Characterization of Stem Rust Resistance in Wheat Cultivar Gage

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ABSTRACT

Wheat (*Triticum* spp.) stem rust, caused by *Puccinia graminis* f. sp. *tritici* Eriks. and E. Henn. (*Pgt*), re-emerged as a devastating disease of wheat because of virulent race Ug99 (TTKSK). Many bread wheat (*T. aestivum* L.) cultivars grown in North America are susceptible to Ug99 or its derivative races that carry additional virulence. 'Gage' was released in 1963 mainly for its excellent field resistance to leaf rust (caused by *Puccinia triticina* Eriks) and stem rust. However, Gage's resistance has not been genetically characterized, which would facilitate its use in breeding programs. To better define the nature of the resistance in Gage, we created an F_2 population and the corresponding $F_{2:3}$ and $F_{4:5}$ families from crosses between Gage and stem rust susceptible cultivar 'Bill Brown'. Inheritance of resistance to *Pgt* race QFCSC and molecular marker analysis indicated that *Sr2* and additional genes explain the stem rust resistance of Gage. Using seedling plant infection types from the F_2, $F_{2:3}$, and $F_{4:5}$ families, we found that at least one dominant and, most likely, one recessive gene are involved in Gage's resistance. Seedling resistance genes acted independently of *Sr2* since *Sr2* is effective only at the adult plant stage.

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Abbreviations: APR, adult plant resistance; CAPS, cleaved amplified polymorphic sequence; CDL, Cereal Disease Laboratory, GBS, genotyping-by-sequencing; CTAB, cetyltrimethyl ammonium bromide; IT, infection type; PCR, polymerase chain reaction; *Pgt*, *Puccinia graminis* f. sp. *tritici* Eriks. and E. Henn.; QTL, quantitative trait loci; SNP, single-nucleotide polymorphism; SSR, simple sequence repeat; UNL, University of Nebraska–Lincoln;

Through the use of resistant cultivars and eradication of the alternate host, wheat (*Triticum* spp.) stem rust, caused by *Puccinia graminis* f. sp. *tritici* Eriks. and E. Henn. (*Pgt*), was controlled for more than five decades in North America (Jin and Singh 2006; Kolmer et al., 2007). In other wheat production areas of the world, the effort to control the disease has also continued through the successful use of resistant cultivars. However, wheat stem rust re-emerged as a devastating disease of bread wheat (*T. aestivum* L.) with the spread from East Africa of virulent race TTKSK (Roelfs and Martens, 1988; Pretorius et al., 2000), commonly known as Ug99. This new race is virulent on previously effective and globally important resistance genes such as *Sr31* (Pretorius et al.,

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2000; Jin et al., 2007). Due to the rapidly evolving nature of Ug99 (eight variants detected; Jin et al., 2008; Jin et al., 2009; Visser et al., 2011; Pretorius et al., 2012) several resistance genes originally described as effective are no longer useful (Singh et al., 2011).

For three decades before 1999, there were no major stem rust disease outbreaks globally except in Ethiopia where a major stem rust epidemic devastated widely grown wheat cultivar Enkoy (Singh et al., 2011). However, 90% of presently grown wheat cultivars worldwide are susceptible to Ug99 and its derivatives with their additional virulence (Jin and Singh, 2006; Fetch, 2007; Singh et al., 2008). While some wheat growers can use fungicides to control the disease, this is not an option for subsistence farmers in Africa or other developing countries as these chemicals are either expensive or not readily available. Furthermore, overreliance on fungicidal control of this disease may lead to the development of stem rust races that are resistant to the fungicide(s). Even for farmers in the developed world, the disease can be devastating if fungicide is not applied frequently, in a timely manner, or at high rates. In addition, using fungicides adds to production costs.

Hard red winter wheat cultivar Gage (Reg. No. 422, C.I. 13532; Johnson et. al., 1965) with the pedigree 'Pon ca'/3/'Mediterranean'/'Hope'//'Pawnee', showed excellent field resistance to leaf rust (*P. triticina*) and stem rust during its testing period in Nebraska starting from the late 1940s until its release in 1963 (Johnson et. al., 1965). It is likely that the parent cultivar Hope contributed the adult plant resistance (APR) gene *Sr2* and other genes to the resistance in Gage. Hope is believed to be a source of *Sr7b*, *Sr9d*, and *Sr17* (Green and Dyck, 1979). The APR from *Sr2* combined with resistance from other unknown minor genes has been called the *Sr2* complex (McIntosh, 1988; Rajaram et al., 1988; Roelfs et al., 1992; Singh et al., 2012) to which earlier cultivars such as Thatcher and Hope may have donated the minor genes. The APR gene *Sr2* is recessively inherited and is the only APR gene conferring resistance against all *Pgt* races since its introgression into hexaploid wheat in 1920s (Mago et al., 2011).

Among other stem rust resistant wheat cultivars grown that share the *Sr2* source cultivar Hope in their pedigrees, Gage is preferred and highly recommended as a source of stem rust resistance due to its more complete field resistance (Alan Roelfs, personal communication, 2009). For example, 'Scout 66' wheat, Reg. No. 487 C.I. 13996 (Schmidt et. al., 1971), is another hard red winter cultivar which was released in 1967 and had field stem rust resistance based on *Sr2*. However, Scout 66 was not as resistant as Gage at seedling stage to Ug99 and some of its derivative races. Scout 66 seedlings also were moderately susceptible to *Pgt* race QFCSC. Despite Gage's superior stem rust resistance compared to other *Sr2* wheat cultivars, its resistance has not been genetically characterized.

The recent development of molecular markers linked to stem rust resistance genes and the advancement of tools that allow marker scans of the whole genome facilitate genetic analysis and breeding in crop plants. The availability of microsatellite and other markers linked to wheat stem rust disease resistance, such as *Sr6, Sr9a, Sr13, SrWeb, Sr22, Sr24, Sr1RS-^{Amigo}, Sr26, Sr28, Sr32, Sr33, Sr35, Sr36, Sr39, Sr40, Sr42, Sr44, Sr45, Sr47, Sr51, Sr52, Sr53, Sr54, Lr19/Sr25* (Prins et al., 2001; Mago et al., 2005; Tsilo et al., 2007; Tsilo et al., 2008; Tsilo et al., 2009; Wu et al., 2009; Olson et al., 2010a, b; Zhang et al., 2010; Hiebert et al., 2010; Niu et al., 2011; Qi et al., 2011; Liu et al., 2011a; Liu et al., 2011b; Simons et al., 2011; Ghazvini et al., 2012; Klindworth et al., 2012; Rouse et al., 2012; Ghazvini et al., 2013) aids identification of known genes for stem rust resistance. Furthermore, the use of genotyping-by-sequencing (GBS) technology to detect and score single-nucleotide polymorphisms (SNPs) simultaneously (Deschamps et al., 2012) is particularly useful to investigate unknown stem rust resistance genes in wheat. Therefore, the objective of this research was to genetically characterize stem rust resistance in Gage by using molecular markers and infection phenotypes at seedling and adult plant stages

MATERIALS AND METHODS

Plant Materials

Gage seed (CI 13532) was obtained from the USDA–ARS National Small Grains Collection, Aberdeen, ID. Seed of Scout 66 was provided by the Nebraska Crop Improvement Association, and seed of stem-rust susceptible 'Bill Brown' (Reg. No. CV-133, PI 653260; Haley et al., 2008) was kindly provided by the Colorado State University wheat breeding program. Bill Brown is known to be susceptible to stem rust at the seedling and adult plant stages. Seedling resistance evaluation of these three lines was performed with 14 *Pgt* pathotypes including variants of Ug99 in the facilities of USDA–ARS the Cereal Disease Laboratory (CDL), St. Paul, MN. Seed of these three lines was planted in the University of Nebraska–Lincoln (UNL) greenhouse and inoculated with *Pgt* race QFCSC to confirm their infection response. The presence of *Sr2* was determined using the *csSr2* molecular marker (Mago et al., 2011). Sixty-four F_1 seeds were obtained from four crosses made between Bill Brown (female) and Gage in spring 2010. The parents, along with F_1 progenies, were screened for disease resistance at the seedling stage and rechecked for the *Sr2* linked molecular markers to ensure the crosses were properly made. Seeds from the F_2 generation were randomly selected, and 238 F_2 individuals were grown to the adult plant stage to produce $F_{2:3}$ seed. Lines were then advanced through single seed descent and $F_{4:5}$ families were developed as indicated in Fig. 1.

Inoculation and Stem Rust Resistance Evaluation

University of Nebraska–Lincoln (UNL)

F_2 seedlings (n = 176), two replications of $F_{4:5}$ seedlings (n = 238, eight to 10 seedlings per family per replication), and two

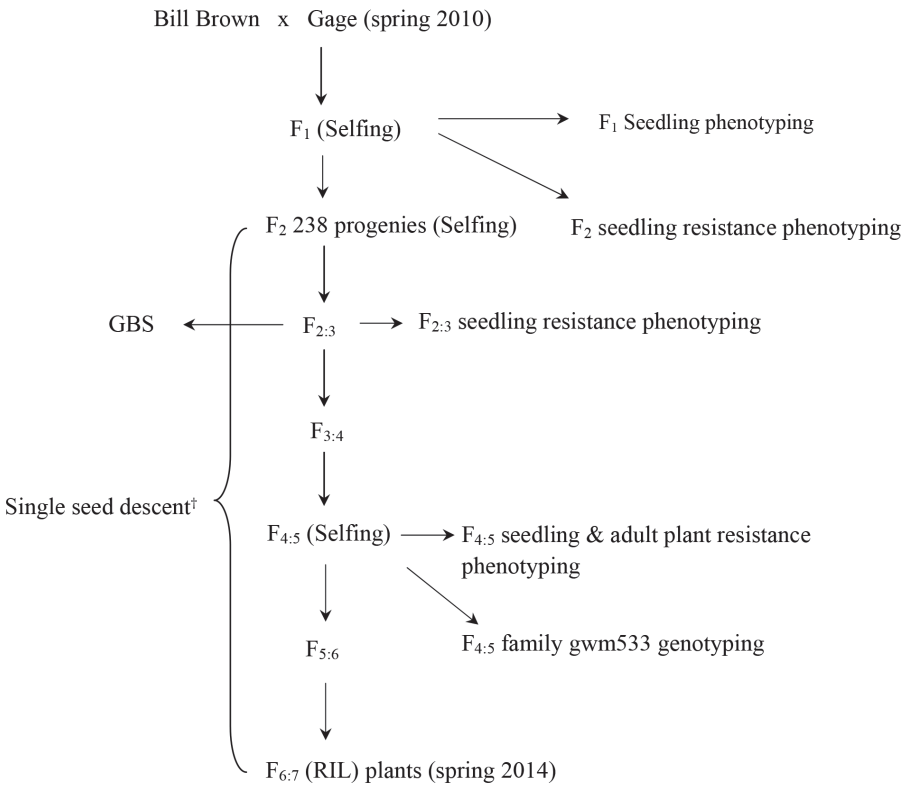

Figure 1. Development scheme of RILs from 'Bill Brown' × 'Gage' crosses. Dagger symbol (†) indicates single seed from single plant was used in each generation to develop $F_{6:7}$ RILs.

replications of $F_{4:5}$ adult plants (n = 196, three to five adult plants per family per replication) were grown and inoculated in the greenhouse with a field collected *Pgt* isolate typed as QFCSC by the CDL. Inoculum was maintained on susceptible cultivar 'McNair 701' and fresh urediniospores were used for inoculation throughout the study. Seedlings at the two-to-three leaf stage and adult plants at anthesis were inoculated by using a pressurized atomizer to uniformly spray an aqueous suspension of freshly harvested urediniospores of race QFCSC (1 mg mL^{-1}) containing Tween 20 (Sigma-Aldrich Corp.) (Pretorius et al., 2007; Mago et al., 2011). Inoculated plants were kept in a dew chamber overnight with 100% humidity at 18°C. Plants were then moved to a greenhouse room (27°C day and 22°C night) with 12-h photoperiod.

Each F_2 plant and $F_{4:5}$ family grown in the greenhouse was scored for disease response 14 d after inoculation, and seedling infection types (IT) were classified on a 0 to 4 scale (Stakman et al., 1962). Families with ITs 0 to 2 were classified as resistant; and ITs 3 and 4 were classified as susceptible. Plant response to infection of $F_{4:5}$ families at the adult plant stage based on lesion size on the stem was recorded 16 d after inoculation as R (resistant), MR (moderately resistant), MS (moderately susceptible), or S (susceptible) (Roelfs et. al., 1992).

St. Paul, Minnesota

The parent lines were screened at the seedling stage to races TTKSK, TTKST, TTTSK, TRTTF, TTTTF, TPMKC, RKQQC, RCRSC, QTHJC, QFCSC, MCCFC, QCCSM, QCCJB, and SCCSC (Rouse et al., 2011). Three separate F_2 populations of Bill Brown × Gage (n = 164, 202, 170) were grown in the greenhouse and evaluated for seedling resistance to *Pgt* race QFCSC, isolate 06ND76C, according to previously described methods (Rouse et al., 2011). For a set of $F_{2:3}$ families evaluated against *Pgt* race TTKSK (Ug99) at seedling stage, 15 to 20 plants were grown in pots containing vermiculite (Sun Gro Horticulture) in a biocontainment safety level-3 facility at the University of Minnesota in February 2012. Urediniospores of *Pgt* races TTKSK (04KEN156/04) and QFCSC were retrieved from storage at −80°C and heat shocked at 45°C for 15 min. Spores were rehydrated by placing the capsules in an air-tight container at 80% humidity maintained by a KOH solution for 2 to 4 h. Urediniospores were then suspended in a light-weight mineral oil (Soltrol 70; Conoco–Phillips Inc.) and sprayed onto seedlings. Inoculated plants were incubated in dew chambers at 22°C ± 2°C and 100% relative humidity overnight. Plants were then moved to greenhouse at 22°°C day and 18°C night ±2°C with 16 h of photoperiod provided by supplemental lighting.

Njoro, Kenya

The adult plant parental lines were screened in the field for stem rust resistance reaction and severity in a coordinated USDA–ARS stem rust nursery in Njoro, Kenya during 2012. The nursery was inoculated with a bulk collection of predominantly, if not exclusively, *Pgt* race TTKST. Race TTKST possesses virulence to stem rust resistance gene *Sr24* (Jin et al., 2008). Ug99 susceptible cultivars were planted as spreader rows and suspension of urediniospores was misted on to the spreader rows to initiate disease development. Based on pustule size and associated necrosis or chlorosis, infection responses were classified into four discrete categories: resistant, moderately resistant, moderately susceptible, and susceptible. Stem rust severity was

measured according to modified 0 to 100% Cobb Scale (Peterson et al., 1948). The severity was estimated visually on a whole-plot basis and scored as a percentage disease cover on internodes and peduncle. Lines were evaluated for stem rust severity two to three times between heading and plant maturity. Stem rust severity at the soft-dough to mid-dough stages of plant growth stages (Zadoks et al., 1974) was used to represent the final disease scores.

DNA Isolation and Polymerase Chain Reaction Conditions

DNA from composite bulked leaves of ten 2-wk-old $F_{2:3}$ seedlings was extracted using a cetyltrimethyl ammonium bromide (CTAB) procedure (Saghai-Maroof et al., 1984). One gram of fresh leaves was placed between the two rollers of a sap extraction apparatus (Ravenel Specialties) and 5 mL of extraction buffer (50 mM Tris-HCL, 25 mM EDTA, 1 M NaCl, 1% CTAB, 1 mM of 1,10-phenanthroline, 0.15% 2-mercaptoethanol) was slowly added to the rollers. The extracted DNA was then resuspended in 200 μL of TE buffer and the DNA concentration quantified by spectrophotometry (Beckman Coulter, DU 730 life science UV spectrophotometer). Composites of four to six leaves of $F_{4:5}$ families and for another set of F_2 plants was extracted using automated DNA extraction methods in which young leaves were collected and lyophilized and DNA was extracted using BioSprint 96 DNA plant kit following the manufacturer's instructions (Qiagen).

Polymerase chain reaction (PCR) for simple sequence repeat (SSR) marker *gwm533*, which is tightly linked to *Sr2*, was performed in 20 μL volume with 4 μL 25 ng DNA template, 7.9 μL ddH_20, 1.6 μL 2 mM $MgCl_2$, 0.4 μL 0.2 mM dNTPs, 1 μL 10 pmol of each primer, 4 μL 1x GoTaq Flexi buffer, and 0.1 μL 0.5U GoTaq Flexi Taq Polymerase (Promega). Amplification conditions included an initial denaturation at 95°C for 5 min followed by 37 cycles of 95°C (45 s), primer annealing temperature 60°C (45 s), and 72°C (45 s), and final extensions of 72°C (5 min) and 15°C (1 min). DNA from the F_2 generation, which was used to create RIL mapping population and the corresponding $F_{4:5}$ families, were genotyped with *gwm533* SSR microsatellite marker (indicative of *Sr2*). The *gwm533* SSR marker product was separated on a 2% (w/v) agarose gel. Polymerase chain reaction for cleaved amplified polymorphic sequence (CAPS) marker *csSr2* was performed in 25 μL volume with 3μL 25ng DNA template, 2.5μL 10x buffer (50 mM KCl, 10mM tris-HCl and 0.1% Triton X), 2 μL 1.5 mM $MgCl_2$, 0.2 mM 2.5 μL dNTP, 10.7 μL dd H_2O, 4 μL Primer, and 0.3 μL1U Taq polymerase. The PCR conditions are the same as described for *gwm533* SSR marker. For CAPS analysis, 5 μL of mix consisting of 2.5 mL of 10x NEB buffer 4 and 0.5 μL of BspHI (10 U μL^{-1}; NEB) was added after the PCR was done and the product was incubated in 37°C water bath for 30 min (Mago et al., 2010). Digestion products were separated using 12% native polyacrylamide gels.

DNA Marker Analysis for Known Stem Rust Genes and Genotyping-by-Sequencing

Gage and Bill Brown were screened with molecular markers for *Sr6, Sr9a, SrWeb, Sr24, Sr1RS^{Amigo}, Sr26, Sr36, Sr42, Sr54,* and *Lr19/Sr25* at the USDA–ARS Eastern Regional Genotyping Laboratory, Raleigh, NC. The PCR for the majority of the SSR and sequence-tagged site markers was performed in a 12 μL volume consisting of 1x PCR buffer (10 mM Tris-HCL, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 0.2 mM of each dNTP, 250 or 500 nM each of forward and reverse primers, 0.45 units Taq polymerase, and 40 to 80 ng genomic DNA. The forward primers were direct-labeled with the fluorescent dye 6-FAM, HEX, or NED at the 5' end. The PCR reaction for the *FSD+RSA* primers consisted of 1x PCR buffer, 0.2 mM of each dNTP, 12 pmol FSD 6-FAM-labelled forward primer, 3.5 pmol RSA reverse primer, 1.0 unit Taq polymerase, and 40 to 80 ng genomic DNA in a final volume of 12 μL (Hiebert et. al., 2011). The reaction for *wmc453* and *cfd43* consisted of 1x PCR buffer, 0.2 mM of each dNTP, 40 nM forward M13-tailed primer, 300 nM reverse primer, 300 nM M13 6-FAM-labelled primer, 0.18 μL Taq polymerase, and 40 to 80 ng genomic DNA in a final volume of 12 μL. The size of the PCR product includes the M13 tail (5'-CACGACGTTGTAAAACGAC-3'). The PCR cycling conditions were 95°C for 2 min, 35 cycles of 94°C for 30 sec, X°C for 30 sec, and 72°C for 45 sec with a final extension at 72°C for 5 min, where X is the annealing temperature for each marker. The annealing temperature was 44°C for *FSD+RSA*, 50°C for *cfd43* and *wmc453*, 55°C for *barc71* and *scm9*, 56°C for *Sr26#43*, 58°C for *barc183*, 60°C for *cfd49*, *cfd270*, *Gb*, *gpw5182*, *gwm47*, and 61°C for *wmc170* and *wmc477*. Amplifications were performed using an Eppendorf Mastercycler (Eppendorf AG). The PCR products were mixed with Hi-Di formamide and GeneScan 500 LIZ size standard (Life Technologies). Sizing of PCR fragments was performed by capillary electrophoresis using an ABI3730xl DNA Analyzer (Life Technologies). Data were analyzed using GeneMarker v1.85 (SoftGenetics, 2009). Screening for *Sr2* and *Sr39* was performed at Department of Agronomy and Horticulture, University of Nebraska–Lincoln. The PCR condition for *Sr39#22r* was the same as PCR condition for markers *gwm533* and *csSr2* described above except the annealing temperature for *Sr39#22r* was 58°C.

Genomic DNA was extracted from bulked leaves of ten 2-wk-old seedlings of $F_{2:3}$ families using the CTAB procedures described above. The resulting DNA was quantified in plates using PicoGreen (Life Technologies) and concentrations were normalized. The GBS libraries were constructed according to Poland et al. (2012) in 95-plex using the restriction enzymes *Pst*I and MspI and the P384A adaptor set. Briefly, genomic DNA was codigested with the restriction enzymes *Pst*I and *Msp*I and barcoded adapters were ligated to individual samples. Samples were pooled by plate into a single library and amplified using PCR. Each library was sequenced on a single lane of Illumina HiSeq 2000 (University of Missouri DNA Core Facility). The SNPs were identified using the custom pipeline of Poland et al. (2012). Briefly, putative SNPs were called by internal alignment of 64 bp tags with an allowable mismatch of up to 3 bp. An F-test for independence between aligned tag pairs with p-value < 0.001 was used to identify SNPs that were allelic.

Statistical Analysis

To predict the number of segregating genes, F_2 generation and $F_{4:5}$ families were qualitatively classified as resistant or susceptible according to ITs. The observed segregation ratios were compared

to expected using Chi-square test of goodness of fit with α = 0.05. When the probability value was less than 0.05 ($P < 0.05$), the observed ratio significantly deviated from expected ratios.

Disease infection type scores for each $F_{2:3}$ family based on Stakman's 0 to 4 scale were linearized according to Zhang's procedure (Zhang et al., 2011) for single marker and other statistical analysis. Categorical Stakman infection types on the 0 to 4 scale (Stakman et al., 1962) were converted to a linearized 0 to 9 scale removing '+', '−' , and ';' notations used in the Stakman scale. The 0 to 4 Stakman scale corresponds to distinct categories of infection types as follows: '0' = no visible uredinia or hypersensitive flecking, ';' = hypersensitive flecking, '1' = small, round uredinia with necrosis or chlorosis, '2' = small- to medium-sized uredinia with green islands surrounded by chlorosis, '3' = medium-sized uredinia with or without chlorosis, '4' = large uredinia without chlorosis. For plants with heterogeneous infection types, all infection types were recorded. For each infection type, '+' or '−' was used to indicate size variation compared to typical infection types. Stakman ITs '0', ';', '1−', '1', '1+', '2−', '2', '2+', '3−', '3', '3+', and '4' were converted to linear values 0, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 9, respectively. For heterogeneous Stakman ITs such as ';13' the ITs were first collapsed to ';3' and then the average of the two corresponding linear values was used. To estimate the number of segregating genes in the $F_{2:3}$ families, the families were qualitatively categorized as resistant if they had Zhang's linearized scores between 5 and 6 (similar to Gage); segregating with scores greater than 6 and less than 8, and susceptible with scores above 8 (similar to Bill Brown).

The GBS-generated SNPs were filtered based on less than 50% missing values, uniqueness, and fit of homozygous segregation ratios to the 1:1 expectation to exclude strongly skewed markers. The resulting set of 596 SNPs was used to construct a linkage map and analyze the association between markers and seedling stem rust resistance. A forward–backward model selection algorithm described in Lorenz (2013) was used to identify the most significant markers associated with seedling resistance in the population. Concisely, all markers were first fit one by one. The marker with the lowest *p*-value was added to the model and all the remaining markers were fitted again one by one. The next marker with the lowest *p*-value was then added to the model and this process repeated. For every forward inclusion step, backward exclusion was done by removing any markers on model refit with significant levels that dropped below the statistical limit. A *p*-value for both marker inclusion and exclusion was set to 0.05. The model selection algorithm was implemented in custom R scripts provided by Lorenz (2013). Correlation analysis was used to assess linkage disequilibrium between the most significant seedling resistance markers and APR marker *gwm533*. The percentage of seedling resistance variance explained by a GBS SNP was estimated as the coefficient of determination (R^2) using single marker analysis from a simple linear regression models procedure (Wang et al., 2006).

MapDisto v1.7.7 (Lorieux, 2012) was used to construct simple linkage groups of GBS SNPs and to identify quantitative trait loci (QTLs) for Zhang's linearized seedling stem rust scores of $F_{2:3}$ families. The GBS-derived SNP tags were analyzed by basic local alignment search tool for nucleotide sequences (BLASTn, www.ncbi.nlm.nih.gov/) using the International Wheat Genome Sequencing Consortium (IWGSC) build 1 (version 20) of the chromosome survey sequence of *Triticum aestivum* (http://plants.ensemble.org, accessed 13 Nov. 2012).

Table 1. The Stakman scale† seedling infection type of Gage (resistant parent), Bill Brown (susceptible parent), Scout 66 (resistant cultivar), and LMPG-6 (susceptible cultivar) tested at USDA Cereal Disease Laboratory.

	Cultivar			
Race (isolate)	Gage	Scout 66	Bill Brown	LMPG-6
TTKSK = Ug99 (04KEN156/04)	2	22+	3+	3+
TTKSK = Ug99 (04KEN156/04)	2	2+/3+	3+	3+
TTKST = Ug99+Sr24 (06KEN19-V-3)	22+	2+	3+	3+
TTTSK = Ug99+Sr36 (07KEN24–2)	22+	2+/3+	3+	3+
TRTTF‡ (06YEM34–1)	3+	3+	3+	3+
TTTTF§(01MN84A-1–2)	4	4	4/0;1	4
TPMKC§ (74MN1409)	4	4/0;1	4	4
RKQQC§ (99KS76A-1)	2+3	3+/32+	4	4
RCRSC§	32+	4	4	4
QTHJC§ (75ND717C)	3	4	4	4
QFCSC§ (03ND76C)	2	3+/0;	4	4
MCCFC§ (59KS19)	4	4	4	4
QCCSM§	13–	33+	33+	4/;3+Y
QCCJB§	3	4/0;1	4	4
SCCSC§	32+	3+	3+	4
TTKSK¶ (predominant race in Njoro, Kenya)	5MR	5MR	50S	

† 0 and ; = resistant, 2 = resistant, 22+ = resistant with different pustule on same plants, 3+ = susceptible, 2+/3+ = resistant and susceptible plants, 4 = very susceptible, Y = bigger postules at tip, smaller postules at base of leaf.

‡ Yemen origin.

§ U.S. origin.

¶ Njoro, Kenya field race where TTKSK and lineage predominate. Evaluation was based on percentage cover of the internode and peduncle. Plant response based on pustule size as MR = moderately resistant, S = susceptible.

RESULTS AND DISCUSSION

Seedling Resistance Genetic Analysis

In the initial parent screen, Gage was resistant to *Pgt* races QFCSC and TTKSK at the seedling stage. Compared to the heterogeneous reaction of Scout 66, which also contains cultivar Hope in its pedigree, Gage was generally more resistant to stem rust including the North American race QFCSC (Table 1). Of the known possible seedling resistance genes that Hope (*Sr7b, Sr9d,* and *Sr17*) could have contributed to the seedling resistance of Gage, the contribution of *Sr9d* and *Sr17* as single gene sources of resistance can be excluded as QFCSC is virulent on both genes and *Sr2* is effective only at the adult plant stage. Although the initial evaluation to Ug99 races indicated seedling resistance, in a subsequent evaluation of $F_{2:3}$ families, Gage and all $F_{2:3}$ families were susceptible (IT = 3) or moderately susceptible (IT = 2+3/3) at the seedling stage to TTKSK. Although we verified the existence of resistance gene *Sr2* in Gage, its effectiveness and phenotypic expression is more reliable at the adult plant stage

Table 2. Segregation of seedling resistance and Chi-square test to determine the probability of the observed data fitting the expected genetic ratios from F_2 plants and $F_{2:3}$ and $F_{4:5}$ families. Stem rust race QFCSC was used at the Cereal Disease Laboratory (CDL). At the University of Nebraska–Lincoln (UNL), plants were infected with a field race believed to be QFCSC as typed by CDL.

Generation/ environment	Number of plants/families			Expected ratio†	χ2	*p*-value
	Resistant	Susceptible				
F_2/CDL Group 1	137	27		3R:1S	6.37	0.01
				13R:3S	0.64	0.43
F_2/CDL Group 2	161	41		3R:1S	2.49	0.11
				13R:3S	0.29	0.59
F_2/CDL Group 3	141	29		3R:1S	5.34	0.02
				13R:3S	0.31	0.58
F_2/CDL Total	439	97		3R:1S	13.62	<0.01
				13R:3S	0.18	0.67
F_2/UNL	128	48		3R:1S	0.48	0.49
				13R:3S	8.39	<0.01
F2:3/CDL	Resistant	Segregating	Susceptible			
	51	114	73	1R:2Seg:1S	4.49	0.12
				7R:8Seg:1S	254.44	<0.01
F4:5/UNL	Resistant	Susceptible				
	185	53		0.563R:0.438S‡	44.6	<0.01
				0.809R:0.191S§	1.50	0.22

† Expected ratio 3R:1S = Single dominant gene, 13R:3S = one dominant and one recessive gene at F_2 generation.

‡ Expected ratio for single dominant gene considering segregating $F_{4:5}$ families as resistant.

§ Expected ratio for one dominant and one recessive gene considering segregating $F_{4:5}$ families as resistant.

(Sunderwirth and Roelfs, 1980). Scout 66 was heterogeneous for reaction to *Pgt* races making its comparison to the resistance in Gage difficult.

All F_1 seedlings of Bill Brown × Gage were resistant to race QFCSC indicating the presence of at least one dominant seedling resistance gene. In three separate F_2 seedling infection type assessments against race QFCSC at the CDL, individual plants were rated according to Stakman ITs and categorized into resistant and susceptible classes. The ratio that best fit the qualitative data was Gage having one dominant and one recessive resistance gene (13 resistant: 3 susceptible) (Table 2). However, seedling F_2 plants tested with the field race QFCSC at UNL classified plants into 128 resistant and 48 susceptible (Table 2), which fits a single dominant gene model ($\chi^2 = 0.48$; $P = 0.49$). Moreover, among the tested 238 $F_{2:3}$ families for race QFCSC seedling infection and classified qualitatively as described above, 51 families were resistant, 114 families segregated, and 73 families were susceptible, which again suggested the involvement of a single dominant resistance gene. The frequency distributions of the average Zhang's linearized IT score of the $F_{2:3}$ families, which we used to classify the families as resistant, segregating, and susceptible, showed that the average value of the majority of the families was between the homozygous resistant and susceptible parental infection types (Fig. 2). This result is consistent with multiple genes (major and minor) being involved in seedling resistance. The $F_{4:5}$ families seedling infection phenotype to race QFCSC was consistent with segregation of one dominant and one recessive gene (Table 2) as was observed in the F_2 generation with field race QFCSC. In summary, our seedling data support the presence of at least one dominant seedling resistance gene, and the replicated F_2 and $F_{4:5}$ data support a second recessive gene. However, the qualitatively classified $F_{2:3}$ data suggested a single dominant gene and possible additional minor genes.

Adult Plant Resistance Genetic Analysis

The observed adult plant disease response of $F_{4:5}$ families in general showed continuous variation ranging between the resistant and susceptible parents. The families were qualitatively classified as R, MR, MS, and S according to lesion size on the stem. Contingency analysis indicated that APR and the qualitative seedling resistance in $F_{4:5}$ families were related in that seedling resistant plants were largely resistant in the adult plant stage. However, the highly significant χ^2 ($\chi^2 = 40.55$; $P < 0.01$; Table 3) suggested that genes) not conditioning resistance at seedling stage also contributed to the APR. This result is best seen in seedling susceptible plants that were adult plant resistant.

Unexpectedly, 25 families with seedling resistance were moderately susceptible (21) or susceptible (4) at the adult plant stage. This result could be due to temperature sensitivity of the resistance gene(s) or the heat and humidity, which favored high stem rust development in the summer greenhouses when this assay was done.

As previously recognized (McIntosh, 1988; Brown, 1997; Eagles et al., 2001; Spielmeyer et al., 2003; Kota et

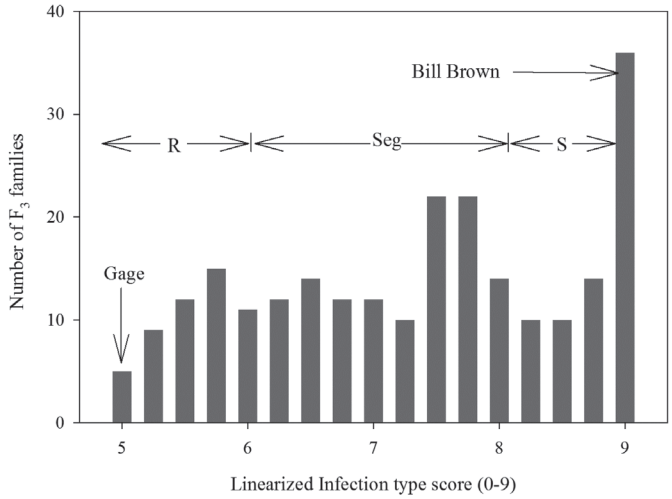

Figure 2. Frequency distribution of $F_{2:3}$ family means of Zhang's linearized stem rust seedling scores (Zhang et al., 2011). Families with mean scores 5 to 6 were categorized as R = resistant, >6 and <8 were categorized as Seg = segregating, and ≥8 were categorized as S = susceptible.

Table 3. Test of independence in $F_{4:5}$ families between seedling and adult plant disease resistance to race QFCSC and between *Sr2*-linked marker *gwm533* with seedling and adult plant resistance.

	Adult plant disease response and respective number of families†			
Seedling phenotype	R	MR	MS	S
R	12	111	21	4
S	1	17	13	13
Total	13	128	34	17
χ2 value	40.55***			

	Seedling disease response		Adult plant disease response			
gwm533	R	S	R	MR	MS	S
G/G	87	21	11	73	9	2
B/G	17	2	0	13	4	0
B/B	67	25	2	43	23	16
Total	171	48	13	129	36	18
χ2 value	3.31ns‡		35.40***			

*** Significant at $\alpha < 0.001$; indicates the marker and disease response are not independent.

† R, resistant; MR, moderately resistant; MS, moderately susceptible; S, susceptible.

‡ ns, not significant; indicates the marker and disease response are independent.

al., 2006; Mago et al., 2011), it was very difficult to follow the adult plant phenotype of *Sr2* in segregating families. That 18 of 44 $F_{4:5}$ families with seedling susceptibility were adult plant resistant is consistent with the presence of a recessive gene for APR (i.e., *Sr2*) segregating in this population ($\chi^2 = 0.14$, $P = 0.70$).

Marker Analysis

Gage was positive for the known *Sr2* stem rust resistance gene marker alleles as indicated by *csSr2* and *gwm533* markers (Table 4). However it was not positive for the other stem rust resistance gene markers including markers indicative of *Sr6, Sr9a, SrWeb, Sr24, 1RS, Sr26, Sr36, Sr39, Sr42, Sr54,* and *Lr19/Sr25.* Hence we do not believe Gage contains *Sr6, Sr9a, SrWeb, Sr24, 1RS, Sr26, Sr36, Sr39, Sr42, Sr54,* and *Lr19/Sr25.* Bill Brown carries the null allele for *XcsSr2*; hence, *csSr2* segregated as a dominant marker in the F_2 progenies as 174 present:71 absent (expected ratio = 3 present:1 absent; $\chi^2 = 2.07$; $P = 0.15$). The SSR marker *gwm533* segregated codominantly and was used for subsequent analyses. *Gwm533* segregated as expected in the Bill Brown × Gage F_2 plants (54 $Xgwm533_{Gage/Gage}$:78 $Xgwm533_{BillBrown/Gage}$:57 $Xgwm533_{BillBrown/BillBrown}$; expected ratio = 1:2:1; $\chi^2 = 5.86$; $P = 0.05$) and $F_{4:5}$ families (108 $Xgwm533_{Gage/Gage}$:19 $Xgwm533_{BillBrown/Gage}$:97 $Xgwm533_{Billbrown/Billbrown}$; expected ratio 0.4375:0.125:0.4375; $\chi^2 = 3.92$; $P = 0.14$).

Table 4. Molecular marker analysis of wheat parents 'Gage' and 'Bill Brown' for known stem rust resistance genes.

		Marker analysis†			
Gene	Marker	Bill Brown	Gage	Positive control	Reference
Sr2	*csSr2, gwm533*	–	+	Hartog, Hope	Mago et al., 2010
Sr6	*wmc453, cfd43*	–	–	Red Egyptian	Tsilo et al., 2009
Sr9a	*gwm47*	–	–	Red Egyptian	Tsilo et al., 2007
SrWeb	*gwm47*	–	–	Webster (RL6201)	Hiebert et al., 2010
Sr24	*barc71*	–	–	McCormick	Mago et al., 2005
1RS	*scm9*	–	–	McCormick (224 bp),	Saal and Wricke 1999
Sr26	*Sr26#43*	–	–	Avocet	Mago et al., 2005
Sr36	*wmc477*	–	–	Neuse	Tsilo et al., 2008
Sr39	*Sr39#22r*	–	–	U5935-2-3	Mago et al., 2009
Sr42/ SrCad	*barc183, cfd49, FSD+RSA, gpw5182*	–	–	Norin40, AC Cadillac	Hiebert et al., 2011 Ghazvini et. al., 2012
Sr54	*cfd270, wmc170*	–	–	Norin40	Ghazvini et al., 2013
Lr19/Sr25	*Gb*	–	–	Wheatear	Prins et al., 2001

† presence (+) or absence (–) of the genes according to corresponding marker alleles.

Two complementary approaches were used for QTL analysis. Genetic linkage construction of GBS generated SNPs using MapDisto v. 1.7.7 software (Lorieux, 2012) resulted in 39 groups of which one of the groups contained a putative major QTL for seedling resistance in $F_{2:3}$ families (Fig. 3). This QTL explained 43% of the variation for seedling stem rust resistance in the $F_{2:3}$ families (Table 5). The QTL identified by composite interval mapping included two (*gbs8942* and *gbs6398*) of three markers that were also

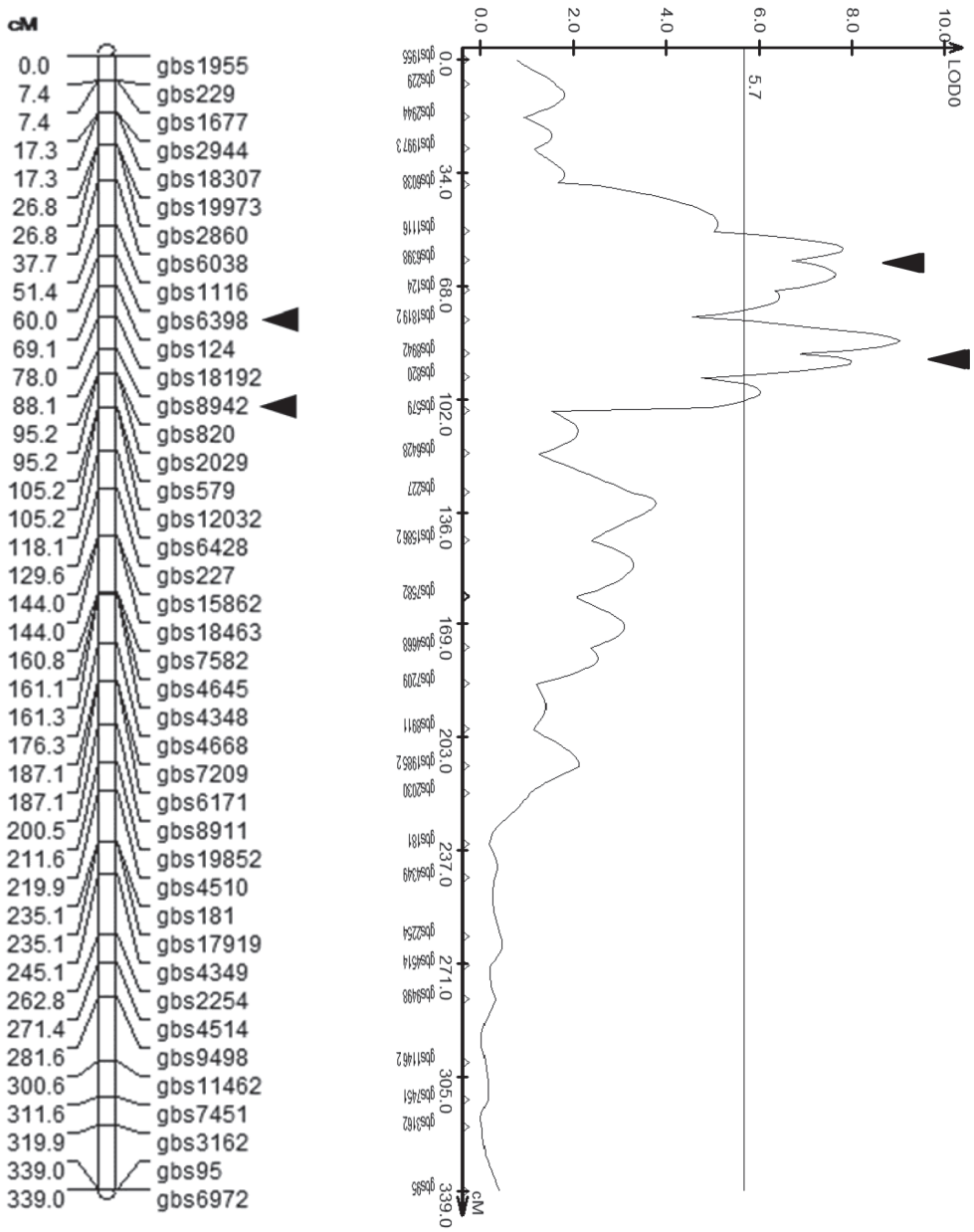

Figure 3. Linkage group containing strong seedling stem rust QTLs for inoculated QFCSC indicated with arrow. The scaffold assigned the markers on chromosome 4AL. Locus marker names are indicated on the right side of the chromosome and values to the left side of the chromosome indicate genetic distance (cM).

identified by the model selection algorithm. Moreover, test of segregation independence in $F_{2:3}$ families indicated that the segregation of seedling resistance is linked to the segregation of the GBS-identified SNPs and is independent of the segregation of the *Sr2*-associated SSR marker, *gwm533* (Table 6), as would be expected for an APR gene. The SNP sequences for *gbs8942* and *gbs6398* both aligned in BLASTn searches with scaffolds derived from chromosome 4AL. Interestingly, *Sr7b* is also mapped to chromosome 4AL and is one of the genes that Hope, the parent line of Gage, is known to carry. Allelism tests are needed to determine if the QTL identified here is allelic to *Sr7b*.

The model selection algorithm also identified a third SNP, *gbs3863*, as associated with a QTL. The QTL analysis also identified this SNP, but it was linked to a minor QTL (R^2 = 0.09). The test of segregation independence indicated that *gbs3863* was related to the seedling resistance (Table 6). The BLASTn search for *gbs3863* did not identify any homologous scaffolds in the wheat genome sequence. Furthermore, the other three markers in the putative linkage group were homologous to scaffolds derived from three different chromosomes.

After two more selfing generations, the APR marker *gwm533* segregated in the $F_{4:5}$ families, again independently from the seedling infection (χ^2 = 3.31; P = 0.19), but was associated with adult plant disease resistance (χ^2 = 35.40; $P <$ 0.01), which confirms *Sr2* as an APR gene in Gage (Table 3).

Table 5. The most significant GBS SNPs linked to seedling QTL and the amount of variation explained

Marker†	R^2 (%)‡	GBS tag sequence
gbs8942	43	TGCAGATTAACGGAGAAGA-CATCCACGCGGAAAACC AGTTCGCTACCGTCTGGATCTTAGTG**[A/G]**
gbs6398	40	TGCAGTGGCAGCG**[A/T]**GCCGCGTTCCATTTC-CAGCCGAGATCGGAAGAGCGGTTCAGCAG-GAATG
gbs3863	9	TGCAGGACATGTGGCGAA**[A/G]** TTCTCTTCCCATACGGGCCAATTGCCACGTT-GACACAAGGGAGA

† Temporary designation; not following formal nomenclature procedure.

‡ Seedling phenotypic variation explained by the best possible QTLs.

Table 6. Chi-square test of segregation independence between $F_{2:3}$ seedling infection phenotype (Zhang's linearized score) and GBS markers that were associated with QTLs for quantitative seedling stem rust resistance.

	$F_{2:3}$ seedling, QFCSC infection			
Marker†	Resistant (5–6)	Segregating (>6 to <8) number of families	Susceptible (≥8)	χ^2
gbs8942				110.55***
G/G	38	22	2	
G/B	7	52	15	
B/B	2	27	41	
gbs6398				117.10***
G/G	37	16	0	
G/B	9	58	24	
B/B	0	34	38	
gbs3863				15.97**
G/G	19	34	8	
G/B	9	21	12	
B/B	8	37	30	
gwm533				1.47ns‡
G/G	11	21	14	
G/B	13	27	22	
B/B	8	21	10	

*** Significant at $\alpha < 0.001$ indicating marker and infection phenotype are not independent.

** Significant at $\alpha < 0.01$.

† G/G, homozygous 'Gage' allele; G/B, heterozygous Gage allele; B/B, homozygous 'Bill Brown' allele.

‡ ns, not significant indicating the marker and disease response are independent.

Forty-five $F_{4:5}$ families with R and MR APR did not have the markers indicative of *Sr2*. Thirty-four of these 45 resistant $F_{4:5}$ families were derived from F_2 families with at least one of the linked GBS SNPs. The high proportion of resistant $F_{4:5}$ families that were derived from earlier generation families with the GBS seedling resistance marker suggested that the seedling resistance gene most likely is the same gene that conveys resistance in the adult plant in addition to *Sr2*. We also observed two adult plant susceptible $F_{4:5}$ families that were homozygous for $Xgwm533_{Gage/Gage}$. This result may be due to recombination between the marker and *Sr2*, which are 1.6 cM apart (Spielmeyer et al., 2003). Eighteen $F_{4:5}$ families were seedling susceptible but adult plant resistant (R and MR). One of these 18 families lacked the *Sr2* linked marker allele. The low frequency of seedling susceptible lines, showing only APR, but lacking APR marker allele $Xgwm533_{Gage/Gage}$ suggested that recombination between the marker and *Sr2* may have occurred or we had an escape in the adult plant evaluation. In an effort to understand the relationship between seedling and adult plant stem rust resistance in 'Thatcher' wheat, Knott (2001) also reported the complexity of field stem rust APR inheritance where he found low frequency of lines with genes only effective in adult plants in the field.

The quantitative APR observed in Gage was similar in complexity to the previous genetic studies (Knott, 1982), which exhibited effective and usually durable APR in parent lines against a wide range of pathogen races. In these studies, few progeny were as resistant as the resistant parent. In our study, both the qualitative and quantitative analyses of stem rust resistance in Gage had similar results, indicating that the resistance in Gage can be explained by a major dominant and possibly a recessive gene(s) at seedling stage and by a combination of *Sr2* and additional gene(s) at the adult plant stage. The additional APR gene is most likely the dominant seedling resistance gene as most seedling resistant $F_{4:5}$ families derived from F_2 plants with the Gage allele for markers *gbs8942* or *gbs6398* were also adult plant resistant and some did not have the $Xgwm533_{Gage/Gage}$ marker allele associated with *Sr2*. We observed a few families with APR but without the detected adult plant marker (e.g., those for *Sr2*) or the seedling stem rust resistance possibly indicating additional APR gene(s) may be involved in the resistance of Gage. The evidence provided in this study helped characterize the stem rust resistance of Gage, but as with all APR studies, verification with replicated field experiments over years will be extremely valuable to fully understand its durability. The detailed identity of the seedling resistance will be revealed with additional markers, allelism tests, and phenotypic data from recombinant inbred lines that are under development.

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