, DV92 was also resistant to TPMKC, RKQQC, and TRTTF, indicating the presence of an additional resistance gene. This second gene was not effective against QFCSC, a characteristic also observed in the *T. monococcum* stock G2919, which is known to carry both *Sr21* and *Sr35*. Both DV92 and G2919 are resistant to TRTTF. Based on the previous results we postulate that the second gene in DV92 is *Sr35*.

The identity of *Sr35* is also supported by its location on the distal region of the long arm of chromosome 3A^m, which agrees with the previous mapping of *Sr35* approximately 40 cM from the centromere using telocentric analysis (McIntosh et al., 1984). The mapping of *Sr35* to the same region in two hexaploid populations derived from the *Sr35* hexaploid genetic stock Marquis*5/G2919 further validated our postulation. The region of *T. monococcum* chromosome 3A^m transferred to the *Sr35* hexaploid genetic stock Marquis*5/G2919 includes the markers flanking the stem rust resistance gene mapped in our diploid wheat segregating population indicating that they carry the same gene. In spite of the five backcross generations used to generate Marquis*5/G2919, the *T. monococcum* segment is still relatively large (between 38 and 80 cM), likely because of the reduced recombination between homeologs caused by the presence of the *Ph1* gene (Dubcovsky et al., 1995; Luo et al., 2000).

The *T. monococcum* origin, resistance profile, 3AL map location, and consistent mapping in two hexaploid populations demonstrate that the stem rust resistance gene mapped in this study is most likely *Sr35*. Our results also show that the *Sr35*-*Sr21* combination is effective against the three races of the TTKS-complex of *P. graminis* f. sp. *tritici* (TTKSK, TTKST, TTTSK) and that *Sr35* is the source of resistance to the TRTTF race from Yemen (Table 1).

Conflicting Mapping Locations of *Sr35*

Sr35 was recently mapped on the long arm of chromosome 3AL in a different hexaploid wheat population but in a different location from the one presented in our current study (Babiker et al., 2009). Babiker et al. (2009) postulated that *Sr35* was 12 cM distal to SSR marker *Xgwm497* and 4.6 cM proximal to *Xgwm155*. Since *Xgwm155* is proximal to *Xwmc153* (Somers et al., 2004) (Fig. 1), *Sr35* would be located proximal to *Xwmc153* (~7 cM) in Babiker et al. (2009) map (Fig. 1). On the contrary, *Sr35* was mapped 5.3 cM distal to *Xwmc153* in our *T. monococcum* population. In the two hexaploid mapping populations, *Sr35* was mapped distal to *Xgwm155,* also contradicting the location in Babiker et al. (2009) map (Fig. 1).

Surprisingly, the two closest *Sr35* flanking markers identified by the Babiker et al. (2009) map were not significantly associated with *Sr35* in their statistical tests, whereas the most distal ones, *Xgwm391* and *Xcfa2076*, were significantly linked (Fig. 1). Even though the authors reported that 94.5% of the F_2 plants that carry the dominant *Xcfa2076* allele from the resistant parent were resistant, suggesting a close linkage, this marker was mapped 28.4 cM distal to

Sr35 (23.8 cM distal to *Xgwm155*). In contrast, in our two hexaploid mapping populations, *Xcfa2076* was mapped only 0.5–1.1 cM distal to *Xgwm155*. Finally, the relative order of markers *Xcfa2076* and *Xgwm391* in the Babiker et al. (2009) map conflicts with their order in the *T. monococcum* map (Fig. 1). A potential source of this conflicting result is the fact that *Xgwm391* and *Xcfa2076* were both mapped as dominant markers in opposite phase, which would provide limited linkage information in an F_2 population. This was not a problem in our *T. monococcum* map since most of the markers were codominant and the dominant ones are equally informative in homozygous RIL lines. The *Sr35* mapping results from our two hexaploid populations are consistent with the more precise mapping of *Sr35* in the two *T. monococcum* segregating populations, supporting the location of *Sr35* distal to *Xgwm155* (Fig. 2).

Comparison of Genetic Distances between Homologous and Homeologous Recombination

The *T. monococcum* map was colinear with the hexaploid wheat microsatellite consensus map (Somers et al., 2004) (Fig. 1) and the distances between markers were similar. These distances were also compared to other hexaploid maps based on experimental rather than consensus data. For example, the distances in the hexaploid population Louise × Penawawa (Carter et al., 2009; GrainGenes CMap comparative map viewer http://wheat.pw.usda.gov/cgi-bin/cmap/viewer [verified 5 Aug. 2010]) and the *T. monococcum* map were both 5 cM between *Xcfa2193* and *Xgwm559*, and 24 and 22 cM between *Xgwm559* and *Xwmc169*, respectively.

Similar genetic distances between *T. monococcum* and *T. aestivum* maps have been reported before (Dubcovsky et al., 1995). However, recombination between *T. monococcum* (A^m genome) and *T. aestivum* chromosomes (A genome) is greatly reduced in the presence of the *Ph1* gene (Dubcovsky et al., 1995; Luo et al., 1996; Luo et al., 2000). In a previous study Luo et al. (1996) found a 3.2-fold reduction in recombination between the $3A^m$ chromosome from *T. monococcum* and the 3A chromosome from wheat, relative to recombination between homologous chromosomes of the same species. However, the same authors reported a ninefold reduction in recombination in the proximal region of the 3L arm, which is similar to the 10-fold reduction observed in our study (Fig. 2). Recombination between $7A^m$ and 7A chromosomes was similarly reduced 3 to sevenfold in the *Sr22* region (Olson et al., 2010b).

In spite of this reduction in recombination, the observed recombination events were sufficient to reduce the length of the *T. monococcum* segment introgressed in hexaploid wheat. The greater number of recombination events in population U5932 provided a better resolution than the U5931 population; this is likely because the number of U5932 F_2 plants was almost double that of the U5931 population. The reduction of the introgressed chromosome segment from *T. monococcum* is useful because it eliminates potentially detrimental alleles of other genes linked to *Sr35* (linkage drag), and also because it reduced the region with limited recombination in the *Sr35* flanking regions.

Colinearity in the *Sr35* Region and Candidate Genes

The incorporation of five sequence-based EST-derived markers to the *T. monococcum Sr35* map facilitated comparisons with the available genomic sequences of *Brachypodium*, rice, and sorghum (Fig. 3). The physical distance between the orthologs of the *Sr35* flanking genes *BF483299* and *CJ656351* in these species was estimated to be 174 kb in *Brachypodium*, 267 kb in rice, and 110 kb in sorghum (Fig. 3). Comparison of the gene order among these regions revealed relatively good colinearity, interrupted by a couple of small inversions in *Brachypodium* relative to rice and sorghum.

Since *Brachypodium* is evolutionarily closer to wheat than rice or sorghum (Faricelli et al., 2010; Kellogg, 2001), we focused on the genes present between the *Brachypodium* orthologs to BF483299 (*Bradi2g60400*) and CJ656351 (*Bradi2g60570*). Starting from the proximal *Bradi2g60400*, there is a group of four linked and related *Brachypodium* genes (*Bradi2g60410–440,* Fig. 3) that code for proteins including a nucleotide binding (NB) domain and a leucine rich repeat (LRR) domain (Table 4). The NB-LRR proteins act mainly as a second line of defense, when plant pathogens overcome pattern recognition receptors (PRR) that form the first line of defense (Jones and Dangl, 2006). The polymorphic NB-LRR proteins are well adapted to recognize specific effectors introduced into the cells by biotrophic or hemi-biotrophic pathogens and to activate the defense responses (Jones and Dangl, 2006). Based on the known function of the NB-LRR proteins in disease resistance, and the characteristic hypersensitive response conferred by *Sr35* to a specific group of stem rust races, the wheat orthologs of these genes are good candidates for *Sr35.* The first step will be to demonstrate that orthologous wheat NB-LRR genes are present in the *Sr35* region, particularly since no orthologous of these genes were found in the rice and sorghum colinear regions (Fig. 3).

Distal to the NB-LRR genes, *Brachypodium* gene *Bradi2g60450* code for a hexokinase protein predicted to be involved in carbohydrate transport and metabolism; and gene *Bradi2g60460* code for a FAD/FMN-containing dehydrogenase predicted to have catalytic activity. Based on their predicted function, the previous two genes are unlikely candidates for *Sr35.* Three other *Brachypodium* genes within this region encode proteins with conserved domains of unknown function, DUF1719 (*Bradi2g60460* and *Bradi2g60470*) and DUF3615 (*Bradi2g60510*), and therefore, it is not possible to infer their putative role on a resistance mechanism. Three additional *Brachypodium* genes (*Bradi2g60490*, *Bradi2g60500,*

and *Bradi2g60560*) encode proteins with significant similarities to β-1,3-glucanases, which cannot be ruled out as candidates for disease resistance genes since transgenic plants overexpressing similar genes have been shown to have enhanced resistance to different pathogens (Logemann et al., 1994; Mackintosh et al., 2007).

The predicted protein for *Bradi2g60520* shows no similarity to any known domain or protein and it is a potential annotation error. *Bradi2g60530* predicted protein includes a serine/threonine kinase, a domain involved in signal transduction in many disease resistance responses. However, serine/threonine kinases involved in disease resistance usually belong to a subclass designated as non-RD kinases, which lack the lysine (R) preceding the invariant aspartate (D) in the catalytic loop (Dardick and Ronald, 2006). *Bradi2g60530* is an RD kinase and, therefore, is less likely to be involved in the *Sr35* hypersensitive response than if it would have been a non-RD kinase.

Bradi2g60540 encodes a metallophosphatase and *Bradi2g60550* a protein with low similarity to a transcription factor including a B3 DNA binding domain (Table 4). Since these proteins regulate the transcription or activity of other genes and proteins, they cannot be ruled out as candidate genes for *Sr35*. Finally, it is possible that the orthologous wheat region includes genes that are absent in *Brachypodium* and that therefore, the wheat *Sr35* gene could be unrelated to any of the genes described in Table 4. In spite of this limitation, this comparative genomics analysis provides useful information to prioritize the next steps in the positional cloning of *Sr35*. Based on the current annotation, we will focus our initial efforts on the *Bradi2g60410 to Bradi2g60440* genes annotated as disease resistance proteins. If none of the wheat homologs of the *Brachypodium* candidate genes is *Sr35*, we will screen the *T. monococcum* DV92 bacterial artificial chromosome (BAC) library (which includes the resistant allele) with the closest *Sr35* flanking markers and initiate a chromosome walk to construct a complete physical map of the candidate gene region. We will then sequence the overlapping BACs and identify potential candidate genes.

CONCLUSIONS

From a practical point of view, the molecular markers identified in this study will be useful to deploy *Sr35* in wheat breeding programs. In polyploid wheat, *Sr35* confers very low infection types to avirulent races of stem rust, which contrasts with the progressive dilution of the resistance conferred by *Sr21* when transferred into polyploid wheats (McIntosh et al., 1984). Although *Sr35* is effective against the TTKS variants (TTKSK, TTKST and TTTSK), races with virulence for *Sr35* have been identified in several regions of the world and therefore, this gene should be deployed in combination with other stem rust resistance genes.

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