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Development of genotyping by sequencing (GBS)- and array-derived SNP markers for stem rust resistance gene *Sr42*

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Abstract The stem rust fungus, particularly race TTKSK (Ug99), poses a serious threat to world wheat production. Gene *Sr42* or *SrCad* (which could be the same gene or an allele of *Sr42*) is effective against race TTKSK. However, known genetic markers for *Sr42* are mostly SSR markers which are generally labor intensive to use. In this study, we mapped a race TTKSK resistance gene derived from PI 595667 at the same locus as *Sr42* on chromosome 6DS. Based on position, pedigree and infection-type information, we propose that this gene is *SrCad* (*Sr42*). We enriched the genetic map for the *Sr42* region using genotyping by sequencing (GBS) and array-derived SNP markers. In total, 21 SNP markers were discovered, spanning a genetic distance of 27.2 cM. Nine of them are derived from GBS and twelve from the Illumina iSelect 90K SNP assay. Ten of the twenty-one SNP markers are closely linked (<2.2 cM, or co-segregating) with *Sr42*. We converted five of the closely linked SNP markers into uniplex KASP assays which will better facilitate marker-assisted selection. We validated the KASP assay in a doubled haploid wheat population derived from a three-way cross between accessions PI 410954, RB07, and Faller that shared an uncharacterized resistance gene mapped at approximately the same locus as PI 595667. The development of closely linked (co-segregating), codominant, sequence-based SNP assays will aid marker-assisted selection and map-based cloning of *Sr42*.

Keywords Wheat (*Triticum aestivum* L.) · Stem rust fungus (*Puccinia graminis tritici*) · *Sr42* · Genotyping by sequencing (GBS) · Kompetitive allele-specific PCR (KASP) · Marker-assisted selection (MAS)

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Introduction

Disease epidemics can cause serious yield losses and are one of the major concerns for global wheat (*Triticum aestivum* L.) production. Rusts are by far the most

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damaging diseases of wheat and have had a great impact on human civilization, with evidence of epidemics dating back more than 3300 years (Kislev 1982; Chaves et al. 2013). Among the three wheat rust pathogens (leaf, stem, and stripe rusts), the stem rust pathogen (*Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn.; *Pgt*) has the capacity to cause the most serious damage to wheat production (Szabo et al. 2014).

Rust pathogens are known to quickly evolve and mutate to overcome plant resistances and cause serious yield losses (Singh et al. 2004; Chen et al. 2009). A recent example is the emergence of the Ug99 (TTKSK) race group of the stem rust fungus in East Africa (Jin et al. 2008; Pretorius et al. 2010; Singh et al. 2011). The TTKSK race group has defeated the resistance provided by stem rust resistance genes (*Sr*) *Sr24*, *Sr31, Sr36*, and *Sr9h* (Jin et al. 2008; Pretorius et al. 2010) and can infect more than 90 % of the wheat crop grown worldwide (Singh et al. 2011). This race group is quickly evolving and migrating throughout Africa and Southwest Asia, posing a serious threat to world wheat production (Singh et al. 2011).

Development of resistant varieties has been the most economical and environmentally sound way to control rust diseases. Stem rust resistance gene *Sr42* showed effective resistance to race TTKSK and was mapped to chromosome 6DS, at approximately the same position as *SrCad,* which is likely the same gene or an allele of *Sr42* (Hiebert et al. 2011; Ghazvini et al. 2012). In the current study, we mapped a TTKSK resistance gene in Canadian accession PI 595667 (syn. GS-117[2965]) to approximately the same locus as *Sr42*. We used an independently developed doubled haploid population (DH) derived from South African accession PI 410954 to validate the markers developed in this research.

The genes from both PI 595667 and PI 410954 showed infection types similar to those displayed by lines possessing *Sr42* or *SrCad* to *Pgt* race TTKSK. The pedigree information for PI 595667 suggests that the race TTKSK resistance gene from this line is *SrCad*, whereas the pedigree information for PI 410954 gave no indication of this gene's presence. PI 595667 was developed as a common bunt resistance source. *Sr42* and the bunt resistance gene *Bt10* are likely closely linked on wheat chromosome 6DS (Knox et al. 1998; Laroche et al. 2000; Hiebert et al. 2011). PI 410954 was developed in South Africa during the 1970s and possesses the cultivar 'Agent' within its pedigree. Agent is the adapted source of *Sr24* in modern wheat (Friebe et al. 1996).

To date, published maps for the 6DS-Ug99 resistance locus, including *Sr42*, *SrCad*, and possibly *SrTmp* (Lopez-Vera et al. 2014), consist of a dominant marker FSD_RSA (Laroche et al. 2000), a few codominant SSR markers (Hiebert et al. 2011; Ghazvini et al. 2012), and a SNP marker BS000010742 that is not closely linked (approximately 6–20 cM) to the gene and is polymorphic in some but not all of the RIL mapping populations developed by Lopez-Vera et al. (2014). In this study, we identified closely linked SNP markers for *Sr42* via genotyping by sequencing (GBS) (Elshire et al. 2011) and a SNP array platform (Wang et al. 2014) and converted some of the SNP markers into easier to use uniplex KASP assays. The uniplex assays are more suitable for marker-assisted selection (MAS) when breeders need to assay one or a few markers on a large collection of germplasm or breeding lines.

Materials and methods

Plant materials

Wheat accession PI 595667 (HY377/SC8021V2//L8474D1) was crossed to susceptible wheat line LMPG-6 to derive 94 F_2 progeny. $F_{2:3}$ seeds were harvested from each F_2 plant and used for testing seedling reactions to stem rust. Line L8474D1 (HY-320*3/BW553) shares the parent line BW553 with AC Cadillac (BW90*3/BW553), which contains the gene *SrCad* (Hiebert et al. 2011; McCallum and DePauw 2008). LMPG-6 is a selection of LMPG, a stem rust susceptible line (Knott 1990). AC Cadillac (BW90*3/BW553) was available through the Triticeae Coordinated Agricultural Project (TCAP). BW553 carries the *Bt10* gene and is the donor of *SrCad* (Hiebert et al. 2011). Norin 40 (donor of *Sr42*) was obtained from the USDA-ARS Cereal Disease Laboratory.

The PI 410954 DH population was created from individual TC_1F_1 plants from the cross RB07//Faller/PI 410954. 'RB07' was developed by the University of Minnesota Agricultural Experiment Station and released in 2007 (Anderson et al. 2009). 'Faller' was released by the North Dakota Agricultural Experiment Station in 2008 (Mergoum et al. 2008). Both Faller and RB07 are high-yielding, popular varieties in the Upper

Midwest of the USA. An LMPG-6/PI 410954 cross was used to determine the genic nature of TTKSK resistance observed in PI 410954.

A collection of 29 wheat parental lines or cultivars known to be positive or negative for the 6DS-Ug99 resistance locus (*Sr42*/*SrCad*/*SrTmp*) was obtained from the USDA-ARS Cereal Disease Laboratory or the University of Minnesota wheat breeding program. These lines were used for validation of newly developed SNP marker assays.

Stem rust disease phenotyping

Pgt urediniospore isolates were retrieved from long-term storage in a −80 °C freezer. Seedling evaluation of *Pgt* races was performed at the USDA-ARS Cereal Disease Laboratory as described previously (Rouse et al. 2011). A total of 15–20 plants of each $F_{2:3}$ family (LMPG-6/PI 595667) and 10 plants of each DH line (LMPG-6/PI 410954) were screened with stem rust race TTKSK (isolate 04KEN156/04). Seedling reactions to stem rust were classified according to the '0–4' infection-type (IT) scale developed by Stakman et al. (1962) with ITs ranging from '0' to '2+' including mixed ITs such as ';13' considered as resistant and ITs ranging from 3− to 4 considered as susceptible.

F_2 plants from LMPG-6/PI 410954 ($n = 346$ and $n = 104$ in two replicates) were screened with *Pgt* race TTKSK. Resistant lines in the PI 410954 DH population were identified in replicate screenings with *Pgt* race TTKSK (*Sr24*-virulent isolate 06KEN19v3; to select against any lines possessing *Sr24*). Chi-square tests were used to infer the genic nature of resistance to *Pgt* race TTKSK.

DNA extraction and genotyping

DNA extraction of F_2 plants derived from LMPG-6/PI 595667 was conducted using a Qiagen Biosprint 96 DNA plant kit. DNA concentrations for 94 progeny and two parents were measured using a PicoGreen method (Ahn et al. 1996). DNA concentrations were adjusted to 10 ng/μL for all samples.

DNA from 48 F_2 plants (LMPG-6/PI 595667) and the two parents were subjected to GBS (Elshire et al. 2011), following the protocol developed by Poland et al. (2012). Specifically, 200 ng of DNA from each sample was double-digested using restriction enzymes *Pst*I and *Msp*I. Barcoded forward adapters and common reverse adapters were ligated to digested fragments and 60 samples (48 F_2 DNA plus two parental DNAs repeating six times each) were pooled into one 60-plex library. PCRs were performed using Illumina primers with sequences complimentary to adapters used in library preparation. PCR products were sent to the University of Minnesota Genomics Center (UMGC) for next-generation sequencing (NGS) with Illumina Hi-Seq 2000 machine. One library was double-loaded onto two lanes of Illumina flow cell, and the two lanes serve as two technical replicates. GBS tag sequences were analyzed using the bioinformatics pipeline UNEAK (Lu et al. 2013). Tag sequences were converted into genotypic calls after UNEAK analysis. SNPs were filtered based on Chi-square test (Chi-square 1:2:1 test $p > 0.05$), missing data percentage (<20 %), and tag counts (≥4 for each SNP call that is homozygous).

As cultivar Agent (*Sr24*) is in the pedigree of PI 410954, all DH progeny and parents were screened with the *Sr24* markers *Xbarc71* and *Sr24#12* following published methods (Mago et al. 2005). DNA was extracted using a PCR microprep method developed by Edwards et al. (1991). All PI 410954 DH lines amplifying *Sr24* amplicons when screened with *Xbarc71* and *Sr24#12* were excluded from further analyses.

A total of 57 F_2 plants and parents (LMPG-6/PI 595667) and 108 DH lines and parents (PI 410954 DH) were genotyped using the Illumina iSelect 90K SNP array (Wang et al. 2014) at the USDA-ARS Biosciences Research Lab in Fargo, North Dakota, USA. DNA extractions of progeny and parents in the PI 410954 DH population were performed using a modified CTAB method (Rouse et al. 2012). The resulting SNP data were manually called using Illumina GenomeStudio software.

DNA from 94 F_2 plants and two parents (LMPG-6/PI 595667) were subjected to SSR marker *Xcfd49* (Somers et al. 2004), marker FSD_RSA (Laroche et al. 2000), and KASP (Semagn et al. 2014) analyses. DNA from 45 DH lines and three parents (RB07, Faller, PI 410954) were also subjected to KASP analyses. Marker FSD_RSA reactions used 1× PCR buffer, 0.2 mM dNTP, 12 pmol FSD forward primer, 3.5 pmol RSA reverse primer, and 1 U Taq DNA polymerase. The thermal cycling conditions were 94 °C 10 min, followed by 35 cycles of 94 °C 30 s,

44 °C 1 min 45 s, 72 °C 2 min, with a final extension step of 72 °C 10 min.

The SSR PCRs included 1x PCR buffer, 0.125 mM dNTPs, 0.4 pmol of each primer, 0.05 U Qiagen Taq polymerase. The thermal cycling conditions for SSR markers were 94 °C 15 min, followed by 35 cycles of 94 °C 1 min, 60 °C 1 min, 72 °C 2 min, with a final extension step of 72 °C for 5 min and 4 °C indefinitely. PCR products were separated by polyacrylamide gel electrophoresis (PAGE) and silver-stained (Bassam et al. 1991) for visualization and genotype scoring. Marker assay csLV34 for examining the possible presence of *Lr34* on PI 595667 was performed according to the published method (Lagudah et al. 2006).

KASP KBD primer assays were designed by LGC Genomics (Teddington, Middlesex, UK, http://lgcgenomics.com). The KASP PCR conditions were adapted from KASP protocol 4.0. Each PCR consisted of 20 ng of wet DNA template, 1X KASP reaction mix, and 0.11 μL of KBD primer assay. Thermal cycling conditions were 94 °C 15 min, followed by 10 cycles of touchdown PCR: 94 °C 20 s, 61–55 °C for 60 s (dropping 0.6 °C per cycle), followed by 26–29 cycles of regular PCR: 94 °C 20 s, 55 °C 60 s, followed by data collection/plate read at 25 °C. Both thermocycling and fluorescence reading were performed on ABI StepOnePlus Real-Time PCR system. For marker validation on the PI 410954 DH population, KASP was performed using the same PCR protocol on a Roche LightCycler 96 real-time PCR machine.

Linkage and marker–trait association analysis

Construction of linkage groups for LMPG-6/PI 595667 population using GBS markers and array-derived SNP markers were all done using JoinMap version 4.0 (Stam 1993). For array-based SNP marker analysis, we utilized the reference map information (Wang et al. 2014) and removed markers that were mapped to other chromosomes. Groupings were done in JoinMap with a LOD threshold of 5.0 and 40 % maximum recombination frequency. Linkage groups were associated with individual wheat chromosomes after aligning the GBS tag sequences to the wheat chromosome survey sequences (Consortium 2014) using command line version of BLAST+ 2.2.8 under Linux environment (with >95 % identity match). After grouping, final maps were generated using MapMaker version 3.0b (Lander et al. 1987). The maximum likelihood mapping algorithm was used to generate the linkage map. Kosambi's mapping function (1944) was used to calculate the interval distances.

The location of TTKSK resistance in PI 410954 was determined by performing a genome-wide association study (GWAS) using the R package rrBLUP (Endelman 2011). The population structure consisting of DH lines derived from several independent TC1F1 plants precluded the use of traditional mapping algorithms. All SNPs mapped by Wang et al. (2014) were exported as a tab-delimited text file ($n = 38{,}800$). SNPs with missing data >10 % (no calls >12) were removed from analysis leaving a total of 36,068 SNPs. The SNP data set was passed through the function 'Amat' in order to develop an appropriate additive relationship matrix for GWAS. 'Amat' automatically removes monomorphic markers, and the data set was trimmed to 27,196 SNPs. Phenotypes for the 111 non-*Sr24* lines were coded as follows: 0 = susceptible and 1 = resistant. All genotypes were converted from the form AA, AB, and BB to 1, 0, and −1, respectively. 'No calls' were coded as NA. The command 'GWAS' conducts a genome-wide association analysis using phenotype and genotype data using a mixed-model approach (Yu et al. 2006).

The command line for GWAS analysis was entered as follows: GWAS (pheno, geno, K = A, n.core = 16, P3D = TRUE, n.PC = 2). 'pheno' and 'geno' are data frames containing the phenotypic and genotypic data, respectively. 'K' is the kinship matrix for covariance between lines and was assigned the matrix, A, developed via the function 'A.mat.' 'n.core' divides the SNPs into 16 groups to allow them to be analyzed in parallel on a single machine. 'P3D' (population parameters previously determined) when TRUE is equivalent to the expedited efficient mixed-model association (EMMAX) is developed by Kang et al. (2010). EMMAX is able to correct for sample structure by taking into account the pairwise relationship between individuals in a population. The final term, 'n.PC,' determines the number of principle components to include as fixed effects. The number used for this setting was determined by conducting an eigenvalue decomposition of matrix A developed via the function 'A.mat.' The first two principal components accounted for ~18 % of the total spectrum and were included as covariates in the GWAS.

Results

A single gene confers resistance to *Pgt* race TTKSK (Ug99) in PI 595667

Seedlings of PI 595667 and LMPG-6 were inoculated with *Pgt* race TTKSK (Fig. 1). The resistant parent PI 595667 displayed '2−' to '2' infection types (ITs) against race TTKSK (Fig. 1), whereas the susceptible parent LMPG-6 showed '3+' to '4' infections types (ITs) (Fig. 1). Segregation ratio (17 resistant:54 segregating:23 susceptible) for seedling response among 94 $F_{2:3}$ families (188 alleles) derived from LMPG-6/PI 595667 indicated that a single gene confers resistance to race TTKSK (Chi-square test for 1:2:1, p value = 0.24).

Two genes confer resistance to *Pgt* race TTKSK (Ug99) in PI 410954: *Sr24* and an unidentified *Sr* gene

F_2 seedlings of an LMPG-6/PI 410954 cross were screened with *Pgt* race TTKSK. LMPG-6 displayed an IT of '3+' and PI 410954 displayed an IT of '2' (Fig. 1). Segregation (327 resistant:29 susceptible and 96 resistant:8 susceptible in two replicates) for seedling response indicated the presence of two genes conferring resistance (Chi-square test for 15:1, p value = 0.40 and 0.84, respectively). As mentioned earlier, PI 410954 might possess *Sr24* through Agent in its pedigree. Amplification of the *Sr24* associated amplicon in PI 410954 when screened with *Xbarc71* and *Sr24#12* indicates that one of the two genes is *Sr24*.

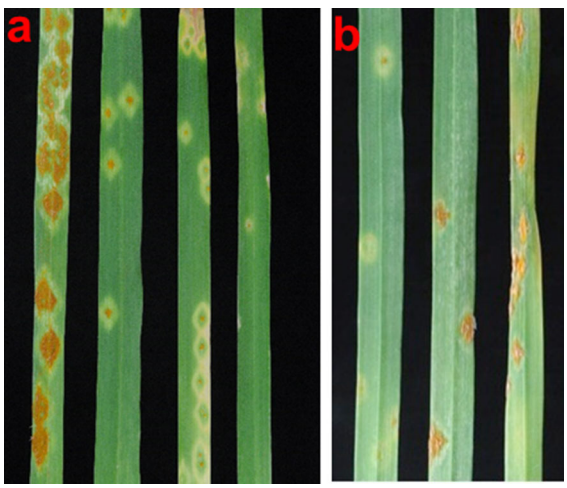

Fig. 1 Seedling reactions of parental lines to *Pgt* race TTKSK. **a** *Left to right* LMPG-6, PI 595667, Norin 40, AC Cadillac. **b** *Left to right* PI 410954, Faller, RB07

Genetic mapping of the race TTKSK resistance gene from PI 595667 using GBS markers

A total of 323 million GBS reads were generated for selected DNA samples (48 F_2s randomly selected and the two parents). Over 70,000 SNPs were identified. After quality filtering, approximately 1200 markers were selected for linkage map construction in JoinMap (Stam 1993). A total of 908 markers were grouped into 31 linkage groups. Through a BLASTn search against wheat chromosome survey sequences (Consortium 2014), we were able to associate each linkage group with a wheat chromosome. Linkage groups were constructed for each chromosome on wheat A and B genomes. Chromosomes from the wheat D genome are not well represented (e.g., chromosomes 4D and 5D were not represented). The D genome is known for low polymorphisms and tends to produce (incomplete) linkage groups that are not spanning whole chromosomes even when genotyped with high-density SNP arrays (Cavanagh et al. 2013). However, we were able to group the stem rust resistance gene derived from PI 595667 with nine GBS SNP markers derived from wheat chromosome 6DS (Table 1; Fig. 2a blue highlighted markers). GBS SNP marker WCSS1_6DS_2123217-1527 (TP43472) co-segregates with the PI 595667-derived TTKSK resistance gene in the F_2 plants subjected to GBS analysis.

The TTKSK resistance gene from PI 595667 is *Sr42*

It was known that *Sr42* and *SrCad* are located in a similar region of chromosome 6DS; therefore, the LMPG-6/PI 595667 mapping population was screened with known *Sr42* markers, FSD_RSA and *Xcfd49*. Our results show that these markers were closely linked to the resistance gene derived from PI 595667 (Fig. 2a). PI 595667 (HY377/SC8021V2//L8474D1) shares a line in its pedigree with several Canadian breeding lines (Knox et al. 1998; Hiebert et al. 2011). The pedigree of L8474D1 (HY-320*3/BW553) contains the donor line (BW553) of *SrCad* (*Sr42*) (Hiebert et al. 2011). In the current study, we mapped the resistance gene derived from PI 595667 to wheat chromosome

Table 1 GBS markers and their BLAST hit information

GBS tag	SNP name	Sequence	Blast_hit	Identity	Length	*E*-value
TP50404	WCSS1_6DS_2064159-2433	TGCAGCGGGGTATCATCTAGTCTAGTTCTCTTATG[C/G]CGAATATCCTCACTTGGCAGTGAATGTG	6DS_2064159	98.44	64	8.00E−25
TP71712	WCSS1_6DS_2086871-8018	TGCAGGCAACAGTAAAAAATGTCACTGTTCATATGCA[A/T]AATACAAAGGAAATTAGGATGTTGGG	6DS_2086871	100	64	2.00E−26
TP96770	WCSS1_6DS_2123349-1302	TGCAGTCATCGACGATGGCA[A/G]TGCCGACAGGAATGTCACGAAGGCTACCGAGATCGGAAGAGCG	6DS_2123349	100	50	1.00E−18
TP43472[a]	WCSS1_6DS_2123217-1527	TGCAGCGACGACGC[A/G]TGGGTGGTGTGAAGCCGTGAGTGAAGAAGACGGGGTCAGAGAATGCACG	6DS_2123217	100	63	6.00E−26
TP61532	WCSS1_6DS_1070043-2325	TGCAGCTGTTCCCATCTTCCTAGCTGCGGTCG[G/A]TAGGCCGAGATCGGAAGAGCGGTTCAGCAGG	6DS_1070043	100	40	4.00E−13
TP93838[a]	WCSS1_6DS_2061773-14642	TGCAGTACGTGGGGCAGTCCTTGCACGCCGTCGTCTCCGT[A/C]TCCGCCTCCGTTGTCTCCGCTGC	6DS_2061773	96.88	64	4.00E−23
TP61320	WCSS1_6DS_2108061-1766	TGCAGCTGTCGCCCATGCTGCTGGAGCGGG[A/G]CACGGAGATCGTCGACATCTCCAACGAGGATTA	6DS_2108061	98.44	64	8.00E−25
TP35740	WCSS1_6DS_2122135-3122	TGCAGCCCAGCCCA[G/T]CCCAGCCCATCAGCGCGTGGGGTCACCTTGTCAGCATCAAGGTCGAACA	6DS_2122135	100	64	2.00E−26
TP27606	WCSS1_6DS_2088891-13300	TGCAGCAGCGATCTG[G/T]GTCGAAGCAAAAGCAGCAAGCAGCGGATTTTGGCCTCTCTCTCTCTCT	6DS_2088891	100	64	2.00E−26

Column headings: GBS tag ID, GBS-derived SNP ID, GBS tag sequence, best Blast_hit, percent identity, length of match, and *E*-value

GBS SNP markers are named according to a naming system suggested by T3 (https://triticeaetoolbox.org/wheat/). 'WCSS1' stands for wheat chromosome survey sequence version 1, '6DS_xxxx' stands for the blast hit contig name, and '–' followed are the actual base pair position of corresponding SNP on the reference (BLAST hit) sequence

[a] GBS SNPs converted to KASP assays

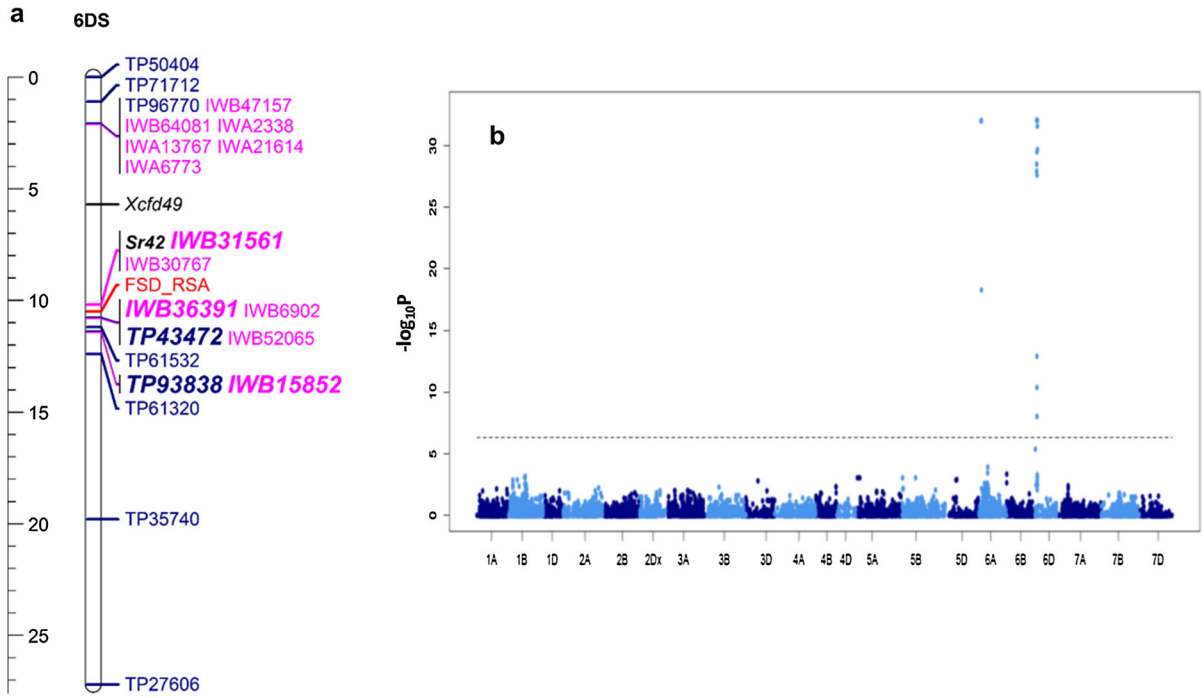

Fig. 2 **a** Genetic linkage map for TTKSK resistance gene *Sr42* using SNP and developed markers based on the LMPG-6/PI 595,667 population. *Blue color* represents GBS derived, *pink color* represents array derived, and *bold italic* highlighted represents KASP assays derived from GBS or array SNPs. Ruler on the *left* indicates genetic distances. **b** Manhattan plot of SNPs associated with non-*Sr24* resistance in the PI 410954 TC1F1 population developed from genome-wide association study in R package 'rrBLUP.' A total of 108 DH lines and three parents were used in the analysis. The *dashed line* represents a q value of 0.05, and all points above this line have q values $\leq$0.05. The X axis represents the haploid chromosome composition ($n = 21$) of *Triticum aestivum*. As discussed in the 'Results' section, the extra *peaks/dots* on chromosome 6A could be due to markers being mapped to both chromosomes 6A and 6D. (Color figure online)

6DS, at the same location as *Sr42* and *SrCad*. It is likely that the race TTKSK resistance gene in PI 595667 is derived from the donor of *SrCad*, BW553. The infection types (ITs) conferred by the resistance gene from PI 595667 ('2−' to '2') (Fig. 1) are similar to those conferred by AC Cadillac (*SrCad*) and Norin 40 (*Sr42*) (Hiebert et al. 2011; Ghazvini et al. 2012). All lines of evidence (pedigree, chromosome arm position, and infection types) suggest that the race TTKSK resistance gene present in PI 595667 is *Sr42*.

Genetic mapping of *Sr42* using array-based markers

A 90K iSelect array platform was used to genotype the two parents and 57 F_2 individuals of the mapping population derived from LMPG-6/PI 595667. In total, 12 SNP markers were found to be linked to the resistance gene derived from PI 595667 (Table S1, Fig. 2, pink highlighted markers). Markers IWB31561 and IWB30767 co-segregated with *Sr42* in this mapping population. Our map orders are largely consistent with published reference consensus maps (Wang et al. 2014). Marker IWB36391 was placed to the distal end of IWB31561 in the reference consensus map, but to the proximal in our map (Fig. 2a). We did a BLAST search using array SNP sequence information (Wang et al. 2014) and found that SNP markers IWB36391 and IWB52065 (mapped proximal to IWB31561 in both the reference and our maps, Fig. 2a) share the same best BLAST hit (contig 6DS_2116396, Table S1), suggesting that our map order probably reflects a true order.

For GWAS analysis in the PI 410954 DH population, a total of 15 SNPs with q values <0.05 (equivalent to a false discovery rate of 0.05) were discovered (Table 2; Fig. 2b, Fig. S1). Two of these SNPs, IWB6072 and IWB6902, map to both

chromosomes 6A and 6D in the consensus map developed by Wang et al. (2014) (Table 2; Fig. 2b). However, best BLAST hits against wheat chromosome survey sequences (Consortium 2014) are to chromosome 6DS. SNP IWB6072 also has significant hits on 6AS. SNP IWB49090 maps to chromosome 6A exclusively in Wang et al. (2014); however, it too has significant BLAST hits on chromosome 6DS. All remaining SNPs are located within a 6-cM region of chromosome 6DS between 18.2 cM and 24.8 cM, using the scaled map distances reported by Wang et al. (2014).

In total, there are five SNPs in common between LMPG-6/PI 595667 and the PI 410954-derived DH populations (Table 2), which are associated with resistance genes from each resistant parental line. As both resistance genes are located on the same region of chromosome 6DS, they are either the same gene, alleles of the same gene, or closely linked genes. We did notice that the PI 410954-derived *Sr* gene seemed to have a higher association (more significant GWAS p values) with markers at position 23.84 cM (Table 2), whereas the PI 595667 derived *Sr* gene was co-segregating with a SNP marker (IWB31561) at 20.75 cM (Table S1).

Conversion of multiplex SNP assays into uniplex KASP assays

To convert multiplex GBS or array-based SNP assays into uniplex KASP assays, we selected a total of eight SNPs (five iSelect array derived and three GBS derived) for KASP assay design through LGC Genomics. Five of the eight KASP assays formed good clusters in the PI 595667 mapping population (Fig. 3). The primer sequences for KASP assays are listed in Table S2. The two alleles are labeled with either FAM or HEX in KASP assays as shown in blue or red colors in allelic discrimination plots (Fig. 3). A final linkage map consisting of SNP based and other known markers was developed for the *Sr42* region in PI 595667 (Fig. 2a). The SNP marker IWB31561 also co-segregated with *Sr42* in the full mapping population.

A subset of the PI 410954 DH population consisting of 48 plants including PI 410954, RB07, Faller, and

Table 2 Single nucleotide polymorphism (SNP) markers from Illumina iSelect 90K assay with significant associations with the resistant phenotype in non-*Sr24* doubled haploid (DH) lines derived from RB07//Faller/PI 410954 TC_1F_1 plants

ID[a]	SNP name	Chr	Position	−log 10 *p* value
IWB49090	Kukri_rep_c105406_308	6A	13.45	32.01
IWB6072	BS00009514_51	6A/6D	16.96/23.84	32.05
IWB6902	**BS00022094_51**	**6A/6D**	**16.96/23.84**	**18.28**
IWB36391[b]	**IACX9471**	**6D**	**18.20**	**32.08**
IWB10744	BS00074495_51	6D	19.00	28.50
IWA6799	wsnp_Ku_c2637_5009091	6D	19.00	27.93
IWB4284	BobWhite_c7090_2001	6D	20.75	10.38
IWB30767	**Excalibur_rep_c66622_1066**	**6D**	**20.75**	**8.03**
IWB31561[b]	**Excalibur_s114066_247**	**6D**	**20.75**	**12.89**
IWB49821	Kukri_rep_c68823_696	6D	20.75	29.49
IWB262	BobWhite_c11808_975	6D	21.83	27.61
IWB6838	BS00021983_51	6D	23.84	31.59
IWB7135	BS00022523_51	6D	23.84	32.07
IWB52065	**Ra_c42576_780**	**6D**	**23.84**	**32.07**
IWB34477	IAAV1942	6D	24.77	29.69

SNPs were identified using the GWAS function in R package 'rrBLUP.' All SNP markers listed have a *q* value ≤0.05, corresponding to a false discovery rate (FDR) of <5 %

[a] Bold highlighted are SNP markers associated with the TTKSK resistance genes in both the PI 410954 and PI 595667 populations through linkage or GWAS analysis. Consensus position are based on 90K reference map (Wang et al. 2014)

[b] Array SNPs converted to KASP assays

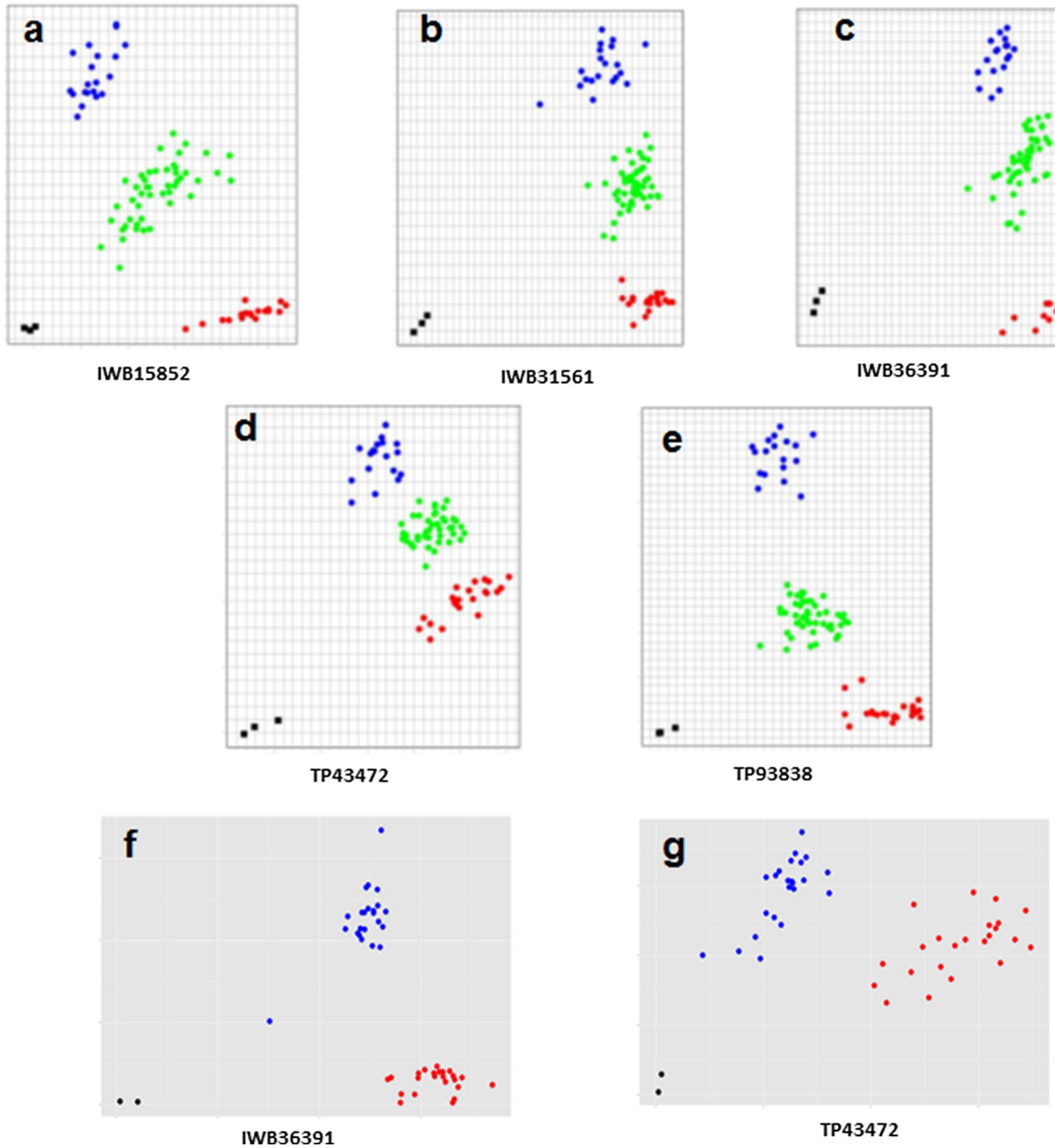

Fig. 3 SNP sequence-based, codominant KASP assays developed for diagnosing and mapping TTKSK resistance gene *Sr42*. In all cases except TP43472, *red* (HEX-labeled) *dots* represent homozygous resistant (R), *blue dots* represent homozygous susceptible (S), and *green dots* represent heterozygous genotypes (H). *Black dots* represent no template controls (NTC). Top five plots (**a**–**e**) show results on PI 595667 population. Bottom two plots (**f**, **g**) with *gray themes* are validation results on the PI 410954 DH population. (Color figure online)

DH individuals was screened with the five KASP markers: IWB15852, IWB31561, IWB36391, WCSS1_6DS_2123217-1527 (TP43472), and WCSS1_6DS_2061773-14642 (TP93838). Markers IWB15852 and TP93838 were monomorphic in this subpopulation. Marker IWB31561 cannot clearly separate alleles from PI 410954 and alleles from RB07 or Faller in the DH population, as all alleles are close to the middle

and difficult to differentiate (data not shown), suggesting that there could be additional sequence variations at the target locus or that there is interference with a possibly homeologous locus. Markers IWB36391 and WCSS1_6DS_2123217-1527 (TP43472) co-segregated with the PI 410954-derived resistance gene (Fig. 3), evidence that this resistance gene is the same or an allele as that in PI 595667.

We screened 29 wheat cultivars or parental lines using three KASP assays developed in this study (IWB31561, IWB36391, and TP43472) (Table S3). Our results show that the lines that possess the 6DS-Ug99 resistance locus fall into three haplotypes: (1) AC Cadillac (*SrCad*), Peace (*SrCad*), and PI 595667 share haplotype of C–C–T, for the three KASP assays, as shown in Table S3, adding further support that PI 595667-derived TTKSK resistance gene is *SrCad*; (2) the genotype of Norin 40 (*Sr42*) had a different haplotype (T*–C–T) that corresponded with lines Eagle 10, Ember, Guard, Ripper, and Shield (Table S3); and (3) the haplotype of Triumph 64 (*SrTmp*) and CnSSrTmp (*SrTmp*) was T–C–T, and this corresponded with lines Blouk, Digalu, Robin, Pfunye, and PI 410954. Lines with the T* allele of KASP marker IWB31561 did not cluster tightly with lines with T or C alleles, but were closer to those with the T allele. This may be caused by an imperfect sequence match of the template DNA with either primer. These results suggest that the sources of resistance (*Sr42*, *SrCad*, *SrTmp*) may correspond to different resistance alleles or genes. All of the resistant and susceptible cultivars or breeding lines (with Chinese Spring and Briggs as possible exceptions) can be successfully distinguished using the selected three KASP assays. Combinations of more SNP assays to form longer haplotypes will likely further empower breeders for more accurate selections. All of the SNP assays could prove to be useful in mapping projects based on biparental populations, as long as the two parents are polymorphic for target SNP markers. These results add further support to the utility of these KASP assays in MAS or molecular breeding.

Discussion

The line BW553 is the donor of *SrCad* (Hiebert et al. 2011), which is likely the same gene as *Sr42* or an allele of it (Ghazvini et al. 2012). BW553 is also the donor of the race TTKSK resistance gene derived from PI 595667. AC Cadillac (*SrCad*) shows different infection types to several non-TTKSK *Pgt* races compared to Norin 40 (*Sr42*) (Hiebert et al. 2011; Ghazvini et al. 2012). These could be due to the presence of background disease resistance genes such as *Lr34* (Hiebert et al. 2011), which is known for broad-spectrum resistance against leaf rust, stripe rust, powdery mildew, and stem rust pathogens (Singh and Huerta-Espino 2003; Lagudah et al. 2006; Lillemo et al. 2007, 2008; Krattinger et al. 2009; Kolmer et al. 2011) and is widely present in Canadian lines and cultivars (McCallum and DePauw 2008) including AC Cadillac (Hiebert et al. 2011).

We did not detect the presence of *Lr34* on PI 595667 (data not shown), based on assay results using a previously published *Lr34* marker csLV34 (Lagudah et al. 2006). The absence of *Lr34* in PI 595667 might help explain why PI 595667 infection type is slightly more severe than AC Cadillac (‘2−’ compared to ‘12−’). However, overall, the TTKSK infection types for AC Cadillac (*SrCad*), Norin 40 (*Sr42*), PI 595667, and PI 410594 are quite similar (12− to 2), and the genes derived from these lines are genetically co-localized within an approximately 3 cM distance according to the consensus map (Table 2; Fig. 2a) developed by Wang et al. (2014). Taking together pedigree, map position, and infection-type information, we feel justified to hypothesize that the stem rust resistance gene derived from AC Cadillac, Norin 40, PI 595667, and PI 410954 is actually *Sr42* or an allele of *Sr42*. Yet, we cannot rule out the possibility that they represent closely linked genes.

The tight coupling linkage between *SrCad* and *Bt10* (Hiebert et al. 2011) together with the potential presence of a few other leaf/stem rust resistance genes in the pedigree lines of PI 595667 (Liu and Kolmer 1997) might present some challenge for allelism tests and further dissection of this 6DS resistance locus. Ongoing allelism test efforts by our group and colleagues (Hiebert et al. 2014) will help further elucidate the relationships among these Ug99 resistance sources. We believe that the SNP markers identified in this study will assist in the construction of an enriched map around the 6DS region of interest.

Single nucleotide polymorphisms (SNPs) are becoming the marker of choice for enriching genetic maps and developing novel markers for MAS. In this

study, we identified over 20 SNP markers for wheat stem rust resistance gene *Sr42*. Development of closely linked, codominant SNP assays for *Sr42* is an important first step in fine mapping and map-based cloning of this gene. The genetic distances between most of the SNP markers identified in this study have a much closer map distance (0–2 cM compared to 6–20 cM) to *Sr42* than a previously validated SNP marker (BS000010724) (Lopez-Vera et al. 2014). The identification of twenty-one (or thirty-one, if counting the additional SNPs detected in GWAS study for PI 410954 population) SNP markers will allow a more efficient tagging and selection of *Sr42* in breeding programs. Some of these KASP assays developed by this study are being utilized at other USDA facilities for marker-assisted screening of different collections.

In GBS analysis, we used 48 samples and the parental lines for initial genotyping and mapping. We were able to construct linkage groups that represented most wheat chromosomes. Our results demonstrate a high success rate in utilizing GBS for target gene tagging, even with a relatively small sample size. Increasing the sample size will likely improve future mapping projects. We did notice that D genome chromosomes were less well represented, which could be due to our limited sample size, or it could be due to the known low polymorphisms for the D genome compared to the A and B genomes (Cavanagh et al. 2013; Wang et al. 2014).

The use of SNP arrays, such as the wheat iSelect 90K assay, will continue to be an important tool for understanding the relationship between resistance loci found in varied wheat accessions. This study represents the first study that associates the 6DS-Ug99 locus to a narrow region (about 3 cM) on the reference 90K map. The high SNP density allows for rapid comparison among genotyped accessions, as shown here. The use of a uniplex genotyping platform will continue to facilitate collaboration among wheat researchers. As genome sequencing efforts move forward (Consortium 2014; Chapman et al. 2015), GBS (Poland et al. 2012; Li et al. 2015) and array-derived SNP markers can be associated with the physical chromosome positions in reference genomes. Sequence-based markers could potentially provide direct insights into the genomic compositions around the target genes, thus facilitating better trait dissection and marker development.

Conclusion

The Ug99 resistance gene(s) derived from PI 595667 is *Sr42* based on pedigree, chromosome position, and infection-type data. We identified more than 20 SNP-based markers linked to the *Sr42* region on wheat chromosome 6DS. Closely linked markers were validated in the PI 410954 DH population, indicating that the markers developed through this research are effective in multiple genetic backgrounds. The development of closely linked (co-segregating) codominant SNP markers will facilitate further fine mapping and map-based cloning of the gene. Uniplex SNP assays will also aid marker-assisted breeding.

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