Molecular Mapping of Stem-Rust-Resistance Gene *Sr40* in Wheat

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ABSTRACT

Stem rust, caused by *Puccinia graminis* f. sp. *tritici,* was historically one of the most destructive diseases of wheat (*Triticum aestivum* L.) worldwide. Deployment of resistant cultivars successfully prevented rust epidemics over the past several decades. Unfortunately, race TTKS (termed Ug99) has emerged in Africa to render several stem-rust-resistance genes ineffective. *Sr40,* a stem-rust-resistance gene from *Triticum timopheevii* ssp. *armeniacum,* was transferred to wheat on translocation chromosome T2BL/2G#2S and provides effective levels of seedling and adult plant resistance against Ug99. Two mapping populations were developed using Ug99-resistant line RL6088 and moderately susceptible to susceptible hard winter wheat cultivars Lakin and 2174. The parents were screened with 83 simple sequence repeats (SSR) from chromosome 2B and the polymorphic markers were analyzed on F_2 populations. F_2 and $F_{2:3}$ populations were inoculated with North American stem rust race RKQQ at the seedling stage. Marker locus *Xwmc344* was most closely linked to *Sr40* (0.7 cM) in the RL6088/Lakin linkage map, followed by *Xwmc474* and *Xgwm374*. Marker locus *Xwmc474* was mapped ~2.5 cM proximal to *Sr40* in the RL6088/2174 population. *Xwmc474* and *Xwmc661* flanked *Sr40* in both populations. Markers linked to *Sr40* will be useful for marker-assisted integration and pyramiding of *Sr40* into elite wheat breeding lines, and reduction in the size of the *T. timopheevii* segment harboring this gene.

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Abbreviations: cM, centiMorgans; ITs, infection types; MAS, marker-assisted selection.

Stem rust, also known as black rust, caused by *Puccinia graminis* f. sp. *tritici* (pgt), has resurfaced as one of the most important threats to wheat (*Triticum aestivum* L.) production worldwide. Severe wheat yield losses due to stem rust epidemics were reported in Europe, Asia, Australia, and the U.S. in the 20th century (Zadoks, 1963; Rees, 1972; Roelfs, 1977; Leonard, 2001; Leonard and Szabo, 2005). In the U.S., stem rust epidemics were frequently damaging in the Great Plains from 1920 to 1960 and caused yield losses of up to 50% in some states (Leonard, 2001). Since 1970, crop losses caused by stem rust have been minimal because of successful deployment of effective stem-rust-resistance genes in commercial wheat cultivars and the success of barberry (*Berberis vulgaris*) eradication in reducing pathogen survival and diversity (Leonard, 2001; Leonard and Szabo, 2005). In 1999, stem rust race TTKS was identified in Uganda and coined Ug99 (Pretorius et al., 2000). Ug99 was discovered in Ethiopia and other East African countries by 2003 (Singh et al., 2006), and recently has reached Middle-Eastern countries, as far as Iran (http://www.fao.org/newsroom/en/news/2008/1000805/ verified 1 July 2009). Therefore, stem rust has reemerged as a threat to wheat production worldwide.

Triticum timopheevii ssp. *armeniacum (*AAGG*;* syn. *T. araraticum),* a wild form of tetraploid wheat *T. timopheevii* (Morris and

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Sears, 1967; Dyck, 1992), has been exploited to transfer several rust resistance genes, such as *Sr36*, *Sr37, Sr40,* and *Lr18* to wheat (McIntosh and Luig 1973; McIntosh, 1988; Yamamori, 1994). *Sr36* has been widely used in Australia and is frequent in soft winter wheat cultivars of the U.S. (Bariana et al., 2001). Recently, *Sr36* was defeated by a new virulent isolate of Ug99 (Jin et al., 2007; http://maswheat.ucdavis.edu/protocols/StemRust/index.htm; verified 1 July 2009). Fortunately, several genes from various wild relatives of wheat, including *Sr22*, *Sr25*, *Sr32, Sr35, Sr39, Sr40,* and *Sr44,* confer resistance to the new Ug99 variants (Jin et al., 2007; Singh et al., 2005). Unfortunately, deployment of these genes in commercial cultivars is still in its infancy (Singh et al., 2005).

Sr40 was transferred into common wheat from accession PGR6195, which was collected in Turkey (Dyck, 1992). Linkage of *Sr40* to rust-resistance genes *Lr13, Lr23*, *Lr16*, and *Sr36* led to a tentative location on the short arm of chromosome 2B (Dyck, 1992), which was supported by C-banding analysis with a designation of the *Sr40*-containing segment as T2BL/2G#2S (Friebe et al., 1996). The G genome of *T. timopheevii* is most closely related to the B genome of common wheat; both the B and G genomes are closely related to the S genome of *Aegilops speltoides* (Dvorak and Zhang, 1990; Talbert et al., 1991), but most likely arose through different lineages (Jiang and Gill, 1994; Kilian et al., 2007). Seedling inoculations with race TTKS showed that *Sr40* confers resistance to Ug99, and the single gene stock with *Sr40*, RL6088, maintained stable resistance over two years in Kenyan field nurseries (Jin et al., 2007). Therefore deployment of this gene in commercial wheat cultivars would ensure protection against stem rust. Pyramiding *Sr40* with other stem-rust-resistance genes would enhance durability of stem rust resistance.

Pyramiding of several genes into one cultivar can be an effective strategy to deploy resistance genes to enhance durability of resistance to stem rust (Leonard and Szabo, 2005). Marker-assisted selection (MAS) is a powerful approach to facilitate new gene deployment and gene pyramiding. Molecular markers are available for only a few resistance genes such as *Sr2* (Hayden et al., 2004), *Sr22* (Paull et al., 1994; Khan et al., 2005), *Sr24* and *Sr26* (Mago et al., 2005), *Sr31* (Mago et al., 2005, Das et al., 2006), *Sr32* (Bariana et al., 2001), *Sr36* (Bariana et al., 2001; Tsilo et al., 2008), and *Sr39* (Gold et al., 1999). Some of these markers have been used in MAS programs, but markers linked to other genes are not diagnostic and/or remain to be improved. New and better markers for stem-rust-resistance genes are urgently needed to expedite deployment in adapted wheat lines. The objective of this study was to identify markers closely linked with *Sr40* for MAS in breeding programs.

MATERIALS AND METHODS

Plant Materials

Two mapping populations developed for this study are RL6088/Lakin and RL6088/2174. RL6088 was derived from the cross RL6071*7/PGR6195 (Dyck, 1992), while Lakin (PI 617032) and 2174 (PI 602595) are both moderately susceptible to susceptible hard winter wheat cultivars. The RL6088 stock was provided by Dr. Yue Jin, USDA-ARS Cereal Disease Laboratory, St. Paul, MN.

Stem Rust Evaluation

Reaction of both populations to stem rust race RKQQ was evaluated at seedling stage in the greenhouse at Kansas State University, Manhattan, KS. The RKQQ isolate used in this study was provided by Dr. Yue Jin, increased at Kansas State University, and tested on the standard *Sr*-gene differential set to verify the race identity. A total of 202 F_2 plants and their 88 randomly selected $F_{2:3}$ families of the population RL6088/Lakin and 107 F_2 plants and $F_{2:3}$ families of population RL6088/2174 were inoculated at the two-leaf stage. Rust rating of $F_{2:3}$ families was performed on over 20 inoculated seedlings. Before inoculation, urediniospores were recovered from a liquid N_2 tank and heat-shocked for 5 min in a water bath at 40°C. Spores were then suspended in Soltrol 170 light oil (Chevron Phillips Chemical Company, The Woodlands, TX) and heavily spray-inoculated. Plants were kept in a mist chamber at 23°C with 100% relative humidity for 16 h after inoculation, and then moved to a greenhouse bench at 20°C with 16 h daylength using supplemental growth lights. The stem rust symptoms were scored 14 d after inoculation when the rust pustules were fully erupted. Rust phenotypes were recorded based on infection types (ITs) using a 0–4 scale (Stakman et al., 1962; Roelfs and Martens, 1988). ITs of 2 or less were classified as *Sr40*-type on the basis of the reaction of RL6088, while ITs ≥ 2+ as 2174 or Lakin-types. Each parent was included as checks in each inoculation experiment.

Marker Analysis

Wheat leaf tissue was collected in a 2-mL tube for DNA isolation after rust evaluation. Collected leaf tissue was immediately dried in a freeze-dryer (ThermoSavant, Holbrook, NY) for 48 h and ground to fine powder using a Mixer Mill (MM300, Rotsch, Germany). Genomic DNA was isolated using a modified CTAB protocol (Saghai et al., 1984). Because *Sr40* was previously located on chromosome 2B, based on genetic analysis (Dyck, 1992), 83 SSR primers on chromosome 2B (Somers et al., 2004) were selected to screen the parents and resistant and susceptible bulks for polymorphism. Resistant and susceptible bulks were constructed separately by mixing genomic DNA of five homozygous resistant and five homozygous susceptible F_2 plants, respectively. Primers polymorphic between bulks were then used to screen the F_2 populations. For SSR analysis, a 13 μL PCR mixture contained 3 uL DNA template (20 ng/uL), 100 nM forward primer, 200 nM of reverse primer, 200 uM of dNTP, 1X ASB buffer (Bioline USA Inc., Taunton, MA), 25 mM $MgCl_2$, 1 U *Taq* polymerase, and 100 nM of fluorescence-dye-labeled M13 primer compatible with ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

PCR amplification was conducted using a touch-down program. Initially, the PCR mixture was denatured at 95°C for 5 min; the first five cycles started with 1 min of denaturing at 96°C, 5 min of annealing at 68°C with a decrease of 2°C for each subsequent cycle, and 1 min of extension at 72°C; in the next five cycles, the annealing temperature started at 58°C for 2 min with a decrease of 2°C for each subsequent cycle; the last 25 cycles ran 1 min at 96°C, 1 min at 50°C, and 1 min at 72°C with a final extension at 72°C for 5 min. The PCR products were analyzed in an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Marker data was scored using GeneMarker version 1.5 (SoftGenetics LLC, State College, PA). All data points were checked twice to remove ambiguous data.

Genetic linkage map was constructed by using JoinMap version 3.0 (Van Ooijen and Voorrips, 2001) with a LOD (logarithm of odds) threshold at 3.0. The genetic distance in centiMorgans (cM) was calculated according to Kosambi function (Kosambi, 1944).

RESULTS

Approximately 10 d after inoculation with stem rust isolate RKQQ, leaves of both susceptible parents were covered with medium to large oval or elongated reddish brown pustules lacking chlorosis with ITs ≥ 2+ for 2174 and > 3 for Lakin, while *Sr40* donor parent RL6088 had small pustules surrounded by necrosis on the infected leaves with an IT 1+. Therefore, rust symptoms in resistant plants were clearly distinct from those in susceptible plants.

Segregation of resistant and susceptible progenies in the population RL6088/2174 fitted well to the expected 3:1 Mendelian single gene ratio in F_2 generation and 1:2:1 (resistant:segregated:susceptible) ratio in the $F_{2:3}$ generation (Table 1). One F_2 plant that was scored as a 2+ (classified as 2174-type) segregated for *Sr40* in the F_3, and was misclassified. Marker analysis identified seven polymorphic SSRs between RL6088 and 2174, and these were mapped on chromosome 2B using the 107 F_2 plants from the cross RL6088/2174. The linkage map covered a total genetic distance of 15.2 cM (Fig. 1). *Sr40* was closest to marker locus *Xwmc474* (2.5 cM) at the proximal end and was flanked by *Xwmc661* on the distal end.

The segregation ratio among 202 F_2 plants from the RL6088/Lakin population was approximately 2 (low IT):1 (high IT), which significantly deviated from the expected 3:1 ratio (Table 1). A total of 88 F_2 plants were arbitrarily selected for F_3 progeny testing and the segregation ratio among the 88 $F_{2:3}$ families was 11 (homozygous resistant):46 (heterogeneous):31 (homozygous

Table 1. Seedling stem rust response segregation of *Sr40* among populations derived from crosses RL6088/Lakin and RL6088/2174 when inoculated with *Puccinia graminis* f. sp. *tritici* isolate RKQQ.

Population	Generation	Total	Observed ratio†	Expected ratio	X^2	*P*-value‡
RL6088/Lakin	F_2	202	133:69	3:1	9.04	0.0026
	F_3	88	11:46:31	1:2:1	9.27	0.0097
RL6088/2174	F_2	107	77:30	3:1	0.53	0.468
	F_3	107	19:57:31	1:2:1	3.15	0.207

†Segregation ratio for F_2 populations indicates *Sr40_* plants: *sr40sr40* plants and the ratio for F_3 segregation includes non-segregating resistant *Sr40* families: segregating *Sr40_* families: non-segregating susceptible *sr40* families.

‡*P*-value significant at 1% level.

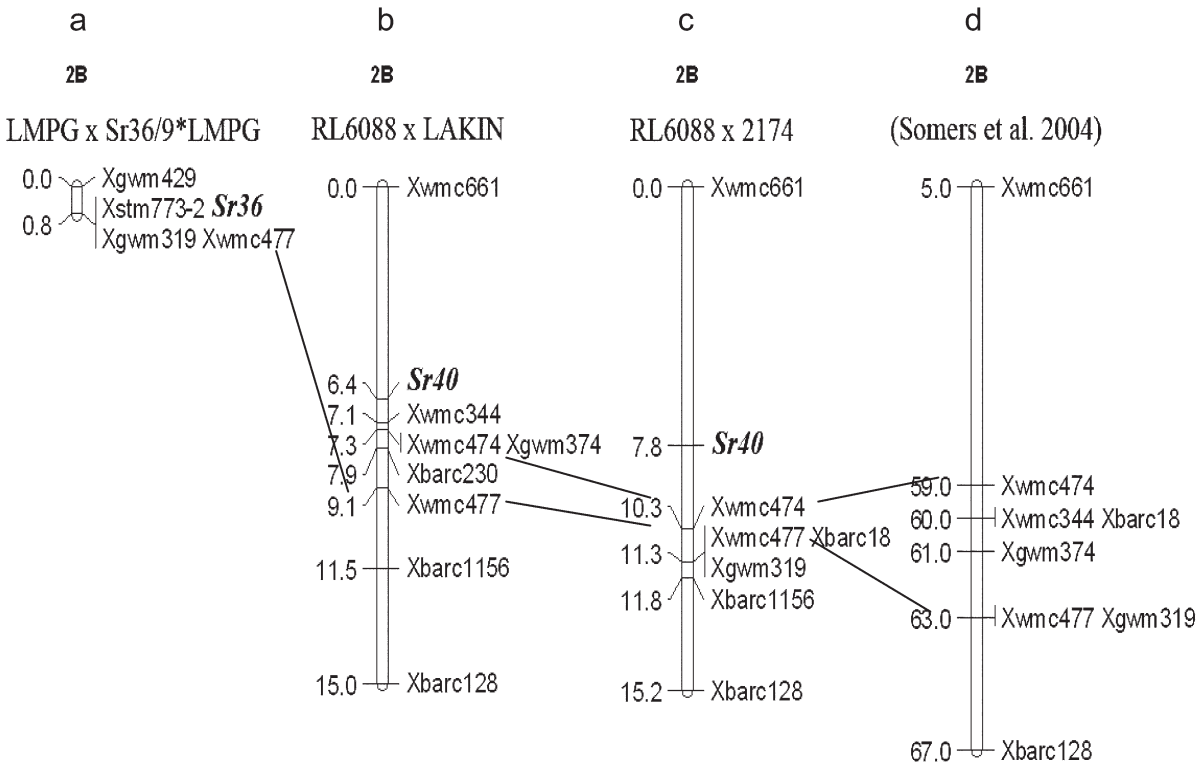

Figure 1. Location of *Sr40* on the wheat chromosome 2B map derived from the two mapping populations. Rust scores of F_2 populations were used to locate the *Sr40* on the linkage map. a) A linkage map of *Sr36* (Tsilo et al. 2008); b) linkage map derived from the RL6088/Lakin population; c) linkage map derived from the RL6088/2174 population; and d) a reference map of chromosome 2B (Somers et al. 2004). Genetic distance (cM) and marker name are listed on the left and right sites of each linkage map, respectively.

susceptible). Therefore, the segregation of resistance in both F_2 and $F_{2:3}$ generations of population RL6088/Lakin significantly deviated from the expected Mendelian ratio (Table 1).

In the population RL6088/Lakin, eight polymorphic markers were analyzed on 202 F_2 plants used for map construction. *Sr40* was located between marker loci *Xwmc661* and *Xwmc344* (Fig. 1). *Xwmc344* was most closely linked at 0.7 cM on the proximal end. *Xwmc474* and *Xgwm374* were also mapped at 0.9 cM proximal to *Sr40*. Segregation of marker alleles linked to *Sr40* also significantly deviated from the expected Mendelian 1:2:1 ratio in the 88 F_3 families tested (Table 2).

To investigate the relationship between *Sr36* and *Sr40*, three SSR markers, *Xgwm271*, *Xgwm319,* and *Xwmc477*, which are closely linked to *Sr36* (Tsilo et al., 2008), were analyzed in the two populations (Fig. 1). Two marker loci, *Xgwm319* and *Xwmc477*, were polymorphic between RL6088 and 2174, but only *Xwmc477* was polymorphic between RL6088 and Lakin (Table 2) and mapped at 2.7 and 3.5 cM proximal to *Sr40* in the two populations.

DISCUSSION

The results demonstrate that *Sr40* was inherited as a single dominant gene in the RL6088/2174 population. Deviation from Mendelian segregation was observed in the RL6088/Lakin population. In agreement with the phenotypic data, DNA markers most tightly linked to *Sr40* also showed segregation distortion (Table 2) in this population. Considering the normal segregation of the *Sr40 T. timopheevii*-derived chromatin in the 2174 background, preferential transmission of gametes with Lakin chromatin in this region is the simplest explanation for the non-Mendelian ratio. A reciprocal cross population (Lakin/RL6088) is being produced to further investigate the mechanism of segregation distortion. Chromosome 2B was identified as the chromosome "most affected by segregation distortion" in an inter-varietal linkage map of wheat (Paillard et al., 2003). Both *Sr36* and *Pm6*, a powdery mildew-resistance gene, were also transferred from the short arm of chromosome 2G of *T. timopheevii* to chromosome 2B and preferential transmission was observed (Allard and Shands, 1954; Brown-Guedira et al., 2003; Tsilo et al., 2008). Brown-Guedira et al., (1998) provided evidence of preferential transmission of chromosome 2G in cytogenetic stocks. Interestingly, the chromosome 2G *T. timopheevii* segment harboring *Sr36* is apparently preferentially transmitted, while the *Sr40*-containing segment is opposite in one of the two populations in this study.

Genetic maps from both populations were very similar with a slight variation in genetic distances between some SSRs and *Sr40* (Fig. 1). Both linkage maps had similar length (~15 cM) and most of the common markers showed the same order in two maps. *Sr40* was flanked by *Xwmc661* and *Xwmc474* or *Xwmc344* in each map; All three loci were mapped on the short arm of chromosome 2B in previous studies. *Xwmc474*, *Xwmc477,* and *Xgwm319* are mapped close to the centromere of chromosome 2B (Somers et al., 2004). The previously reported location of *Sr40* on chromosome 2BS (Dyck, 1992) was validated through molecular mapping. A total of 10 markers showed linkage to *Sr40* in the two populations. Among them, five markers were common between two populations and the other five markers were polymorphic in only one population (Table 2). Although the marker order is consistent with existing genetic maps (Somers et al., 2004), the ~15 cM map length represents significant suppression of recombination. This is not surprising given the origin of this resistance gene from the wild relative *T. timopheevii*. A similar compression of map distance was found for the *Sr36*-containing chromosome segment when crossed to homeologous chromosome 2B (Bariana et al., 2001, Tsilo et al., 2008).

Classical genetic mapping of *Sr40* indicated close linkage to the gene cluster of *Lr13*, *Lr23*, and *Sr36* (Dyck, 1992). *Xwmc477*, which co-segregates with *Sr36* (Tsilo et al., 2008), was mapped ~3 cM away from *Sr40* in the present study. This inferred genetic distance (~3 cM) between *Sr36* and *Sr40* is much closer than the genetic distance of 21.9 cM between *Sr40* and *Sr36* determined by Dyck (1992). The differences in map distance are due to the nature of the respective populations. By intercrossing *Sr36* and *Sr40* lines, homologous *T. timopheevii* 2G chromosomes are pairing at a high level and recombining frequently, yielding map distances that would be comparable to an inter-varietal map of hexaploid wheat chromosome 2B (Dyck, 1992). The decreased homology between 2B and 2G results in substantial recombination suppression.

Table 2. Segregation behavior of SSR markers on RL6088/Lakin and RL6088/2174-derived populations.

Population	Gene/ Marker	Total	RR†	RS	SS	Expected Ratio	X^2	*P*-Value
RL6088/Lakin	*Xwmc661*	88	15(190)	46(190/192)	27(192)	1:2:1	3.455	0.178
	Xwmc344	88	12(264)	46(264/248)	30(248)	1:2:1	7.545	0.023
	Xwmc474	88	12(150)	46(150/148)	30(148)	1:2:1	7.545	0.023
	Xgwm374	88	12(234)	46(234/217)	30(217)	1:2:1	7.545	0.023
	Xbarc230	88	12(218)	46(218/216)	30(216)	1:2:1	7.545	0.023
	Xwmc477	88	12(180)	45(180/182)	31(182)	1:2:1	8.25	0.016
RL6088/2174	*Xwmc661*	107	24(190)	56(190/214/228)	27(214/228)	1:2:1	0.402	0.818
	Xwmc474	107	20(150)	57(150/130)	30(130)	1:2:1	2.327	0.312
	Xwmc477	107	20(180)	57(180/176)	30(176)	1:2:1	2.327	0.312
	Xgwm319	107	20(192)	57(192/196)	30(196)	1:2:1	2.327	0.312
	Xbarc18	107	20(260)	57(260/236)	30(236)	1:2:1	2.327	0.312

†RR were homozygous for RL6088 allele; RS were heterozygous with both RL6088 and either Lakin or 2174 allele; SS were homozygous for Lakin or 2174 alleles.

Although *Sr40* has been transferred into several wheat backgrounds, it has not been deployed in commercial cultivars to date. The lack of focus on stem-rust-resistance breeding over the past few decades is one possible explanation for *Sr40* not being deployed. It is also possible that the *Sr40* carrying the *T. timopheevii* segment is associated with linkage drag. The widespread use of *Sr36,* also from the short arm of chromosome 2G, in commercial cultivars is not consistent with 2G being intrinsically associated with linkage drag. Assuming that linkage drag is not associated with the *Sr36–Sr40* interval, if *Sr36* and *Sr40* are intentionally recombined in coupling on the 2G segment, the linkage block will likely be maintained in future crosses in breeding programs due to decreased recombination with 2B. Recombining *Sr36* and *Sr40* into a single linkage block should be straightforward given the availability of *Puccinia graminis* f. sp. *tritici* races that differentiate the two genes and the molecular markers reported currently and previously (Tsilo et al., 2008).

Another limitation in most breeding programs has been the inability to detect *Sr40* in genetic backgrounds stacked with other effective resistance genes and no "super races" that would allow for gene postulation. The presence of *Sr40* could not be postulated by molecular markers, as they were not documented until the present study. We report markers closely linked to *Sr40* that should be effective tools for quick deployment of this gene in elite breeding materials, for gene pyramiding, and to aid in development of an *Sr36* linkage block.

To enhance the durability and effectiveness of a single resistance gene, gene pyramiding is an important strategy in breeding programs. Stacking of *Sr31, Sr24,* or *Sr25* in one genetic background expressed a high level of resistance to stem rust (Tomar and Menon, 2001). Although *Sr2* is a durable resistance gene, *Sr2* alone does not provide sufficient protection in stem rust epidemics, and a combination of *Sr2* with *Sr23* in the cultivar Selkirk demonstrated enhanced resistance to Ug99 (Singh et al., 2006). Therefore, a combination of *Sr40* with *Sr2* and/or other genes is an advisable strategy for stem rust control under epidemic conditions. The markers for *Sr40* developed in this study should be a useful tool to speed up deployment of *Sr40* in modern wheat cultivars.

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