Identification and Validation of a Common Stem Rust Resistance Locus in Two Bi-parental Populations

Mandeep S. Randhawa*, Ravi P. Singh2, Susanne Dreisigacker2, Sridhar Bhavani2, Julio Huerta-Espino3, Matthew N. Rouse4, Jayaveeramuthu Nirmala4 and Maricarmen Sandoval-Sanchez2,5

1 International Maize and Wheat Improvement Center (CIMMYT), Nairobi, Kenya, 2 International Maize and Wheat Improvement Center (CIMMYT), Mexico City, Mexico, 3 Campo Experimental Valle de México INIFAP, Mexico City, Mexico, 4 Cereal Disease Laboratory, United States Department of Agriculture-Agricultural Research Service, St. Paul, MN, United States, 5 Colegio de Postgraduados, Texcoco, Mexico

Races belonging to Ug99 lineage of stem rust fungus *Puccinia graminis* f. sp. *tritici* (*Pgt*) continue to pose a threat to wheat (*Triticum aestivum* L.) production in various African countries. Growing resistant varieties is the most economical and environmentally friendly control measure. Recombinant inbred line (RIL) populations from the crosses of susceptible parent ‘Cacuke’ with the resistant parents ‘Huhwa’ and ‘Yaye’ were phenotyped for resistance at the seedling stage to *Pgt* race TTKSK (Ug99) and in adult plants in field trials at Njoro, Kenya for two seasons in 2016. Using the Affymetrix Axiom breeders SNP array, two stem rust resistance genes, temporarily designated as *SrH* and *SrY*, were identified and mapped on chromosome arm 2BL through selective genotyping and bulked segregant analysis (BSA), respectively. Competitive allele specific polymorphism (KASP) and simple sequence repeat (SSR) markers were used to saturate chromosome arm 2BL in both RIL populations. *SrH* mapped between markers *cim109* and *cim114* at a distance of 0.9 cM proximal, and *cim117* at 2.9 cM distal. *SrY* was flanked by markers *cim109* and *cim116* at 0.8 cM proximal, and *IWB45932* at 1.9 cM distal. Two Ug99-effective stem rust resistance genes derived from bread wheat, *Sr9h* and *Sr28*, have been reported on chromosome arm 2BL. Infection types and map position in Huhwa and Yaye indicated that *Sr28* was absent in both the parents. However, susceptible reactions produced by resistant lines from both populations against *Sr9h*-virulent race TTKSF confirmed the presence of a common resistance locus *Sr9h* in both lines. Test of allelism is required to establish genetic relationships between genes identified in present study and *Sr9h*. Marker *cim117* linked to *SrH* was genotyped on set of wheat lines with Huhwa in the pedigree and is advised to be used for marker assisted selection for this gene, however, a combination of phenotypic and genotypic assays is desirable for both genes especially for selection of *Sr9h* in breeding programs.

**Keywords:** QTL mapping, SNP, *Puccinia graminis* f. sp. *tritici*, *Triticum aestivum* L., adult plant resistance
INTRODUCTION

Stem rust, caused by *Puccinia graminis f. sp. tritici* (Pgt), is one of the most damaging fungal diseases of bread and durum wheat (*Triticum aestivum* L. and *Triticum turgidum* ssp. *durum*, (Desf.) Husnot., respectively) (Roelfs et al., 1992). It can cause complete yield losses under severe epidemics if susceptible cultivars are grown in rust hot-spot areas (Saari and Prescott, 1985; Dean et al., 2012). Global wheat production was threatened by stem rust when a highly virulent *Pgt* race known as Ug99 or TTKSK that combined virulence to *Sr31* and various other commonly deployed resistance genes was detected in 1998 in Uganda (Pretorius et al., 2000; Jin et al., 2007, 2008). After its appearance, evaluations of international wheat germplasm and varieties in both field and greenhouse screenings revealed the predominance of wheat susceptibility to race TTKSK (Jin and Singh, 2006; Singh et al., 2006, 2011). In subsequent years, new variants of Ug99 emerged that carry additional virulence to *Sr24* (Jin et al., 2008; Pretorius et al., 2010; Visser et al., 2011), *Sr36* (Jin et al., 2009), and *SrTnp* (Newcomb et al., 2016) placing an even greater number of wheat varieties at risk. Races of the Ug99 race group have already spread over a wide geographical area including 13 countries in the East African highlands, Southern Africa, Yemen, Egypt and Iran, and there is a high chance of further spread into large wheat growing belts of Asia and beyond (Singh et al., 2015). Consequently, the new variants belonging to Ug99 race group and their geographical spread have further reduced the number of effective genes that can be used by breeding programs.

Sixty wheat stem rust resistance genes have a designated gene symbol and a few more carry temporary designations (McIntosh et al., 2016). Five genes, namely *Sr2*, *Sr55* (*Lr67/Yr46/Pm46*), *Sr56*, *Sr57* (*Lr34/Yr18/Pm38*), and *Sr58* (*Lr46/Yr29/Pm39*), confer adult plant resistance (APR) (Singh et al., 2015), and the 34 genes that impart all stage resistance (ASR) to *Pgt* race TTKSK are *Sr9h, Sr13a, Sr13b, Sr15, Sr21, Sr22, Sr24, Sr25, Sr26, Sr27, Sr28, Sr32, Sr33, Sr35, Sr36, Sr37, Sr39, Sr40, Sr42, Sr44, Sr45, Sr46, Sr47, Sr50, Sr51, Sr52, Sr53, Sr59, SrCad, SrTA10171, SrTA10187, SrTA1662, SrTnp, and Sr1RS* (Singh et al., 2006; Jin et al., 2007; Faris et al., 2008; Hiebert et al., 2010; Kolmer et al., 2011; Liu et al., 2011a,b; Qi et al., 2011; Rouse et al., 2011a,b; Rouse and Jin, 2011; Ghazvini et al., 2012; Olson et al., 2013a,b; Bansal et al., 2014; Moore et al., 2015; Singh et al., 2015; Rahmatov et al., 2016; Zhang et al., 2017). Interestingly, *Sr8155B1* does not confer resistance to race TTKSK, but does confer resistance to the more recent variants in the Ug99 race group (Nirmala et al., 2017). Only a few resistance genes (*Sr2, Sr13a, Sr13b, Sr25, Sr26, Sr57, Sr58, SrCad, SrTnp, SrND643, Sr1RS* (Singh et al., 2006; Jin et al., 2008, 2015; Njau et al., 2010; Hiebert et al., 2011; Basnet et al., 2015; Nirmala et al., 2017; Zhang et al., 2017). The continuous search for new resistance sources and following through with their genetic characterization and strategic deployment is necessary for long-term control of the fast-evolving races of the stem rust fungus.

Bringing widely effective gene combinations into new cultivars is important to avoid the evolution of Ug99 races and minimize potential yield losses. Multiple gene combinations can be achieved more efficiently if DNA markers, tightly linked to target genes, are available. Markers linked to some stem rust resistance genes have been identified which can be useful in marker assisted selection (MAS). Cloning of genes *Sr13a, Sr21, Sr22, Sr33, Sr35, Sr45, Sr50, Sr55*, and *Sr57* has not only enhanced the knowledge of resistance mechanisms but also resulted in the development of diagnostic gene-based markers (Krattinger et al., 2009; Periyanann et al., 2013, 2014; Saintenac et al., 2013; Mago et al., 2015; Moore et al., 2015; Zhang et al., 2017; Chen et al., 2018). Several genes effective against Ug99 were tagged using DNA markers, to name some; *Sr2* (Hayden et al., 2004; Mago et al., 2011), *Sr9h* (Rouse et al., 2014), *SrND643* (Basnet et al., 2015), *Sr22* (Periyanann et al., 2011), *Sr26* (Mago et al., 2005), *Sr25* and *Sr26* (Liu et al., 2010), *Sr28* (Rouse et al., 2012), *Sr35* (Saintenac et al., 2013), *Sr45* (Periyanann et al., 2014), *Sr48* (U. Bansal, University of Sydney, personal communication), *Sr56* (Bansal et al., 2014), *Sr55* (Moore et al., 2015), and *Sr57* (Lagudah et al., 2006). In the last decade, new and cost-effective marker technologies emerged as powerful tools, which are very helpful in identifying rust resistance genes and their strategic deployment in breeding.

Observations on 'Huhwa' (H), 'Yaye' (Y) and their progenies in International Maize and Wheat Improvement Center (CIMMYT) germplasm indicated the presence of *Asr* to *Pgt* races belonging to the Ug99 group. Furthermore, several high yielding wheat lines derived from Huhwa and Yaye have been distributed in CIMMYT international nurseries. Despite the fact that Huhwa and Yaye carry stem rust resistance genes effective to Ug99 race group, their genetic and molecular characterization remains unclear. Therefore, the present study was undertaken to characterize stem rust resistance in these cultivars using recombinant inbred lines (RIL) populations, and to develop and validate SNP markers closely linked to these genes.

MATERIALS AND METHODS

Plant Material and Development of Mapping Populations

The two RIL populations were developed by crossing stem rust resistant bread wheat lines, Huhwa [CIMMYT germplasm identification number (GID): 5552006] and Yaye (GID: 5343322) each with the susceptible parent Cabuc (GID: 5347441) as the female parent during wheat seasons 2007-08 and 2008-09 at Obregon, Mexico, respectively. The Pedigrees of Huhwa and Yaye are ‘HUU234+Lr34/Prinia/Kronstad F2004’ and ‘Yanacc/3/Parula/Sara/TSH/Veery#5/4/Croc_1/Ae. squarrosa’ (224)//Opata, respectively. The pedigrees of HUU234 and Yanac are ‘HUU212/SPRW’//HUUW1’ and ‘Jabiru/M-5392-1//M-5392/3/Cook’, respectively, whereas Cabuc is ‘Canadian/Cunningham’//Kennedy.’ Hereafter, Cabuc × Huhwa and Cabuc × Yaye populations are abbreviated as C × H and C × Y, respectively. The F₃ RIL populations C × H (148 RILs) and C × Y (198 RILs) were developed using single spike descent method modified from the single seed descent method. To develop the population, a single spike from each F₂ plant, generated from three different F₁ plants, was harvested.
under fungicide-protected conditions and advanced to the F₄ generation by harvesting a single spike in successive generations. The F₅ plots, derived from single F₄ spikes were then harvested in bulk to obtain sufficient seed of the F₅ RILs. C × H (3 RILs) and C × Y (27 RILs) did not germinate when populations were planted for seed multiplication at El Batan in 2015. Finally seeds of C × H (145 RILs) and C × Y (171 RILs) were used for all genotypic and phenotypic analyses.

**Greenhouse Evaluations**

The parents and RILs of the two populations, C × H and C × Y, were tested against Pgt race TTKSK (isolate 04KEN156/04) at the seedling stage in a greenhouse at the United States Department of Agriculture- Agricultural Research Service (USDA-ARS) Cereal Disease Laboratory (CDL), St. Paul, MN, United States following the procedure described in Rouse et al. (2011b). The avirulence/virulence formula of race TTKSK is Sr24,36,Tmp/Sr5,67,7b,8a,9a,9b,9d,9e,9g,10,11,17,30,31,38,McN (Jin et al., 2007). Between 20 and 30 seeds of each RIL and the parents were grown for seedling tests. Eight-day old seedlings were inoculated with urediniospores of Pgt race TTKSK. Infection types (ITs) on seedlings were recorded 14 days post-inoculation using 0 to 4 scale as described by Stakman et al. (1962). Infection types ‘0’, ‘1’, and ‘2’ were considered resistant, whereas ITs ‘3’ to ‘4’ were considered susceptible. RILs were classified in three categories homozygous resistant (HR), homoyzogous susceptible (HS) and segregating. Two HR and HS RILs from both populations along with respective parents were also tested against Sr9h-virulent race TTKSK+ (isolate 09ZIM01-2; Pretorius et al., 2012; Rouse et al., 2014). Tests were repeated twice to avoid any chances of misclassification of RILs in each population.

**Field Evaluations**

The two RIL populations and the parents were phenotyped for response to stem rust in field trials at the Njoro research station of Kenya Agricultural and Livestock Research Organization (KALRO) during two wheat growing seasons (2016- off and main seasons). About 5 g seed of each entry was planted in 0.7 m long paired-row flat beds, with 0.3 m spacing between them. Spreaders comprised of a mixture of stem rust susceptible cultivars ‘Cacuke’ and ‘Robin,’ and six-Sr24 carrying lines (GIDs: 5391050, 5391052, 5391056, 5391057, 5391059, and 5391061) were planted as hill plots on one side of each test plot in the middle of the 0.3 m-wide pathways. Spreaders were also planted along the borders of the experimental field in 1 m plots. Spreaders were inoculated with a field collection of races TTKST and TTKTT belonging to the Ug99 race group (Jin et al., 2008; Singh et al., 2015; Newcomb et al., 2016) by spraying a mixture of urediniospores suspended in water plus Tween 20 suspensions, and needle inoculations with the same suspension as described in Njau et al. (2013). Stem rust responses of each RIL and parents were recorded at post-flowering stage when the susceptible parent displayed moderately susceptible to susceptible (MS to S) responses (Roelfs et al., 1992) with 70% disease severity (DS) following the Modified Cobb’s Scale (Peterson et al., 1948). Responses were recorded a second time about 8 days later.

**Molecular Marker Analysis**

Leaf tissues were harvested from young leaves and genomic DNA was extracted using CIMMYT’s laboratory protocols (Dreisigacker et al., 2015). The quality and quantity of DNA was assessed on 1% agarose gels and using a NanoDrop 8000 spectrophotometer (Thermo scientific). Selective genotyping was carried out for parents, 47 resistant and 47 susceptible RILs of C × H population, whereas bulk-segregant analysis (BSA; Michelmore et al., 1991) was performed on pooled DNA of 10 resistant and 10 susceptible RILs along with parents of the C × Y population. Genotyping was conducted using the 35K Affymetrix Axiom Breeders array (Allen et al., 2017) by outsourcing to TraitGenetics GmbH (Germany). The SNPs closely linked to stem rust resistance locus in each population were converted into Kompetitive allele-specific polymorphic (KASP) markers using the automated bioinformatics pipeline, PolyMarker (Ramirez-Gonzalez et al., 2015). KASP markers were then used to verify the targeted SNP polymorphism in the parents and polymorphic markers were applied on the respective mapping population. In addition, 3 KASP markers derived from the SNP markers IWB45296, IWB3891 and IWB45932 (Wang et al., 2014), linked to Sr9h were tested on the respective parents of both populations. Oligos were synthesized in 0.025 µM concentration from Sigma-Aldrich, United States. SNP markers were carried out in a final volume of 8 µl consisting of 3 µl of genomic DNA (30 ng/µl), 4 µl of 2x KASP mix [a mixture of FAM and HEX Fluorescence Resonance Energy Transfer (FRET) cassettes, ROXTM passive dye, an optimized buffer containing MgCl₂, Taq polymerase and dNTPs], 0.11 µl primer mix containing two allele specific forward primers each with 12 and 30 µM of common primer and 0.89 µl of sterile water. PCR amplification was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using the cycling conditions: 95°C for 15 min for hot-start Taq DNA polymerase activation, followed by a touchdown profile of 9 cycles at 94°C for 20 s and 61°C for 1 min with a 0.6°C reduction per cycle, followed by 38 cycles at 94°C for 20 s and 55°C for 1 min. The reactions were held at 12°C for 3 min. Endpoint fluorescent images were visualized using the PHERASTarTM Plus (BMG LABTECH, Germany), and the data were analyzed using ClusterCallerTM software (LGC genomics).

Similarly, 22 simple sequence repeat (SSR) markers namely; barc101 and barc159 from Song et al. (2005); cjf70 and cjf73 (Sourdille et al., 2001); gwm16, gwm47, gwm120, gwm382, gwm388, gwm501 and gwm526 (Roder et al., 1995, 1998); wmc51, wmc149, wmc175, wmc317, wmc332, wmc356, wmc361, wmc441, wmc500, and wmc627 (Somers et al., 2004), located on chromosome arm 2BL were also tested on parents, Cacuke, Huhwa and Yaye, to survey polymorphisms between parents and the respective RIL population was genotyped with polymorphic SSRs using respective PCR protocols. In addition, Sr26-linked marker Sr26#43 (Mago et al., 2005) was tested on ten RILs each of populations C × H and C × Y along with respective parents.

**Data Analyses and Genetic Mapping**

Chi-squared analyses were performed to determine the goodness-of-fit of observed segregation with the expected genetic ratios.
for 1 gene segregation in F_{4.5} RIL population using only the homozygous lines (HR: HS = 1:1). Genetic linkage maps for the stem rust resistance loci were constructed using genotypic data of molecular markers and seedling stem rust data using Inclusive Composite Interval Mapping (ICIM) software (Li et al., 2008). The Kosambi mapping function (Kosambi, 1943) was applied to convert recombination fractions into centimorgans (cM). Markers and respective resistance loci were grouped using a logarithm of odds (LOD) threshold of 10, the linkage maps were ordered and rippled using nearest-neighbor two-opt (nnTwoOpt) and sum of adjacent recombination (SAR) fraction algorithms, respectively.

Associations between markers on chromosome arm 2BL and quantitative resistance were revealed using the DS data recorded during 2016 off and main seasons using the ICIM software. A quantitative trait locus (QTL) was designated to be significant with a LOD value above a threshold calculated through 1000 permutation tests. Linkage map diagrams were prepared and aligned for a visual inspection of map order using MapChart 2.2 software (Voorrips, 2002).

Validation of Flanking Markers for Marker-Assisted Selection

Closely linked markers to stem rust resistance were used in validation studies by testing on a set of wheat genotypes carrying Huhwa and Yaye in their pedigree, respectively, and the efficacy of the reported markers for marker assisted selection (MAS). Sixty-seven and 44 lines with Huhwa and Yaye in their pedigree respectively, were chosen for validation of flanking markers. These lines were selected from the CIMMYT 9th and 10th Stem Rust Resistance Screening Nursery (SRRSN); the 26th High Rainfall Wheat Screening Nursery (HRWSN); 32nd, 33rd, and 34th Semi-Arid Wheat Screening Nursery (SAWSN); 47th, 48th, and 49th International Bread Wheat Screening Nursery (IBWSN) (Supplementary Tables 1, 2). Seedling IT’s in response to races TTKSK, TTKSF+ and TTKTT (isolate 14KEN58-1, avirulent to Sr9h) were assessed at the USDA-ARS Cereal Disease Laboratory (CDL), St. Paul, MN following procedures described in Rouse et al. (2011b). Infection type data were recorded on the 0 to 4 scale of Stakman et al. (1962), where ITs 0, 1, and 2 (or combinations thereof) were considered resistant and ITs 3 and 4 were susceptible. For all wheat lines, stem rust infection response and severity data were also obtained from the international stem rust nurseries evaluated in 2013, 2014, and the 2015 off season in Kenya. The marker haplotype and stem rust phenotype in each line were compared to evaluate the efficiency of the markers in selecting resistance gene.

RESULTS

Greenhouse Evaluations

Huhwa and Yaye displayed resistant ITs 22+ and 2, respectively, in contrast to susceptible IT 3+ for the susceptible parent ‘Cacuke’ when tested at the seedling stage with Pgt race TTKSK (Figure 1A). Although both populations were classified into three categories (C × H: 52 HR, 60 HS and 33 segregating; C × Y: 81 HR, 65 HS and 25 segregating) based on seedling ITs, only two categories namely HR and HS, were used for analyses. Monogenic segregation of stem rust resistance was confirmed in C × H \( \chi^2_{(1:1)} = 0.58 \) non-significant and \( p = 0.45 \) and C × Y \( \chi^2_{(1:1)} = 1.76 \) non-significant and \( p = 0.19 \) (Table 1). Two HR and HS lines along with respective parents of both populations showed susceptible IT 3+ when tested against Sr9h-virulent race TTKSF+ at the seedling stage (Figure 1B). We refer to the single
stem rust resistance genes segregating in the C × H and C × Y populations as “SrH” and “SrY,” respectively.

Field Evaluations
Uniform disease development was observed in the field nurseries in both seasons in 2016. Terminal disease severity (TDS) and infection response for Cacuke was 70 S, whereas it ranged 10–20 MR for Huhwa and 5–15 RMR for Yaye over two seasons in the adult plant stage. A wide range of infection responses (MR to S) was observed among the RILs in both populations. TDS of RILs in C × H population ranged from 5 to 70% and 10 to 70% during 2016 off and main seasons, respectively (Figures 2A,B). In C × Y population, TDS varied from 10 to 100% and 10 to 80% during 2016 off and main seasons, respectively (Figures 2C,D). Only seedling stem rust data were used to classify lines carrying or not carrying the respective resistance locus in the two populations to avoid any chances of misclassification.

Mapping of Stem Rust Resistance Locus
C × H Population
Selective genotyping using 47 each resistant and susceptible RILs along with the parents identified a total of 2,122 polymorphic SNPs distributed across 21 wheat chromosomes. Eighty-three SNPs located on chromosome arm 2BL showed close linkage with stem rust resistance in population C × H using a genetic map of 65.0 cM constructed using ICIM software. Nineteen SNPs linked to SrH within 10.0 cM distance were converted into KASP markers designated as cin (CIMMYT) namely; cin101, cin102, cin103, cin104, cin105, cin106, cin107, cin108, cin109, cin110, cin111, cin112, cin113, cin114, cin115, cin116, cin117, cin118, and cin119 (Table 2). In addition, 7 KASP markers (BS00004405, BS00003597, BS00009864, BS00003589, BS00003673, BS00010081, and BS00009460) previously mapped on chromosome arm 2BL (Wilkinson et al., 2012), 3 Sr9h-linked KASP markers, and 9 KASP markers developed for SrY (Table 2) were tested on parents, Cacuke and Huhwa, to verify their polymorphism. A total of 60 markers were

![Figure 2](https://example.com/figure2.png)

**FIGURE 2** | Distribution of stem rust disease severity among recombinant inbred lines of Cacuke × Huhwa with (+) or without (−) SrH (A,B); and Cacuke × Yaye with (+) or without (−) SrY (C,D). Data were collected during two seasons viz. 2016 off (A,C) and main (B,D) seasons at Njoro, Kenya.
screened (the 38 KASP above mentioned and 22 genomic SSRs). Out of them, 19 markers (cim102, cim103, cim104, cim106, cim107, cim109, cim114, cim116, cim117, cim128, BS00009864, barc159, cfd70, gwm47, gwm388, wmc149, wmc317, wmc332, and wmc627) showed clear differences between parents and were genotyped on the entire mapping population, C × H. Sr9h-linked markers were found to be monomorphic between Cauke and Huhwa. Using the ICIM mapping software, a partial genetic map of chromosome arm 2BL spanning a distance of 49.8 cM was constructed in which SrH was flanked by markers cim109 and cim114 at 0.9 cM proximal, and a marker cim117 at 2.9 cM distal, respectively (Figure 3B). Independent assortment of seven markers (cim128, barc159, cfd70, gwm47, gwm388, wmc149, and wmc317) with respect to SrH was observed. The Sr9h map from previous study (Rouse et al., 2014) was included for comparison (Figure 3A).

### C × Y Population

RSA results indicated association of 24 SNPs located on chromosome arm 2BL with stem rust resistance in population C × Y. Nine SNPs were converted into KASP markers cim120, cim121, cim122, cim123, cim124, cim126, cim127 and cim128 (Table 2). In total, 38 KASP markers (9 designed for SrY, 19 for SrH, 3 linked to Sr9h, 7 selected from Wilkinson et al. (2012) and 22 SSRs were tested on the parents, Cauke and Yaye. Finally, 14 markers (cim103, cim109, cim114, cim116, cim117, cim128, BS00009864, barc159, cfd70, gwm47, gwm388, wmc149, wmc317, wmc332, and wmc627) showed clear polymorphism and were genotyped on the entire population Cauke × Yaye. A partial genetic map of chromosome arm 2BL spanning a distance of 49.8 cM with SrY flanked by markers cim109 and cim114 at 0.9 cM proximal, and marker IWB45932 at 1.9 cM distal (Figure 3C). Seven markers (cim128, BS00009864, IWB3891, IWB4545926, IWB4545932, IWB3891, and BS00009864, barc159, cfd70, gwm47, gwm388, wmc149, and wmc317) showed clear polymorphism and were genotyped on the entire population Cauke × Yaye.

### Table 2

| Gene | Marker | 3SK SNP id | cM<sup>a</sup> | Allele 1 primer<sup>b</sup> | Allele 2 primer<sup>c</sup> | Common/reverse primer |
|------|--------|------------|----------------|-----------------------------|-----------------------------|-----------------------|
| SrH  | cim101 | AX-96562712 | 103.81 | agatacaacgaacacgtaaggt  | agatacaacgaacacgtaagggc  | gcgtgtgccggtgtgtagtg|
|      | cim102 | AX-94401999 | 104.59 | attttgggcaacagcaagcag  | attttgggcaacagcaacag  | acacctccaaggctactactc|
|      | cim103 | AX-94048176 | 104.59 | cagcccgttgtctcaaggg  | cagcccgttgtctcaagggaa  | acagaaacactcgcacgaaagt|
|      | cim104 | AX-9478727 | 104.59 | tagatagcggatgcacagag  | tagatagcggatgcacagaa  | tggctgtctggcttgtaa|
|      | cim105 | AX-95859966 | 104.59 | tctgctgtagaactgctaatcfc | tctgctgtagaactgctaat  | gggaagacagactaatcctctt|
|      | cim106 | AX-9461362 | 104.59 | gaacactctactcctacactc | gaacactctactcctacactc  | gcggcagcagcagactcag|
|      | cim107 | AX-94664270 | 104.59 | gagaaaatcgggtgttgtgctc | gagaaaatcgggtgttgtgccc | gacccgcatttaaggctatga|
|      | cim108 | AX-94787485 | 104.59 | agatacaacgaacacgtaaggt  | agatacaacgaacacgtaagggc  | gcgtgtgccggtgtgtagtg|
|      | cim109 | AX-94884026 | 104.59 | gcaccaaatcggctccatca  | gcaccaaatcggctccatcg  | gcagctgtccacggttagg|
|      | cim110 | AX-94873992 | 104.59 | cgatattctgctactaggg  | cgatattctgctactaggggg  | gtcgtattcctagcagggac|
|      | cim111 | AX-94904089 | 104.59 | cagcggctttaagatgcgcg  | cagcggctttaagatgcgcga  | gtcgtattcctagcagggac|
|      | cim112 | AX-94939666 | 104.59 | tgfctgtctggtctcttaccc | tgfctgtctggtctcttacc  | gtcgtattcctagcagggac|
|      | cim113 | AX-94992638 | 104.59 | tctccggggtcacttgcgc  | tctccggggtcacttgcgc  | gtcgtattcctagcagggac|
|      | cim114 | AX-95008732 | 104.59 | acatcactacactacactcaca  | acatcactacactacactcaca  | gtcgtattcctagcagggac|
|      | cim115 | AX-95023630 | 104.59 | gacactagctctgtgccgc  | gacactagctctgtgccgc  | gtcgtattcctagcagggac|
|      | cim116 | AX-95023630 | 104.59 | gacactagctctgtgccgc  | gacactagctctgtgccgc  | gtcgtattcctagcagggac|
|      | cim117 | AX-96260999 | 104.59 | ggcgcgtgtcagcaagtgatca  | ggcgcgtgtcagcaagtgatcg  | gcgcgtgtcagcaagtgatcg|
|      | cim118 | AX-94399009 | NA | cgcaccaacgtcttaacttct | cgcaccaacgtcttaacttcc | gcgcgtgtcagcaagtgatcg|
|      | cim119 | AX-95662871 | NA | ccaatattctcttacttaaggt  | ccaatattctcttacttaaggt  | gcgcgtgtcagcaagtgatcg|
| SrY  | cim120 | AX-94433285 | 72.22 | agtcctactatctccgtaacctc | agtcctactatctccgtaacctc  | gcgcgtgtcagcaagtgatcg|
|      | cim121 | AX-94414057 | 103.81 | tggatggatgcacagacactc  | tggatggatgcacagacactc  | gcgcgtgtcagcaagtgatcg|
|      | cim122 | AX-94433997 | 103.81 | cgtgtgctctctctctctc  | cgtgtgctctctctctctc  | gcgcgtgtcagcaagtgatcg|
|      | cim123 | AX-94842524 | 104.59 | tcaagtctagctgtgctcag  | tcaagtctagctgtgctcag  | gcgcgtgtcagcaagtgatcg|
|      | cim124 | AX-95107632 | 153.87 | ccatctctctaatatctggtctca  | ccatctctctaatatctggtctc  | gcgcgtgtcagcaagtgatcg|
|      | cim125 | AX-95242045 | 153.87 | gcgcgtctacgtactcatac  | gcgcgtctacgtactcatac  | gcgcgtgtcagcaagtgatcg|
|      | cim126 | AX-95142803 | 255.63 | aagttgcatgaggaacacag  | aagttgcatgaggaacacag  | gcgcgtgtcagcaagtgatcg|
|      | cim127 | AX-94897857 | 295.13 | gcgcgtgtcagcaacgcactc  | gcgcgtgtcagcaacgcactc  | gcgcgtgtcagcaagtgatcg|
|      | cim128 | AX-94432664 | NA | acacgctgtcgaactaactc  | acacgctgtcgaactaactc  | gcgcgtgtcagcaagtgatcg|
| Sr9h | IWB38891 | NA | NA | NA | NA | NA|
|      | IWB4545926 | NA | NA | NA | NA | NA|
|      | IWB4545932 | NA | NA | NA | NA | NA|

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*<sup>a</sup>Consensus genetic map position (Allen et al., 2017); <sup>b</sup>A1 primer labeled with FAM: GAAGGTGACCAAGTTCATGCT; <sup>c</sup>A2 primer labeled with HEX: GAAGGTGACCAAGTTCATGCT, and NA, not available.*
burc159, cfd70, gwm47, and wmc149) showed independent assortment with respect to SrY. The marker Sr26#43 linked to Sr26 did not amplify any product in Cacuke, Huhwa, and Yaye.

**Effect of SrH and SrY on Stem Rust Disease Severity**

To study the effect of SrH on disease severity, all RILs of the population C × H were reclassified into resistant (+SrH) and susceptible (−SrH) groups, based on the flanking markers cim109 and cim117. Among 112 RILs, two recombinants were observed between SrH and cim109 whereas 11 recombinants were identified between SrH and cim117. Based on the flanking markers, 100 non-recombinant RILs, (46 +SrH and 54 −SrH) were used for this analysis. In all cases, +SrH lines displayed MR or M infection responses in the field. Conversely, −SrH RILs displayed compatible responses (MS, MSS, or S). The disease severity of +SrH was 10–50% (mean = 31%) and 10–40% (mean = 17.2%) in off and main seasons in 2016, respectively, whereas it was 30–100% (mean = 66.8%) and 20–80% (mean = 39.4%) in −SrH lines (Figures 2C,D). The mean differences between the two groups were highly significant (t = 17.1, P < 0.0001 and t = 9.13, P < 0.0001 for 2016-off and 2016-main seasons, respectively) showing that SrY also confers significant effects on disease severity reduction in addition to conferring a resistant infection response.

**Validation of Flanking Markers**

Five markers (cim103, cim109, cim114, cim116, cim117) closely linked to SrH were used to genotype 67 CIMMYT advanced breeding lines derived from Huhwa (Supplementary Table 1). Based on the seedling data, 35 and 32 lines were postulated to carry and not-carry SrH, respectively. Marker cim117 amplified SrH-linked positive allele A in 32 lines postulated to carry SrH whereas 3 lines showed negative allele G. Positive validation of marker cim117 in 32 lines (out of 35) lines indicated 91.4% efficiency of this marker to identify lines with SrH. The seedling ITs of the SrH postulated lines ranged from 2- to 2 IT response. Out of 32 lines not postulated to carry SrH, 31 lines amplified negative allele G except one line with positive allele A. The other 4 markers

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**FIGURE 3** Genetic maps of the Sr9 region of chromosome 2BL from our data and previously published studies: (A) Rouse et al. (2014), (B) Cacuke × Huhwa and (C) Cacuke × Yaye. Genetic distances between the markers are given in centiMorgan (cM), LOD, Logarithm of odds.
were not associated with SrH in the advanced breeding lines.

We tested six markers *cim103, cim109, cim114, cim116, IWB45932* and *IWB45296* closely linked to SrY on a set of 44 wheat lines derived from Yaye (*Supplementary Table 2*). Marker *IWB45932* amplified SrY-linked positive allele T in 14 lines postulated to carry SrY whereas one line showed negative allele C. Positive validation of marker *IWB45932* in 14 of the 15 lines showed 93.3% efficiency of this marker to identify lines carrying SrY. When tested on 29 lines not postulated to carry SrY, marker *IWB45932* amplified SrY-linked negative allele C in 13 lines whereas 14 lines carried SrY-linked positive allele T. Therefore, *IWB45932* alone cannot be used to accurately predict the presence of SrY.

**DISCUSSION**

In this study, stem rust resistance genes SrH and SrY were identified in wheat lines Huhwa and Yaye, respectively, and mapped on chromosome arm 2BL using selective genotyping (SG) and bulked segregant analysis (BSA) methods, respectively. Both Huhwa and Yaye have been utilized as common parents in CIMMYT bread wheat improvement program. Therefore, it is expected that SrH and SrY are already widely present in recently developed wheat lines resistant to *Pgt* races in the Ug99 group.

To this date, five stem rust resistance genes Sr9, Sr16, Sr28, Sr47, and SrWeb have been mapped on chromosome arm 2BL. There are seven alleles characterized at the Sr9 locus namely; Sr9a, Sr9b, Sr9d, Sr9e, Sr9f, Sr9g, and Sr9h, and each allele demonstrates unique race specificities to *Pgt* races (Green et al., 1960; Knott, 1966; McIntosh and Luig, 1973; Loegering, 1975; Rouse et al., 2014). The gene designation Sr9c was originally reserved for a gene that was subsequently designated as Sr36 on chromosome arm 2BS (McIntosh et al., 1995). *SrWeb* was mapped to chromosome arm 2BL near the Sr9 locus (Tsilo et al., 2007; Hiebert et al., 2010) and named as *Sr9h* (Rouse et al., 2014).

Six characterized alleles at the Sr9 locus (*Sr9a, Sr9b, Sr9d, Sr9e, Sr9f, and Sr9g*) and *Sr16* are not effective against Ug99 race, whereas *Sr9h, Sr28*, and *Sr47* confer resistance (Jin et al., 2007; Rouse et al., 2014). Three *Sr9h*-linked KASP markers (*IWB45296, IWB3891*, and *IWB45932*) showed no polymorphism between Cacuke and Huhwa. Although SSR marker *gwm47* closely linked to *Sr9h* was polymorphic between Cacuke and Huhwa, the marker could not be integrated into a genetic linkage map with *Sr9h* due to lack of linkage. No variation shown by *Sr9h*-linked markers between parents and independent assortment of marker *gwm47* with respect to *SrH* suggested that the stem rust resistance locus in Huhwa was unlikely to be *Sr9h*. The *Sr28*-linked marker *wmc332* (Rouse et al., 2012) was mapped more than 30 cM distal to *SrH*. Moreover, IT differences between *Sr28* and *SrH*, indicate potential unlikeness of *SrH* to *Sr28* *SrH* displayed IT 22+ whereas *Sr28* displayed IT 13+ (as reported in Rouse et al., 2014).

In contrast, *Sr9h*-linked markers (*IWB45296* and *IWB45932*) and two SSR markers (*wmc175* and *gwm47*) were found polymorphic between Cacuke and Yaye. Upon genotyping the entire RIL population *C × Y*, markers *IWB45932* and *IWB45296* were mapped at 1.9 cM proximal and 2.6 cM distal to *SrY*, respectively. Association of *Sr9h*-linked markers with *SrY* indicated possible similarity of *SrY* to *Sr9h*. *Sr28*-linked marker *wmc332* was monomorphic in the parents Cacuke and Yaye, therefore indicating that segregation at the stem rust resistance locus was possibly not associated with *Sr9h*. An *Agelas speltoides* introgression carried *Sr47* which ruled out potential similarity of either *SrY* or *SrH* with *Sr47* (Klindworth et al., 2012) since neither parent carry *Agelas speltoides* in their pedigree. Both *SrH* and *SrY* were mapped on chromosome arm 2BL. There is no common marker between the *SrY* and *SrH* maps that suggests that the position of the genes is different. Both genes are slightly distal to *cim114*. Advanced wheat lines carrying *HUV234* and Yanac in their pedigree showed stem rust resistance indicating the most likely source of *SrH* is HUW234 and *SrY* is Yanac. Based upon pedigree information, Yanac could carry stem rust resistance gene *Sr26*, however, marker *Sr26g43* did not amplify any product in resistant parent Yaye, indicating that the gene in Yaye is different from *Sr26*.

Susceptible reactions displayed by seedlings of resistant lines from both populations when tested with *Sr9h* virulent pathotype TTKSF+ confirmed a common resistance locus in both cultivars Huhwa and Yaye which is most likely *Sr9h* identified in wheat cultivar Gabo 56 (Rouse et al., 2014). Gene *Sr9h* is effective against *Pgt* race TTKSK whereas it is ineffective against race TTKSF+. Race TTKSF+, detected in South Africa and Zimbabwe (Pretorius et al., 2012), is virulent on *Sr9h* (Rouse et al., 2014). Similar response shown by Huhwa and Yaye indicated presence of *Sr9h* in both lines.

Association of KASP marker *cim117* on a set of advanced wheat lines postulated to carry *SrH* indicates that this marker could be useful for marker-assisted selection of this gene in breeding. During marker validation process, we concluded that previously known *Sr9h*-linked markers can be used for identification of *Sr9h* or *SrY* together with phenotypic markers as the behavior of *Sr9h* linked markers vary with genetic backgrounds.

RILs carrying *SrH* showed disease severities of 5–40% and 10–40% during 2016 off and main seasons, respectively. However, these lines still displayed incompatible reactions to *Pgt* with infection responses of MR or M. In contrast, in the absence of *SrH*, disease severities of the RILs were 15–70% with corresponding infection responses of MS or S during 2016 off and main seasons. Similar, results were also found for *SrY*-carrying and *SrY*-lacking RILs. As reported by Basnet et al. (2015), disease severity per se is not useful for determining the presence or absence of race-specific stem rust resistance genes. Basnet et al. (2015) reported *SrND643*-carrying lines expressing 5–40% disease severities accompanied by R, MR or M infection responses. Similarly, Singh et al. (2011) observed that CIMMYT advanced lines with race-specific genes *Sha7* and *SrTmp* displayed 1–30 and 5–60% disease severities accompanied by R or MR infection responses. Stem rust variation
observed in the present study may be due to the presence of quantitative adult plant resistance genes that interact with SrH or SrY.

CONCLUSION

This study identified a common stem rust resistance locus located on chromosome arm 2BL in wheat cultivars Huhwa and Yaye. A robust SNP marker cim117 closely linked to SrH was identified and validated on advanced wheat lines carrying Huhwa in their pedigree. This marker can serve as a breeder friendly tool for marker assisted selection of SrH in breeding programs to generate new gene combinations in breeding Ug99 resistant cultivars.

KOMPETITIVE ALLELE-SPECIFIC POLYMORPHIC (KASP) MARKERS DATA SUBMISSION

We attempted to submit KASP markers generated in this study to European Variation Archive (EVA). EVA needs specific requirements including variant quality, filter, information, format etc. for each assay/variant that will only be available if we could have conducted axiom assay in our laboratory. As we outsourced genotyping using 35K axiom breeders’ arrays, this specific information is not available to be included in the submission file to EVA. Markers were generated from publicly available contig sequences developed by International wheat Genome Sequencing Consortium (IWGSC) and available on http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/axiom_download.php. Markers developed in this study are PCR based markers representing SNPs of contig sequences from 35K axiom breeder’s arrays. Therefore, we decided not to submit these markers to avoid any conflict of interest among wheat scientists.

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AUTHOR CONTRIBUTIONS

MSR phenotyped the population, did analysis, and wrote the manuscript. MSR, SD, JN, and MS-S genotyped the population with SSR and SNP markers. MSR, RS, SB, and JH-E phenotyped the population at adult plant stage. MNR phenotyped the population at seedling stage.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01788/full#supplementary-material

TABLE S1 | List of advanced wheat lines with Huhwa in their pedigree, their adult plant stem rust response, seedling infection types against Puccinia graminis f. sp. tritici (Pgt) races and haplotypes of SrH-linked markers.

TABLE S2 | List of advanced wheat lines with Yaye in their pedigree, their adult plant stem rust response, seedling infection types against Puccinia graminis f. sp. tritici (Pgt) races and haplotypes of SrY (Sr9h)-linked markers.

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