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RESEARCH

Development and Validation of KASP Markers for Wheat Streak Mosaic Virus Resistance Gene *Wsm2*

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ABSTRACT

Wheat streak mosaic virus (WSMV) can cause significant yield loss in wheat (*Triticum aestivum* L.) in the Great Plains of North America. A recently identified WSMV resistance gene, *Wsm2*, was mapped to chromosome 3BS in germplasm line 'CO960293–2'. Effective genetic markers tightly linked to the gene will enhance the selection of WSMV-resistant lines through marker-assisted selection. We have mapped *Wsm2* using a high-density map developed from the wheat 90K Infinium iSelect single-nucleotide polymorphism (SNP) array with recombinant inbred lines from the cross between CO960293–2 and susceptible cultivar 'TAM 111'. Array-based SNPs that mapped within 4 cM of *Wsm2* on chromosome 3BS were converted to Kompetitive Allele Specific Polymerase Chain Reaction (KASP) assays in this study. Six KASP SNPs were validated in two doubled haploid populations developed from crosses of 'RonL' × 'Ripper' and 'Snowmass' × 'Antero'. RonL and Snowmass possess the *Wsm2* gene from CO960293–2. Three closely linked KASP SNPs, converted from IAAV6442, BS00018764_51, and wsnp_Ra_c16264_24873670, showed high sensitivity and specificity ($0.83 \leq$ sensitivity ≤ 0.97, $0.89 \leq$ specificity ≤ 0.99). The latter two were also validated in six F_2 breeding populations. These three KASP SNPs were effective in differentiating resistant and susceptible genotypes. Comparative mapping was performed using sequences of SNPs flanking *Wsm2* and identified candidate genes and regions in *Brachypodium* and rice (*Oryza sativa* L. ssp. *japonica*). The KASP SNPs developed in this study should be useful for marker-assisted selection of *Wsm2* in wheat breeding programs, and the newly constructed map will also facilitate map based cloning of *Wsm2*.

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Abbreviations: BAC, bacterial artificial chromosome; BLASTN, nucleotide–nucleotide basic local alignment search tool; DH, doubled haploid; EST, expressed sequence tag; IWGSC, International Wheat Genome Sequencing Consortium; KASP, Kompetitive Allele Specific Polymerase Chain Reaction; LOD, logarithm of odds; MAS, marker-assisted selection; RILs, recombinant inbred lines; RR, 'RonL' × 'Ripper'; SA, 'Snowmass' × 'Antero'; SNP, single-nucleotide polymorphism; SSR, simple sequence repeat; WSMV, wheat streak mosaic virus.

Wheat streak mosaic virus (WSMV, genus *Tritimovirus*) has been found in all major wheat (*Triticum aestivum* L.) growing regions in the world. It is vectored by the wheat curl mite (*Aceria tosichella* Keifer) and can cause a devastating disease in wheat, especially with infections on winter wheat prior to dormancy in the fall. In the Great Plains of North America, the average annual losses due to WSMV were approximately 2.6 to 5% (Christian and Willis, 1993; French and Stenger, 2003). For the 2009 wheat season, Velandia et al. (2010) estimated reductions in grain yield ranging from 27.8 to 47.7% in a field study in the Texas High Plains. There is no effective chemical treatment available for this disease. Host resistance is a cost-effective and environmentally safe approach for combating this disease. Wheat breeding programs worldwide have relied on two primary sources of resistance, designated as *Wsm1* and *Wsm2*,

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for development of WSMV-resistant wheat cultivars. Since *Wsm2* was discovered in a winter wheat germplasm line, 'CO960293–2' (PI 615160; Haley et al., 2002), it is easier to use in breeding programs than *Wsm1* from alien fragment (Friebe et al., 1991). *Wsm2* has been recently incorporated into several cultivars, including 'RonL' (PI 648020; Seifers et al., 2007), 'Snowmass' (PI 658597; Haley et al., 2011), 'Clara CL' (PI 665948; Martin et al., 2014), and 'Oakley CL' (PI 670190; Zhang et al., 2015a).

Marker-assisted selection (MAS) is a powerful tool for a trait like WSMV resistance that is difficult to evaluate phenotypically (Ribaut et al., 2002). Effective molecular markers (closely linked to the target genes, highly accurate, and easily implemented) are the key for the success of MAS. Evolution of marker systems has primarily been driven by throughput, data turnaround time, genotyping cost, and performance (reliability and reproducibility). Simple sequence repeats (SSRs) were the most widely used markers for genetic mapping and MAS studies in wheat. However, this gel-based polymerase chain reaction marker has the limitations of low throughput and being time-consuming and labor intensive, which make it impractical for use in MAS. Therefore, it is important to select an appropriate in-house genotyping platform that can achieve high throughput in a timely and cost-effective manner. Previous studies have mapped *Wsm2* on chromosome arm 3BS, with SSR marker *Xbarc102* being the closest (2.4 cM) (Lu et al., 2011, 2012; Liu et al., 2014). However, this SSR marker is difficult to amplify and was not diagnostic across diverse genetic backgrounds. Novel and more effective sources of markers closely linked to *Wsm2* are therefore needed in wheat breeding programs. Recent advances in high-throughput genotyping technology have enabled the discovery of thousands of molecular markers in wheat, mainly single-nucleotide polymorphisms (SNPs). The development of high-density SNP markers in hexaploid wheat are widely used to detect the association between markers and traits in genetic mapping experiments (Cabral et al., 2014; Forrest et al., 2014; Jighly et al., 2015; Li et al., 2015; Assanga et al., 2016). A recent study used the 90K Infinium iSelect SNP array to generate a high-resolution genetic map for identification of SNPs tightly linked to *Wsm2* in a recombinant inbred line (RIL) population derived from a cross of CO960293–2 × 'TAM 111' (Assanga et al., 2016). In that study, a total of eight SNPs linked to *Wsm2* were mapped within 1 cM. For MAS, however, those SNPs need to be converted to breeder-friendly and high-throughput markers and be validated for their effectiveness.

Chromosome 3B of 'Chinese Spring' has been sequenced based on the bacterial artificial chromosome (BAC) libraries (Paux et al., 2008). A region with a high level of synteny was found among genomes of *Pooideae* subfamilies, including wheat, *Brachypodium*, and rice (*Oryza sativa* L.), with wheat more closely related to *Brachypodium* than to rice. Conservation of gene order and structural similarity of orthologous genes have been reported in wheat and *Brachypodium* (Vogel et al., 2010). The similarities in gene content and gene family structure among wheat, *Brachypodium*, and rice may be beneficial for map-based cloning and gene discovery in wheat through genome sequencing and comparative mapping approaches.

In this study, we developed breeder-friendly Kompetitive Allele Specific Polymerase Chain Reaction (KASP) assays for those SNPs tightly linked to *Wsm2* and validated them in multiple breeding populations. Moreover, sequences of those SNPs flanking *Wsm2* were subjected to a nucleotide–nucleotide basic local alignment search tool (BLASTN) search against genomic sequences of *Brachypodium* and rice to identify possible candidate genes for *Wsm2*.

MATERIALS AND METHODS

Plant Materials and Phenotyping

A set of 214 $F_{5:7}$ RILs derived from a cross of CO960293–2 × TAM 111 (CT111) and the Infinium iSelect 90K SNP array were previously used for fine mapping of *Wsm2* in CO960293–2 (Assanga et al., 2016). Moderately susceptible cultivar TAM 111 is clearly distinguishable from CO960293–2 for its response to WSMV infection. In the present study, the same population was used to validate the KASP method with matching genotype calls of Infinium SNP data. Two independent populations of 207 and 143 doubled haploid (DH) lines, developed by Colorado State University from the crosses of RonL × 'Ripper' (RR) and Snowmass × 'Antero' (SA), respectively, were used to validate SNPs flanking the *Wsm2* gene. Among the parental lines of these two crosses, RonL and Snowmass are WSMV-resistant with the *Wsm2* gene, while Ripper (PI 644222; Haley et al., 2007) and Antero (PI 667743; Haley et al., 2014) are WSMV-susceptible.

In addition, six F_2 breeding populations from the Agricultural Research Center–Hays at the Kansas State University were used to validate the accuracy of the assay for the association between the SNP alleles and their corresponding phenotypes for *Wsm2* resistance (Table 1). Among them, Cltr 9358, PI 225288, PI 245526, PI 321730, PI 243753, and PI 250041 are WSMV-resistant while KS04HW87 and T81 are susceptible to WSMV (Seifers et al., 2013; Zhang et al., 2015b).

Phenotyping used a similar method as described in Zhang et al. (2014). Briefly, seeds were planted in metal flats and inoculated with the virus strain Sidney 81 at the two-leaf stage. Each plant in the experiments was rated for disease severity on a scale of 1 to 5 (1 = no chlorosis; 2 = a few chlorotic streaks; 3 = moderate mosaic; 4 = severe mosaic; 5 = severe mosaic, necrosis, and yellowing) at 3, 4, and 5 wk after inoculation. Plants with scores $\leq$2 were categorized as resistant; otherwise, they were considered susceptible. About 10 seeds were planted for each parental line, RIL, and DH line and their disease scores were averaged for further analysis. For F_2 populations, the same protocol was followed to differentiate resistant and susceptible plants.

DNA Extraction and KASP Assay

Genomic DNA of CT111 was isolated from leaf tissue of 10-d-old seedlings following the cetyl trimethylammonium bromide (CTAB) protocol with minor modifications (Liu et

Table 1. Segregation of wheat streak mosaic virus resistance and Kompetitive Allele Specific Polymerase Chain Reaction (KASP) single-nucleotide polymorphisms (SNP) linked to *Wsm2* in six F_2 populations derived from crosses between resistant and susceptible lines.

	Phenotype†			BS00018764_51‡					wsnp_Ra_c16264_24873670				
Pedigrees of population	R	S	Total	AA (AA)	AB (AG)	BB (GG)	SP	SE	AA (CC)	AB (CT)	BB (TT)	SP	SE
Cltr9358/KS04HW87	69	20	89	23, 0	45, 2	1, 18	0.90	0.99	23, 0	45, 2	1, 18	0.90	0.99
PI225288/KS04HW87	136	32	168	46, 1	85, 1	5, 30	0.94	0.96	43, 1	88, 0	5, 31	0.97	0.96
PI245526/T81	54	38	92	–	–	–	–	–	20, 0	34, 6	0, 32	0.84	1.00
PI321730/T81	58	32	90	24, 0	32, 12	2, 20	0.63	0.97	NP	NP	NP	–	–
PI243753/T81	64	28	92	–	–	–	–	–	32, 4	30, 4	2, 20	0.71	0.97
PI250041/T81	65	24	89	–	–	–	–	–	24, 0	40, 4	1, 20	0.83	0.98

† R, resistant; S, susceptible; disease score is based on a single plant.

‡ AA, homozygous resistant; AB, heterozygous; BB, homozygous susceptible; SP, specificity, measures the true negative rate; SE, sensitivity, measures the true positive rate; NP, no polymorphism; –, no data. The genotypes in parenthesis are the mutations. A set of two numbers are the number of R and S plants. For F_2 populations, the phenotype of the heterozygote was indistinguishable from the phenotype of the homozygous resistant plants. A resistant plant with AB genotype is considered true positive. The KASP SNP BS00018764_51 results were presented in Zhang et al. (2015b).

al., 2013). DNA concentration was estimated by running the samples in an agarose gel and was adjusted to 20 ng μL^{-1}. Samples of genomic DNA of RR and SA were provided by Colorado State University. DNA of 620 F_2 plants derived from six breeding populations was extracted and the KASP assay was conducted at the USDA–ARS genotyping Center at Manhattan, KS.

The KASP assay was used to detect and distinguish resistant and susceptible alleles for the *Wsm2* gene. Each KASP reaction was performed in a volume of 10 μL with 5 μL DNA and 5 μL of the prepared genotyping mix (2× KASP master mix and primer mix). A total of six representative SNPs flanking the *Wsm2* gene were converted to the KASP platform. Sequences from the 90K array-based SNPs flanking the *Wsm2* gene were used for primer design using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi, accessed on 15 Jan. 2015). Sequences of allele-specific and common primers were designed and tested (Table 2). Protocols for the preparation and running of KASP reactions are given in the KASP manual (http://www.kbioscience.co.uk, accessed on 1 Feb. 2015). Amplification was performed using the ABI 7500 instrument (Applied Biosystems, Foster City, CA), starting with 15 min at 94°C, followed by 40 cycles of 94°C for 20 s and 60°C for 1 min. Endpoint detection of the fluorescence signal was acquired for 1 min at 30°C using the same instrument. The specificity (SP) and sensibility (SE) of all tested markers were determined using equations described in Rosas et al. (2014). The calculations were performed as follows:

$$SP = \frac{TN}{TN + FP}$$

$$SE = \frac{TP}{TP + FN}$$

where TP = true positive, TN = true negative, FP = false positive, and FN = false negative. The classification of TP, TN, FP, and FN were also described in Rosas et al. (2014).

Linkage Mapping

Six converted KASP SNPs were analyzed together with other markers previously used in the RIL population derived from

Table 2. Kompetitive Allele Specific Polymerase Chain Reaction (KASP) single-nucleotide polymorphisms (SNPs) tightly linked to *Wsm2* and their primer sequences.

SNP name	KASP primers	Primer sequences (without tail sequences)
wsnp_Ex_c3005_5548573	SNP77649_T	5′-GCGAAACTCACACACAGAGT-3′
	SNP77649_C	5′-GCGAAACTCACACACAGAGC-3′
	Common reverse	5′-GACATGGTTTGAGGAAACACCA-3′
wsnp_Ra_c16264_24873670	SNP80940_T	5′-CCTTCCTTCTCGTGGTGGTT-3′
	SNP80940_C	5′-CTTCCTTCTCGTGGTGGTC-3′
	Common reverse	5′-TTATTTGACGGAGCGGCCA-3′
GENE-1856_1005	SNP32688_A	5′-TTGTACGCTTAGCCTAAGGACATA -3′
	SNP32688_G	5′-TGTACGCTTAGCCTAAGGACATG-3′
	Common reverse	5′-GGGCTTCAAATGCAAGACAT-3′
IAAV6442	SNP35202_A	5′-ACCCCGGTGTTTTTCAAGTACA-3′
	SNP35202_C	5′-CCCCGGTGTTTTTCAAGTACC-3′
	Common reverse	5′-TGATGGCACTGAAGCTATCG-3′
BS00018764_51	SNP6660_A	5′-GCGGTTTCGGTCTTCCCA-3′
	SNP6660_G	5′-CGGTTTCGGTCTTCCCG-3′
	Common reverse	5′-ACTGCAGCCAGTATGAGCA-3′
BS00026471_51	SNP7629_T	5′-GAAGCAAGGATAGAGTAGGTGT-′
	SNP7629_G	5′-AAGCAAGGATAGAGTAGGTGG-3′
	Common reverse	5′-TGTCGTAGTAGCGTGACAGT-3′

the cross of CT111 for constructing a linkage map. The KASP SNPs were also used to construct genetic maps using 207 and 143 DH lines derived from the cross of RR and SA, respectively. Linkage analysis was performed using maximum likelihood with JoinMap 4.0 (Van Ooijen, 2006). Linkage groups were created using a logarithm of odds (LOD) score of 30.0. The map distances in centiMorgans (cM) were estimated by using the Kosambi mapping function (Kosambi, 1943).

Identification of Candidate Genes and Regions

Sequences of SNPs flanking the *Wsm2* gene on chromosome 3BS were used as queries for a BLASTN search on the International Wheat Genome Sequencing Consortium (IWGSC) database (http://wheat-urgi.versailles.inra.fr/Seq-Repository, accessed on 2 Apr. 2015). Each marker position was compared with the wheat reference sequence assembly on chromosome 3B. Best survey sequence hits determined in the IWGSC database were subjected to a BLASTN search on Gramene (www.gramene.org) against both *Brachypodium* and rice (*O. sativa* ssp. *japonica*) databases to obtain possible candidate genes for *Wsm2*. Further, the sequences of those SNPs closely linked to *Wsm2* were also searched in expressed sequence tag (EST) databases in GenBank to determine if any marker hit any wheat EST that has been mapped in the corresponding wheat chromosome bin.

RESULTS

Linkage Maps of KASP SNPs in Different Populations

Sequences of six representative SNPs closely linked to *Wsm2* on chromosome arm 3BS (Assanga et al., 2016) were converted to KASP assays. The resulting KASP SNPs replaced array-based SNPs to reconstruct the genetic map of *Wsm2* using 214 RILs of the CT111 population (Fig. 1A). The results showed that the genotypic data obtained from the KASP assays were consistent with the genotypic calls of Infinium SNP data from 152 RILs (data not shown). To increase the resolution of the genetic map, these KASP SNPs were genotyped on the rest of the 62 lines of the same population. The newly generated genetic map with 30 closely linked SNPs spanned a genetic distance of 7.6 cM. This saturated genetic map represents an average density of 0.27 cM marker^{-1}.

In this high-resolution map, four KASP SNPs converted from array-based SNPs IAAV6442, BS00018764_51, wsnp_Ra_c16264_24873670, and GENE-1856_1005 were mapped closer to *Wsm2* (within 1 cM), whereas wsnp_Ex_c3005_5548573 was 2.6 cM distal and BS00026471_51 was 3.9 cM proximal from *Wsm2*. To validate the associations between the *Wsm2* gene and converted KASP SNPs, five KASP SNPs were genotyped in both RR and SA populations. Because the two SNPs GENE-1856_1005 and IAAV6442 hit the same wheat gene on survey sequence contig10416721, only one KASP SNP converted from array-based SNP IAAV6442 was used for linkage analysis. A 3BS linkage map was constructed for both populations with five KASP SNPs (Fig. 1B and 1C). Linkage analysis indicated that three KASP SNPs converted from array-based SNPs (IAAV6442, BS00018764_51, and wsnp_Ra_c16264_24873670) were mapped between the flanking markers wsnp_Ex_c3005_5548573 and BS00026471_51 for *Wsm2* locus (Fig. 1A and 1B). In the CT111 population, wsnp_Ra_c16264_24873670 was 1 cM from *Wsm2*, while IAAV6442 and BS00018764_51 were 0.5 cM from *Wsm2*. In the RR population, wsnp_Ra_c16264_24873670 was 0.5 cM from *Wsm2*, whereas IAAV6442 and BS00018764_51 were 0.8 cM and 1.9 cM from *Wsm2*, respectively. The

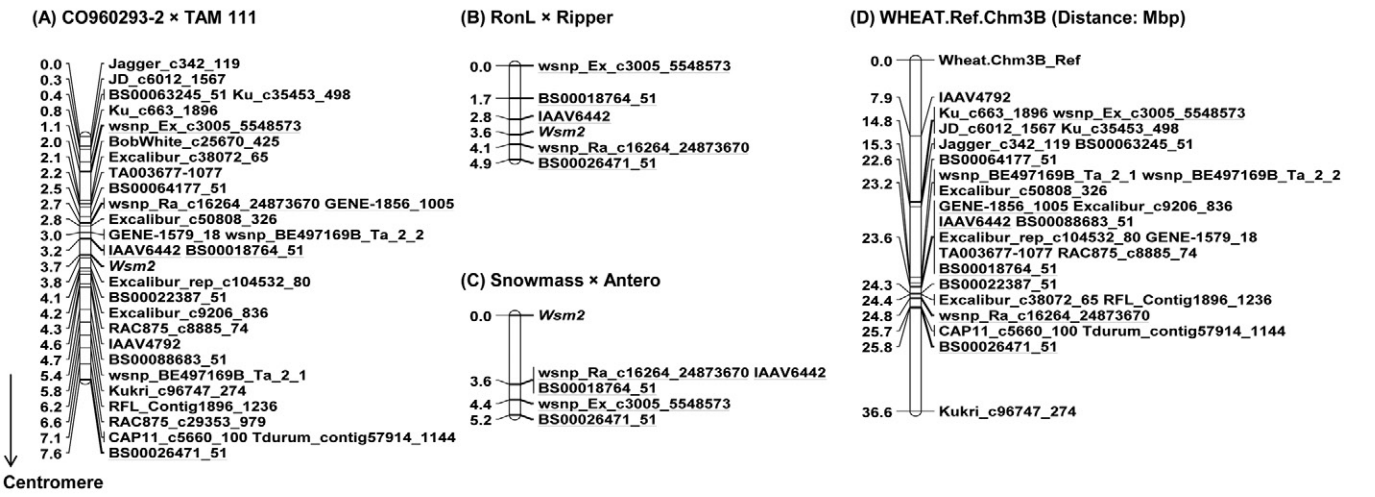

Fig. 1. Linkage maps of single-nucleotide polymorphisms (SNPs) flanking *Wsm2* on chromosome arm 3BS in three advanced breeding populations and their corresponding physical map locations on chromosome 3B of Chinese Spring. (A) CO960293–2 × TAM 111 RIL, (B) RonL × Ripper doubled haploid (DH), (C) Snowmass × Antero DH, numbers to the left of the chromosome are the genetic distances of SNPs in centiMorgans. (D) Diagram of wheat chromosome 3B reference map showing the physical location of the SNPs mapped on the high-resolution genetic map.

relative order of KASP SNPs converted from array-based SNPs wsnp_Ra_c16264_24873670, IAAV6442, and BS00018764_51 in the CT111 map conflicts with their order in the RR map (Fig. 1A and 1B). A possible source of this conflict is the fact that more missing data from array-based SNPs were observed in the CT111 population (data not shown). In the SA population, however, all five KASP SNPs were mapped proximally to *Wsm2*, with wsnp_Ra_c16264_24873670, IAAV6442, and BS00018764_51 being the closest (3.6 cM from *Wsm2*) (Fig. 1C). This can be partly explained by the segregation distortion and smaller population size in the SA population.

Our results from the CT111 and RR maps show that the *Wsm2* gene is located in a genetic interval defined by the flanking KASP SNPs converted from array-based SNPs wsnp_Ex_c3005_5548573 and BS00026471_51. The genotypes and phenotypes of the 214 CT111 RILs were further analyzed for the interval between wsnp_Ex_c3005_5548573 and BS00026471_51 and identified few critical recombinant lines (Fig. 2). Among the RILs, one line (#122) showed a crossover between wsnp_Ex_c3005_5548573 and the *Wsm2* gene. Similarly, four lines (#10, #96, #110, and #210) had a crossover between BS00026471_51 and the *Wsm2* gene. Therefore, KASP SNPs converted from array-based SNPs wsnp_Ex_c3005_5548573 and BS00026471_51 are not tightly linked to *Wsm2*. The sequence of each flanking SNP was searched in the IWGSC database, and the physical locations of the SNPs were identified based on wheat reference sequence assembly on chromosome 3BS (Fig. 1D). A proximal SNP, RAC875_c29353_979, physically apart from Kukri_c96747_274 by 718.6 Mbp, was not shown in the reference map.

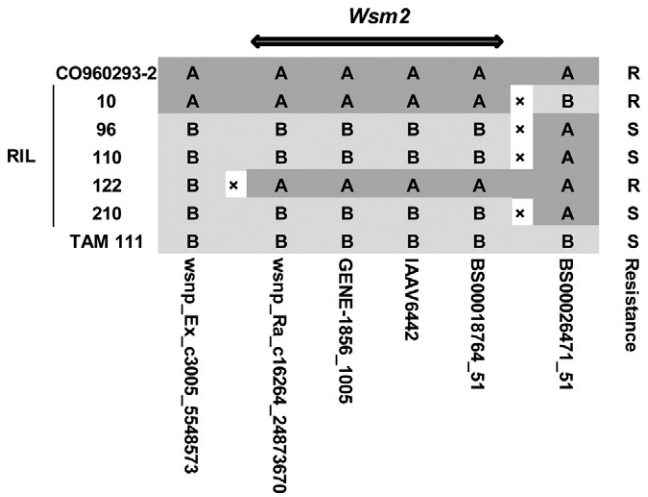

Fig. 2. Genotypes and phenotypes of the five critical recombinant inbred lines from the cross of CO960293–2 × TAM 111 at the *Wsm2* locus. The crossover is denoted by "×" between markers. The "A" genotype represents the CO960293–2 allele and the "B" genotype represents the TAM 111 allele. The gene *Wsm2* is defined by the genetic interval between Kompetitive Allele Specific Polymerase Chain Reaction (KASP) single-nucleotide polymorphisms (SNPs) converted from array-based SNPs wsnp_Ra_c16264_24873670 and BS00018764_51.

Validation of KASP SNPs in Different Genetic Backgrounds

To validate the usefulness of the KASP SNPs for MAS, three KASP SNPs converted from array-based SNPs (BS00018764_51, wsnp_Ra_c16264_24873670, and IAAV6442) that mapped closer to *Wsm2* were evaluated in three advanced breeding populations (DH and RIL) derived from the crosses of CT111, RR, and SA (Table 3). All three KASP SNPs segregated in the three populations. In the SA population, however, fewer susceptible individuals were observed. This segregation distortion could be partly explained by the method used to accelerate the attainment of homozygous lines in the SA population (Adamski et al., 2014). It is also possible that Snowmass is not very pure (Haley et al., 2011).

For BS00018764_51, the resistant allele 'A' was present in 96.5% of the resistant lines and the susceptible allele 'G' was present in 96.2% of the susceptible lines. For wsnp_Ra_c16264_24873670, the resistant allele 'C' was present in 96.9% of the resistant lines and the susceptible allele 'T' was present in 98.7% of the susceptible lines. For IAAV6442, the resistant allele 'A' was present in 96.5% of the resistant lines and the susceptible allele 'C' was present in 98.7% of the susceptible lines. There were a few lines with the resistant allele that were still susceptible to WSMV, and some lines with the susceptible allele were resistant to WSMV. The remaining lines were heterozygous. All three KASP SNPs can effectively distinguish resistant alleles from susceptible alleles with relatively high specificity ($0.89 \leq SP \leq 0.99$) and sensitivity ($0.83 \leq SE \leq 0.97$) in the advanced breeding populations and can be used for selection of *Wsm2* in breeding programs. However, the KASP SNP converted from array-based SNP wsnp_Ra_c16264_24873670 showed tighter linkage to *Wsm2* than BS00018764_51 and IAAV6442 (Fig. 1B). A critical recombinant line (#202, resistant line) identified in the RR population had the RonL allele for wsnp_Ra_c16264_24873670 but the Ripper allele for IAAV6442 and BS00018764_51.

To further validate the usefulness of the KASP SNP converted from array-based SNP wsnp_Ra_c16264_24873670, it was screened on six F_2 populations derived from the crosses between WSMV-resistant lines and susceptible lines (Table 1). The genotyping data showed polymorphism between the parental lines and segregated within each F_2 population, except for the cross of PI 321730 × T81. The KASP SNP converted from the array-based SNP wsnp_Ra_c16264_24873670 mapped closer to *Wsm2*, but this marker did not segregate in the PI 321730 × T81 population, suggesting that resistance in PI 321730 may be controlled by another gene (Zhang et al., 2015b). Another KASP SNP converted from the

Table 3. Segregation of wheat streak mosaic virus resistance and Kompetitive Allele Specific Polymerase Chain Reaction single-nucleotide polymorphisms linked to *Wsm2* in three advanced mapping populations (recombinant inbred line and doubled haploid) derived from crosses between resistant lines and susceptible lines.

	Phenotype†			BS00018764_51‡						wsnp_Ra_c16264_24873670						IAAV6442					
Crosses§	R	S	H	AA (AA)	AB (AG)	BB (GG)	M	SP	SE	AA (CC)	AB (CT)	BB (TT)	M	SP	SE	AA (AA)	AB (AC)	BB (CC)	M	SP‡	SE‡
CT111	107	97	10	106,1,0	1,7,1	0,2,96	0	0.98	0.97	106,0,0	1,8,1	0,2,96	0	0.99	0.97	106,1,0	1,7,1	0,2,96	0	0.98	0.97
RR	101	100	6	99,1,5	0,1,2	2,4,92	1	0.92	0.94	100,1,1	–	1,4,98	2	0.98	0.95	99,2,1	–	2,4,98	1	0.97	0.94
SA	86	45	12	72,4,1	–	7,8,41	10	0.89	0.83	72,4,1	–	7,8,41	10	0.89	0.83	72,4,1	–	7,8,41	10	0.89	0.83

† R, resistant; S, susceptible; H, heterozygote. Disease score is based on an average of 10 plants.

‡ AA, homozygous resistant; AB, heterozygous; BB, homozygous susceptible; M, missing data; –, no data. The genotypes in parenthesis are the mutations. A set of three numbers are the number of R, H, and S plants. SP, specificity, measures the true negative rate; SE, sensitivity, measures the true positive rate.

§ CT111, CO960293–2 × TAM 111; RR, RonL × Ripper; SA, Snowmass × Antero.

array-based SNP BS00018764_51 segregated in the population derived from PI 321730 but showed lower specificity (SP = 0.63). The F_2 populations that showed segregation with the KASP SNP converted from the array-based SNP wsnp_Ra_c16264_24873670 were excluded for further screening using either SNP BS00018764_51 or IAAV6442. Similarly, the KASP SNP converted from the array-based SNP wsnp_Ra_c16264_24873670 showed relatively high sensitivity ($0.96 \leq SE \leq 1.00$) and specificity ($0.83 \leq SP \leq 0.97$) when analyzed in the F_2 segregating populations, except in the PI 243753-derived population that showed lower specificity (SP = 0.71). Zhang et al. (2015b) reported that PI 243753 may carry a resistance gene different from *Wsm2*. Because *Wsm2* is a single dominant gene, a 1:2:1 (F_2) and 1:1 (DH or RIL) allele segregation ratio was expected for the tightly linked KASP SNP converted from the array-based SNP wsnp_Ra_c16264_24873670, except in the F_2 population from the cross of PI 243753 × T81, where more homozygous resistant and fewer heterozygous genotypes were observed than expected (Table 4). Based on the results from our allelic tests, three KASP SNPs that were mapped closer to *Wsm2* provide more alternate markers to use in MAS when some commonly used markers are not polymorphic between genotypes.

Six haplotypes were identified based on three KASP SNPs across three advanced breeding populations (Table 5) with three haplotypes, each associated with resistance (R1 = A/C/A; R2 = G/C/C; R3 = G/T/C) and susceptibility (S1 = G/T/C; S2 = A/T/C; S3 = A/C/A). In the CT111 population, all of the resistant and susceptible wheat lines were of haplotype R1 and haplotype S1, respectively, except that one line each associated with resistance (#202) and susceptibility (#145) was heterozygous for all three loci. In the RR population, the resistant wheat lines were of haplotypes R1, R2, and R3 (98% of haplotype R1, 1% haplotype R2, and 1% haplotype R3), while the susceptible wheat lines were of haplotypes S1, S2, and S3 (93% of haplotype S1, 6% of haplotype S2, and 1% of haplotype S3). In the SA population, the resistant wheat lines were of haplotypes R1 and R3 (91% of haplotype R1 and 9% of haplotype R3), while the susceptible wheat lines were of haplotypes S1 and S3 (98% of haplotype S1 and 2% of haplotype S3).

Table 4. Chi-square test for allele segregation of Kompetitive Allele Specific Polymerase Chain Reaction (KASP) single-nucleotide polymorphism (SNP) wsnp_Ra_c16264_24873670 in a panel of eight segregated populations.

Crosses	Type†	Expected ratio‡	χ^2 (P)
Cltr9358 × KS04HW87	F_2	1AA:2AB:1BB	0.640 (0.726)
PI225288 × KS04HW87	F_2	1AA:2AB:1BB	1.142 (0.565)
PI245526 × T81	F_2	1AA:2AB:1BB	4.696 (0.096)
PI243753 × T81	F_2	1AA:2AB:1BB	10.521 (0.005)§
PI250041 × T81	F_2	1AA:2AB:1BB	0.214 (0.899)
CO960293–2 × TAM111	RIL	1AA:1BB	0.314 (0.575)
RonL × Ripper	DH	1AA:1BB	0.004 (0.949)
Snowmass × Antero	DH	1AA:1BB	3.315 (0.069)

† RIL, recombinant inbred lines; DH, doubled haploid.

‡ Genotype scores for KASP SNP wsnp_Ra_c16264_24873670. "AA" represents resistant genotype, "AB" represents heterozygous genotype, and "BB" represents susceptible genotype.

§ The significant χ^2 deviated from the 1:2:1 Mendelian segregation ratio for one gene model.

Candidate Regions for *Wsm2* in *Brachypodium* and Rice

A total of 30 SNPs linked to *Wsm2* were physically anchored and ordered based on recently published reference sequence assembly of the 3B chromosome of cultivar Chinese Spring (Fig. 1D). The 30 SNPs, spanning 7.6 cM in the genetic map, covered a physical distance of between 7947,939 and 755,297,527 bp in 24 survey sequence contigs on chromosome 3B of cultivar Chinese Spring (Table 6). The results showed that two flanking SNPs, BobWhite_c25670_425 and Tdurum_contig57914_1144, encompassing a 5.1-cM genetic interval containing *Wsm2* on chromosome arm 3BS corresponded to a ~52-kb region (1,267,176–1,319,594 bp) on chromosome 2 of *Brachypodium* (BRADI2) and a ~125-kb region (1,413,839–1,539,601 bp) of rice chromosome 1 (OS01). The findings revealed that wheat chromosomes of group 3 are syntenic with chromosome 2 of *Brachypodium* and chromosome 1 of rice, which concurs with a previous study (Vogel et al., 2010).

Table 5. Haplotypes of the three single-nucleotide polymorphisms associated with wheat streak mosaic virus (WSMV) resistance in three advanced breeding populations derived from the crosses of CO960293–2 × TAM 111, RonL × Ripper, and Snowmass × Antero.

Haplotypes	BS00018764_51	wsnp_Ra_c16264_24873670	IAAV6442	WSMV resistance
R1	A	C	A	Resistant
R2	G	C	C	Resistant
R3	G	T	C	Resistant
S1	G	T	C	Susceptible
S2	A	T	C	Susceptible
S3	A	C	A	Susceptible

Table 6. Genetic map and physical contigs of single-nucleotide polymorphisms (SNPs) flanking *Wsm2* on chromosome 3BS in the CO960293–2 × TAM 111 RIL population and their corresponding candidate regions in *Brachypodium* and rice.

Group†	SNP name	Map	Survey sequence contig	Wheat EST‡	BLASTN hits to *Brachypodium* genes§	BLASTN hits to rice genes
		cM				
1	Jagger_c342_119	0.0	9107402	BF484398	–	–
2	JD_c6012_1567	0.3	10687875, 10762670, 10762671, 10762672	–	–	OS11G0490200 (17,290,977–17,294,288)¶
	Ku_c35453_498	0.4		–	–	
	Ku_c663_1896	0.8		–	–	
	wsnp_Ex_c3005_5548573	1.1		–	–	
3	BS00063245_51	0.4	10509849, 10509850	–	BRADI2G01770 (1243,617–1,246,007)	–
4	BobWhite_c25670_425	2.0	10469750, 10469751	–	BRADI2G01890 (1297,039–1,299,518)	OS01G0127400 (1508,213–1,510,771)
5	Excalibur_c38072_65	2.1	10406459	BE496017	BRADI2G01870 (1290,132–1,293,202)	OS01G0127300 (1504,037–1,508,057)
6	TA003677–1077	2.2	10665467	–	BRADI2G01840 (1274,442–1,279,915)	OS01G0125900 (1421,693–1,427,650)
	GENE-1579_18	3.0		–		
	BS00018764_51	3.2		–		
	Excalibur_rep_c104532_80	3.8		–		
	RAC875_c8885_74	4.3		–		
7	BS00064177_51	2.5	10406379	–	–	–
8	wsnp_Ra_c16264_24873670	2.7	10400994	–	–	–
9	GENE-1856_1005	2.7	10416721	BG314551	BRADI2G01827 (1267,176–1,271,693)	OS01G0125800 (1413,839–1,418,982)
	IAAV6442	3.2				
	Excalibur_c9206_836	4.2				
	BS00088683_51	4.7				
10	Excalibur_c50808_326	2.8	10437805	–	–	–
11	wsnp_BE497169B_Ta_2_2	3.0	10759219, 10764407	BE497169	BRADI1G29260 (24,791,148–24,793,381)¶	OS11G0661300 (26,549,883–26,552,469)¶
	wsnp_BE497169B_Ta_2_1	5.4				
12	BS00022387_51	4.1	10683613	–	–	–
13	IAAV4792	4.6	10699569	–	BRADI5G10130 (13,379,114–13,382,644)§	OS04G0429600 (21,288,252–21,293,643)¶
14	Kukri_c96747_274	5.8	10463446	–	–	–
15	RFL_Contig1896_1236	6.2	10406459	BE496017	BRADI2G01870 (1292,475–1,292,822)	OS01G0127300 (1504,037–1,508,057)
16	RAC875_c29353_979	6.6	10720489	–	–	–
17	CAP11_c5660_100	7.1	6672380	–	BRADI2G01950 (1315,653–1,319,594)	OS01G0127900 (1534,135–1,539,601)
	Tdurum_contig57914_1144	7.1		–		
18	BS00026471_51	7.6	10590363	–	BRADI3G23000 (22,264,069–22,266,497)¶	OS10G0195000 (6577,603–6,581,966)¶

† The SNP groups correspond to best survey sequence hits on chromosome 3B.

‡ Mapped wheat expressed sequence tag, –, no hits.

§ BLASTN, nucleotide–nucleotide basic local alignment search tool; genomic regions in parenthesis.

¶ Hits that do not correspond to the synteny region in *Brachypodium* or rice.

In this map, SNPs GENE-1856_1005 and Tdurum_contig57914_1144, located between the BRADI2G01827/OS01G0125800 and BRADI2G01950/OS01G0127900 orthologs, were physically mapped on subtelomeric chromosome 3BS deletion bin 3BS8–0.78–0.87 in two BAC contigs, Ctg714 and Ctg1683, respectively. Sequences of flanking SNPs hit wheat ESTs that have been mapped in wheat bin further validated the defined *Wsm2*-containing candidate region. *Brachypodium* and rice candidates for *Wsm2* orthologous to the colinear region in wheat chromosome 3B were identified (Supplemental Table 1). However, we found that a large proportion of the predicted genes in the candidate region are nonsyntenic with the orthologous chromosomes of *Brachypodium* and rice, suggesting inter- and intrachromosomal gene duplications in wheat (Glover et al., 2015). Of all the annotated genes on the candidate region of the 3B wheat reference sequence within the bin, 45 were protein-coding genes and the remaining 12 were classified as pseudogenes. There are 15 genes in the *Brachypodium* synteny region and 20 in the rice region. These results indicated that *Brachypodium* and rice have similar numbers of genes (an average of 18), whereas the candidate region in wheat carries 68% more genes (57) than what would be expected by comparison with other grasses used in this study. Comparative analysis shows substantial modifications and rearrangement of the wheat gene space, suggesting high inter- and intrachromosomal gene duplication activities in wheat.

Brachypodium and rice genes orthologous to the candidate region for *Wsm2* were found to be involved in the signaling pathway of plant defense regulation, including Bradi2g01827/Os01g0125800 (66-kDa stress protein), Bradi2g01840/Os01g0125900 (a ubiquitin-conjugating enzyme-like protein), BRADI2G47480/OS01G0699100 (a mitogen-activated protein kinase), BRADI2G01910/BRADI2G01920/BRADI2G01927/OS01G0127600 (a Bowman–Birk type inhibitor), and BRADI2G08707/OS01G0248300/OS01G0248500 (a putative pathogenesis-related protein). Among nonsyntenic genes in wheat, two pseudogenes containing a NB-ARC (a nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4) domain were similar to a well-documented disease resistance RPM1-like (RESISTANCE TO PSEUDOMONAS SYRINGAE 3) protein.

DISCUSSION

Single-nucleotide polymorphisms have been extensively used as markers in marker-assisted breeding in recent years due to their abundance in genomes and their amenability for high-throughput detection. In a previous study, several SNPs tightly linked with the WSMV resistance locus *Wsm2* were identified in the CT111 RIL mapping population using the 90K Illumina iSelect SNP array (Assanga et al., 2016). Such array-based SNPs have facilitated the fine genetic mapping of WSMV-resistant genes and improved map density on chromosome arm 3BS with eight SNPs mapped within 1 cM from the *Wsm2* gene, which supported previous observations that genome B harbors the greatest amount of sequence diversity (Paux et al., 2008; Choulet et al., 2014; Liu et al., 2016). However, SNP arrays are not well suited for marker-assisted breeding because they are not cost effective for evaluating a few SNPs on tens of thousands of plants in segregating generations. Low-cost, breeder-friendly predictive markers are needed for marker-assisted selection in small-scale breeding programs.

Among different platforms for SNP detection, the KASP assay is an ideal platform for genotyping a few markers on a large number of samples. The uniplex KASP assay offers medium-throughput genotyping and quick turnaround time at low cost per sample and is suitable to screen thousands of genotypes in days with low error rates. In this study, all six SNPs derived from the 90K array were successfully converted to KASP SNPs, indicating a high successful conversion rate, as previously reported (Semagn et al., 2013). Further, codominant KASP SNPs are capable of determining both alleles in a single assay, which is very important for identification of heterozygotes in early-breeding generations. Three closely linked KASP SNPs converted from array-based SNPs BS00018764_51, wsnp_Ra_c16264_24873670, and IAAV6442 mapped closer to *Wsm2* and were evaluated for their usefulness in marker-assisted breeding. For the KASP SNP converted from the array-based SNP wsnp_Ra_c16264_24873670, the resistant allele 'C' showed higher correlation with WSMV resistance (haplotypes R1 and R2), indicating that wsnp_Ra_c16264_24873670 was closer to *Wsm2* than BS00018764_51 and IAAV6442, and thus it is a more effective marker for MAS of *Wsm2*. This result was confirmed by linkage mapping in the RR DH population, in which the KASP SNP converted from the array-based SNP wsnp_Ra_c16264_24873670 is mapped as the nearest marker to *Wsm2*. Therefore, the KASP SNP converted from the array-based SNP wsnp_Ra_c16264_24873670 is the best marker for MAS of *Wsm2*n and another two KASP SNPs are also useful for *Wsm2* selection in cases where there is no polymorphism of wsnp_Ra_c16264_24873670. However, it should be noted that wsnp_Ra_c16264_24873670 is not the functional SNP for WSMV resistance because the susceptible allele for wsnp_Ra_c16264_24873670 exists in resistant lines and vice versa (haplotypes R3 and S3).

A comparative mapping approach using *Brachypodium* and rice genomes helped to identify the wheat *Wsm2* syntenic region in *Brachypodium* and rice and enabled a targeted focus for identification of the candidate genes. A reference sequence of Chinese Spring chromosome 3B has been recently published, and the physical interval for *Wsm2* spans roughly 2.1 Mb if no big insertions or deletions occurred

in CO960293–2. The findings of orthology among wheat, *Brachypodium*, and rice chromosomes revealed five syntenic genes involved in plant immune responses, including a 66-kDa stress protein, an ubiquitin-conjugating protein, a mitogen-activated protein kinase, a Bowman–Birk type inhibitor, and a putative pathogenesis-related protein. Within the sequenced 2.1 Mb proximal to *Wsm2* in the variety Chinese Spring, perhaps the most promising candidates for *Wsm2* could be two nonsyntenic pseudogenes with NB-ARC domains similar to a known function of the RPM1-like protein in disease resistance. However, it should be noted that the sequence was derived from the susceptible Chinese Spring and that this is not the complete *Wsm2* physical interval. Therefore, obtaining the sequence of the resistant cultivar CO960293–2 may be essential for eventual determination of the candidate gene.

Previous studies have identified several effective resistant genes to multiple diseases on chromosome 3B in wheat, including *Sr2* to stem rust (caused by *Puccinia graminis* f. sp. *tritici*), *Lr27* to leaf rust (caused by *Puccinia triticina* Erikss.), *LrSV2* for race-specific adult plant leaf rust (caused by *P. triticina*), *Pm13* to powdery mildew (caused by *Blumeria graminis* f. sp. *tritici*), *Fhb1* to Fusarium head blight (caused by *Fusarium graminearum* Schwabe), and *Wsm2* to WSMV (Mago et al., 2011; Hao et al., 2012; Lu et al., 2012; Diéguez et al., 2014; Liu et al., 2014). Research has shown that a higher number of gene loci are on the B genome compared with A and D genomes (IWGSC). In addition, these genes were all mapped on the same deletion bin on the short arm of chromosome 3B, except for *Lr27* on 3BS3–0.87–1.00 (Paux et al., 2008), suggesting that this genomic region may contain a cluster of resistance genes against multiple diseases. Therefore, chromosome 3B will serve as a promising hub to study the interaction of *Wsm2* with other genes to decipher the defense mechanism of resistance to WSMV. Interestingly, *Wsm2* was mapped in a different BAC contig, as were *Sr2* and *LrSV2* (Ctg11), *Lr27* and *Pm13* (Ctg344), and *Fhb1* (Ctg954), suggesting that different genes may be involved in the regulation of WSMV resistance. Recent completion of the reference sequence assembly of chromosome 3B of hexaploid bread wheat enables researchers to have access to a complete gene set with gene order and to identify candidate genes between markers associated with important traits in wheat (Choulet et al., 2014). More than 7000 protein-coding genes have been identified on chromosome 3B, and 171 genes have been putatively associated with disease resistance. The distribution of these resistance genes is highly uneven, with 135 (79%) of them located in the distal regions, and such distribution was supported by several resistance genes that have been repeatedly mapped to the end of chromosome 3BS.

Our study used closely linked SNPs from the 90K array for KASP SNP conversion to predict WSMV resistance. The validated KASP SNPs are a novel resource of markers for MAS for WSMV resistance in wheat. Further, the identification of candidate genes and regions for *Wsm2* in wheat, *Brachypodium*, and rice will enable us to target candidate genes with biological functions linked to WSMV resistance.

CONCLUSION

In this study, we utilized SNP sequences from 90K SNP array that are tightly linked to *Wsm2* to develop and validate breeder-friendly KASP SNPs for MAS in wheat breeding programs. These KASP SNPs were tested on a panel of nine wheat breeding and mapping populations with diverse genetic backgrounds and found to be very effective in differentiating resistant and susceptible genotypes for WSMV. Further, SNP sequences flanking *Wsm2* were used to locate syntenic regions in *Brachypodium* and rice and identified putative candidate genes that may be associated with WSMV resistance. Identification of closely linked genic markers provides an excellent opportunity for MAS and future genetic studies on functional analysis and cloning of the WSMV resistance gene in CO960293–2.

Conflict of Interest

The authors declare there to be no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

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