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ORIGINAL ARTICLE

Physical mapping of DNA markers linked to stem rust resistance gene *Sr47* in durum wheat

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Abstract
Key message **Markers linked to stem rust resistance gene*Sr47* were physically mapped in three small *Aegilops speltoides* chromosomal bins. Five markers, including two PCR-based SNP markers, were validated for marker-assisted selection.**
Abstract In durum wheat (*Triticum turgidum* subsp. *durum*), the gene *Sr47* derived from *Aegilops speltoides* conditions resistance to race TTKSK (Ug99) of the stem rust pathogen (*Puccinia graminis* f. sp. *tritici*). *Sr47* is carried on small interstitial translocation chromosomes (Ti2BL-2SL-2BL·2BS) in which the *Ae. speltoides* chromosome 2S segments are divided into four bins in genetic stocks RWG35, RWG36, and RWG37. Our objective was to physically map molecular markers to bins and to determine if any of the molecular markers would be useful in marker-assisted selection (MAS). Durum cultivar Joppa was used as the recurrent parent to produce three BC_2F_2 populations. Each BC_2F_2 plant was genotyped with markers to detect the segment carrying *Sr47*, and stem rust testing of BC_2F_3 progeny with race TTKSK confirmed the genotyping. Forty-nine markers from published sources, four new SSR markers, and five new STARP (semi-thermal asymmetric reverse PCR) markers, were evaluated in BC_2F_2 populations for assignment of markers to bins. *Sr47* was mapped to bin 3 along with 13 markers. No markers were assigned to bin 1; however, 7 and 13 markers were assigned to bins 2 and 4, respectively. Markers *Xrwgs38a, Xmag1729, Xwmc41, Xtnac3119, Xrwgsnp1*, and *Xrwgsnp4* were found to be useful for MAS of *Sr47*. However, STARP markers *Xrwgsnp1* and *Xrwgsnp4* can be used in gel-free systems, and are the preferred markers for high-throughput MAS. The physical mapping data from this study will also be useful for pyramiding *Sr47* with other *Sr* genes on chromosome 2B.

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Introduction

Stem rust (caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. and E. Henn., *Pgt*) is a historically important fungal disease of common wheat (*Triticum aestivum* L., $2n=6x=42$, AABBDD genome) and durum wheat (*T. turgidum* L. subsp. *durum*, $2n=4x=28$, AABB) (Singh et al. 2006). Since the emergence of the highly virulent *Pgt* races of the Ug99 race group, including *Pgt* races TTKSK (Jin and Singh 2006), TTKST (Jin et al. 2008), TTTSK (Jin et al. 2009), and others (Visser et al. 2011; Newcomb et al. 2016), researchers have worked to reduce the threat posed by these virulent races. Attempts to find new sources of host resistance to the Ug99 races have included screening of older un-released wheat breeding lines (Rouse et al. 2011b), landraces (Newcomb et al. 2013), relatives of

wheat (Rouse and Jin 2011; Rouse et al. 2011a), and evaluation of genetic stocks derived from wide crosses for transfer of genes from related wild species to wheat (Turner et al. 2013; Xu et al. 2009; Kielsmeier-Cook et al. 2015). The gene *Sr47*, derived from goat-grass *Aegilops speltoides* Tausch ($2n=2x=14$, SS), provides resistance to the Ug99 race group (Faris et al. 2008). In its original durum germplasm, *Sr47* was carried on a T2BL-2SL·2SS translocation chromosome in which the wheat segment comprised less than 10% of the long arm (Faris et al. 2008). To reduce the size of the *Ae. speltoides* segment, Klindworth et al. (2012) used a durum 5D(5B) substitution to induce homoeologous recombination and recovered progeny carrying *Sr47* in small interstitial translocations (Ti2BL-2SL-2BL·2BS) in which the *Ae. speltoides* segments accounted for only 6–8% of the long arm.

Despite *Ae. speltoides* likely being the closest living relative of the wheat B-genome donor (Kilian et al. 2007), the B and S genomes exhibit a low affinity for chromosomal pairing (Fernández-Calvín and Orellana 1994). In the absence of recombination, 2B/2S translocations are transmitted intact through heterozygotes. As a result, accurate maps based on genetic recombination between B and S homoeologous segments are not possible, but physical mapping is possible. In wheat, deletion stocks induced by gametocidal chromosomes have extensively been used to map breakpoint intervals or bins (Qi et al. 2003; Sourdille et al. 2004; Kumar et al. 2013), and radiation hybrid-induced deletion stocks have been used to facilitate production of wheat physical maps as well (Hossain et al. 2004; Kalavacharla et al. 2006, Pu et al. 2015; Song et al. 2016). Translocation breakpoints have also been physically mapped in wheat (Sepsi et al. 2009). In reporting the Ti2BL-2SL-2BL·2BS introgression lines carrying *Sr47*, Klindworth et al. (2012) reported the positions of the proximal and distal breakpoints in ten lines. Of these lines, we mapped the three (Fig. 1) originally identified as 0406, 0696, and 0717, but subsequently assigned germplasm identification numbers RWG35, RWG36, and RWG37, respectively. The composite map (Fig. 1) indicates that four bins span the three RWG lines. Only bin 3, which is the largest bin and spans the distance from fraction length (FL) 0.828 to 0.876, was common to all three RWG lines, and therefore, *Sr47* lies within this bin.

Mapping studies are most easily conducted using crosses specifically designed for that purpose. For disease resistance traits, susceptible and resistant parental genotypes are crossed and marker data and phenotypic data are often collected in the F_2 generation. In the case of mapping a race TTKSK-resistance gene, this process is complicated by the necessity to quarantine and destroy plants prior to maturity. In such instances, rust testing can be delayed to the F_3 where F_2-derived families may be used to determine the F_2 genotype. Alternatively, molecular marker analysis conducted on the F_2 plants may be used to determine genotype. This is especially true where low affinity for chromosomal pairing of homoeologous B/S segments results in the absence of recombination over relatively large physical blocks and complete linkage of markers that are physically remote from the gene of interest.

Mapping studies need not be confined to pre-determined crosses. Crosses between advanced breeding lines may also be used, though sometimes with complications. In the case of race TTKSK-resistance, the analysis becomes complicated, because a major breeding objective is durable resistance achieved through combining multiple resistance genes (Singh et al. 2011; Rouse et al. 2014) which are often found in advanced breeding lines. The race TTKSK-effective gene *Sr13* (Jin et al. 2007) is widely found in durum wheat of North America (Klindworth et al. 2007) and

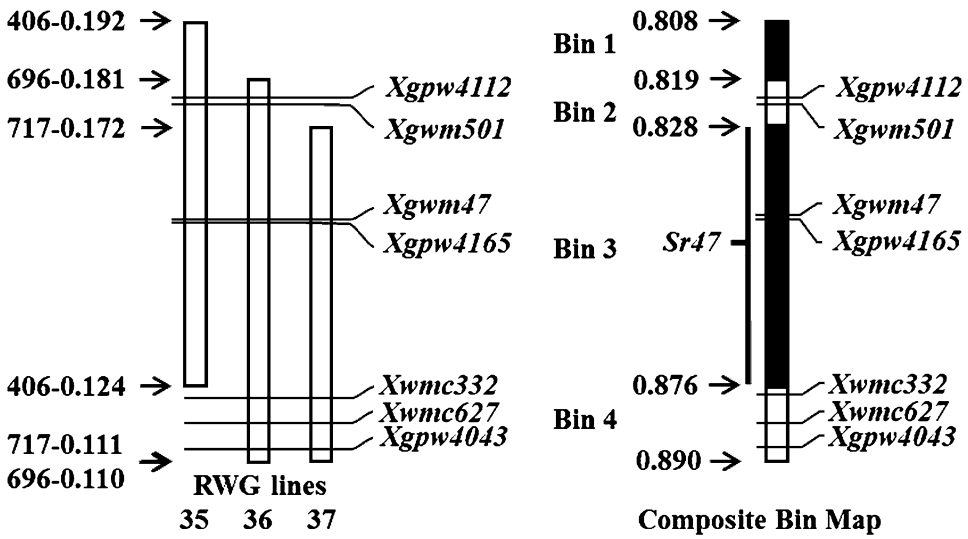

Fig. 1 Maps of three durum RWG lines, RWG35, RWG36, and RWG37, based on breakpoints and molecular markers reported by Klindworth et al. (2012). The composite map (*right*) of *Ae. speltoides* chromosome 2S segment carrying *Sr47* is composed of four bins. *Sr47* is located within bin 3, because that is the only bin common to all three RWG lines. Note that a bin between breakpoints 696-0.110 and 717-0.111 is not included in the composite map due to its size

the Mediterranean (Letta et al. 2014). While not effective against race TTKSK, the gene *Sr9e* is also very common in durum cultivars (Klindworth et al. 2007; Letta et al. 2014) and complicates analyses by limiting the choices of races that can be used to detect other genes.

Based on linkage to *Xgwm47*, Klindworth et al. (2012) inferred that *Sr47* may be homoeoallelic to *Sr9*. The *Sr9* gene is a complex locus that presently has seven known alleles (Rouse et al. 2014) and additional putative alleles (Zurn et al. 2014). Tsilo et al. (2007) found that the *Sr9a* allele was tightly linked to single sequence repeat (SSR) markers *Xwmc175* and *Xgwm47*. Zurn et al. (2014) noted that *SrWLR* was located to an 8.8 cM region flanked by *Xgwm47* and *Xwmc332*, and that *Sr9* and *SrWeb* also located to this region. *SrWeb* was determined to be an allele of the *Sr9* locus and was designated *Sr9h* (Rouse et al. 2014). The *Sr9* region is important not only because *Sr9* is multi-allelic, but also because of the close linkage of *Sr9* to *Yr7* and *Yr5* (McIntosh et al. 1981; Zhang et al. 2009), genes conditioning resistance to stripe rust (*Puccinia striiformis* Westend. f. sp. *tritici* Eriks.).

Although markers useful for marker-assisted selection (MAS) of *Sr47* have been reported, all have some limitations. The only co-dominant markers reported are *Xgpw4043* and *Xrwgs38* (Klindworth et al. 2012; Yu et al. 2015), and these markers are unable to detect *Sr47* in RWG35 and RWG37, respectively. The only dominant marker in coupling-phase is *Xgwm501* (Klindworth et al. 2012), but it is unable to detect *Sr47* in RWG37. All the remaining markers reported by Klindworth et al. (2012) are dominant markers in repulsion phase. Repulsion phase markers have limited value in breeding, because they can identify individuals that are homozygous-recessive for the marker allele, but cannot distinguish between homozygous-dominant vs. heterozygous genotypes. However, results from dominant coupling-phase and dominant repulsion-phase markers can be combined to produce a co-dominant test. Based on the limitations of the *Sr47* markers, additional co-dominant or dominant coupling-phase markers linked to *Sr47* in bin 3 are needed.

A further consideration in identifying new markers that detect *Sr47* is the trend among breeders towards gel-free genotyping based on abundant single-nucleotide polymorphisms (SNPs). Currently, several PCR-based methods, such as Tm/Ta (melting temperature/annealing temperature)-based genotyping approaches (Syvänen 2001; Taylor et al. 2010) and allele-specific PCR (Semagn et al. 2014), are available for gel-free genotyping of individual SNPs. However, they have certain drawbacks in accuracy, operational costs, and compatibility with different genotyping platforms. Long et al. (2017) recently developed a new SNP genotyping method designated as semi-thermal asymmetric reverse PCR (STARP), which is compatible with multiple genotyping systems including gel-free fluorescence signals and gel-based size separation. Our objective was to evaluate additional SSR markers for their usefulness in MAS of *Sr47*, investigate STARP and SNP-associated SSR markers for their usefulness, and to physically map those markers to the four 2B/2S bins.

Materials and methods

Plant materials and backcross breeding

Three lines, RWG35, RWG36, and RWG37 (Klindworth et al. 2012), were used as sources of *Sr47* on small interstitial Ti2BL-2SL-2BL·2BS translocations. The durum cultivar Joppa (Elias and Manthey 2016) was used as the recurrent parent in a backcross breeding program. DAS15 carrying *Sr47* on an unmodified T2BL-2SL·2SS chromosome (Faris et al. 2008), Rusty (Klindworth et al. 2006), Rusty 2D(2A), and Rusty 2D(2B) were used as checks in the marker analysis. Rusty is a near-universal stem rust susceptible durum line that was used in the breeding of RWG35, RWG36, and RWG37. Rusty 2D(2A) and Rusty 2D(2B) are durum aneuploids in which chromosome 2A or chromosome 2B, respectively, has been substituted with chromosome 2D (Klindworth and Xu 2008).

RWG35, RWG36, and RWG37 were backcrossed to Joppa and MAS was initiated in the BC_1 generation. Leaves were sampled and DNA extracted as described below. Markers *Xgwm501* and *Xgpw4043* were used to select plants having the *Ae. speltoides* segments carrying *Sr47*. Selected plants were backcrossed to Joppa to initiate the next backcross cycle. In the BC_2F_1 generation (pedigrees: Joppa*3/RWG35, Joppa*3/RWG36, or Joppa*3/RWG37), plants were self-pollinated to produce BC_2F_2 generations, henceforth referred to as the RWG35, RWG36, and RWG37 populations, which were composed of 39, 45, and 41 plants, respectively. Because RWG36 carries the longest *Ae. speltoides* segment, spanning all bins except bin 1 (Fig. 1), the RWG36 population was the primary population for analysis and included all 45 plants. The RWG35 and RWG37 populations were reduced to a subset of 22 plants each, which were primarily designed to test whether the markers performed similarly on these populations compared to the RWG36 population. DNA was extracted from the BC_2F_2 plants and used for analysis of new molecular markers. Plants classified as homozygous for the presence or absence of *Sr47* were grown to maturity in the greenhouse to produce seed for rust testing.

Stem rust testing

Twenty seedling plants per homozygous BC_2F_2 family were tested at the Cereal Disease Laboratory (USDA-ARS, St. Paul, MN) with race TTKSK (isolate 04KEN156/04). Planting, inoculation, and greenhouse conditions followed the procedure of Rouse et al. (2011b). Plants were inoculated by spraying urediniospores suspended in mineral oil (Soltrol 70; Conoco-Phillips Inc., Houston, TX) on 7–10-day-old seedlings. Seedlings remained in a mist chamber for 12 h and were then removed to a greenhouse maintained at 18 °C ± 2 °C. Infection types were scored 14 day post-inoculation using the 0–4 scale of Stakman et al. (1962), where 0 = immune, ; = necrotic flecks, 1 = small necrotic pustules, 2 = small to medium sized chlorotic pustules with green island, 3 = medium sized chlorotic pustules, and 4 = large pustules without chlorosis. Minus ($^-$) and plus ($^+$) indicated smaller or larger pustules than normal within a class, e.g., 2^- indicated small IT 2 and 2^+ indicated large IT 2. For combinations of ITs, order indicated predominant type; e.g., IT 12 is predominantly IT 1 with decreasing amounts of IT 2. Plants with IT 2 or lower were considered resistant and plants with IT 3 or greater were considered susceptible.

Marker analysis

Leaf tissue from approximately 3-week-old seedlings was collected in 96-well plates and DNA was extracted following the procedure of Niu et al. (2011). A total of 47 SSR, STS, eSSR, and eSTS markers, and two PLUG markers, from published sources were evaluated (Supplemental Table S1). In considering markers that may detect *Sr47*, we included markers linked to *Yr7* and *Yr5* (Bariana et al. 2001; Crossa et al. 2007; Smith et al. 2007; Yao et al. 2006), and markers possibly linked to *Sr9* due to their reported linkage to either *Xwmc175, Xgwm501, Xgwm47*, or *Xwmc339* (Torada et al. 2006; Yu et al. 2014; Xue et al. 2008; Quirin 2010). Additional *gpw* markers that map to chromosome arm 2SL (Dobrovolskaya et al. 2011) were also included in the study.

In addition to published markers, we used two approaches to develop new markers. In the first approach, we developed and evaluated 17 new SNP-associated SSR markers. Five SNPs (Table 1), lying between SSR markers *Xgwm388* and *Xwmc332*, were selected from the *Sr47* specific region of the Ug99 2B consensus map (Yu et al. 2014). The flanking sequences of the selected SNPs were retrieved from the cereals database (Wilkinson et al. 2012; http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/indexNEW.php) and they were used in a BLASTn (Altschul et al. 1997) query to search the wheat sequence survey chromosome arm 2BL (http://wheat-urgi.versailles.inra.fr/Seq-Repository). Survey sequence contigs with high e-values were used to design SSR primers (Table 1) using the software Batchprimer3 v3.1 (You et al. 2008). The buffer used to conduct PCR was either Green GoTAQ® Reaction Buffer (Promega Biosciences, Madison, WI, USA) or NEB (New England Biolabs, Ipswich, MA) PCR buffer. In general, PCR additives were avoided; however, there were three instances where better results were obtained using other buffers or with PCR additives, and these are reported in supplemental Table S1 along with PCR cycling conditions for each primer. Electrophoresis was conducted on 6% polyacrylamide—large format gels (33 × 42 cm); however, whenever the initial tests indicated that the polymorphism was sufficiently robust, subsequent electrophoresis was conducted using 8% polyacrylamide - minigels (33 × 10 cm). Following electrophoresis, gels were stained with Gel-Red (Biotium Inc., Hayward, CA) and scanned with a Typhoon 9410 imager (GE Healthcare, Waukesha, WI). Amplicon sizes were analyzed from large format gels using a 100 bp molecular weight standard and the Carestream Molecular Imaging software (Bruker Biospin, Billerica, MA).

In the second approach, we developed STARP markers based on SNPs or INDELs (INsertions/DELetions) following the procedure described by Long et al. (2017). The STARP assay was performed using two universal priming element-adjustable primers (PEA-primers) and two asymmetrically modified allele-specific primers (AMAS-primers) in combination with one common reverse primer. The sequences of the two PEA-primers

Table 1 Sequences, annealing temperatures, and sources of four new SNP-associated SSR markers

Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temp/# cycles[a]	SNP[b]	IWGSC_chr2BL
Xrwgs39	AGACAGATCCAATTGTCTTCC	GCTAAACCTAGATGCCACAC	61–54 °C/35	BS00110209	8020970
Xrwgs40	GGACAGGGACCTATTTATGAC	CGTCCCAAAAATAGAAATTGT	58 °C/35	BS00110209	8020970
Xrwgs41	GTGGAAAAGAAGCTGTCATC	GAGTTGTGATTGGTTGAGCTA	58 °C/40	BS00022064	8000154
Xrwgs42	GCTGGAGATGCTCTTAGCTC	AATGCAATACCAATCCACTAA	61–54 °C/35	BS00009461	8091682

[a]Entries with a temperature range for annealing temperature were tested using touchdown PCR

[b]SSRs derived from SNPs BS000009989 and BS00003585 were either monomorphic or unreliable and were discontinued

used in this study were the same as reported by Long et al. (2017). To design the AMAS-primers and reverse primers, we had first determined that PLUG (PCR-based landmark unique gene) markers *Xtnac3119* and *Xtnac9215* (Table 2), developed by Chao et al. (2016), were located in bin 3. Therefore, SNP markers closely linked to these PLUG markers (Chao et al. 2016), including *Xiwa5145, Xiwa4096, Xiwa5051, Xiwa3741*, and *Xiwa3742*, may also be located in bin 3. We used these sequences that contain these SNP or PLUG sites in a BLASTn query to search the scaffolds on chromosome 2B of Chinese Spring (CS) at https://urgi.versailles.inra.fr/blast_iwgsc/blast.php. Then, the sequences of the hit scaffolds were used in a BLASTn query to search the whole genome sequence of CS to identify low-copy (three copies or less) sequences. These low-copy sequences were further used in a BLASTn query to search the survey genome of *Ae. speltoides*. The sequences with SNP and INDEL variations between wheat and *Ae. speltoides* were used to design the AMAS-primers and reverse primers based on Long et al. (2017). To prevent spurious amplifications from wheat chromosomes 2A and 2D, the reverse primers were designed with more than two bases at the 3′ end which were different from the non-target sequences. The STARP markers were initially evaluated on 6.5% denaturing polyacrylamide gels in an IR2 4300/4200 DNA Analyzer (LI-COR, Lincoln, NE, USA). The polymorphic STARP markers were further analyzed using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Foster City, CA, USA).

Prior to evaluating new markers, we genotyped each plant, which was also tested for reaction to stem rust, with co-dominant markers *Xgpw4043* (Klindworth et al. 2012) and *Xrwgs38* (Yu et al. 2015). Markers *Xgwm501* (Klindworth et al. 2012) and *XcsSrAes1t* (Mago et al. 2013) were used as checks in testing dominant coupling-phase markers. Marker *Xwmc175*, which was a robust marker located to bin 3, was selected as a check dominant repulsion-phase marker. An initial analysis of each marker was conducted using a population of 11 plants. The population consisted of Rusty, Rusty 2D(2A), Rusty 2D(2B), DAS15, Joppa, and, based on the marker analysis, one homozygous (*Sr47Sr47*) and one heterozygous (*Sr47sr47*) plant for each of the three RWG lines. If the initial analysis indicated that the marker-locus was within the translocated segment, then the marker was analyzed on the complete population of 45 plants for the RWG36 population and 22 plants each for the RWG35 and RWG37 populations.

Marker validation and co-dominance of *Xwmc41*

A diverse set of 52 cultivars or genetic stocks was used for marker validation. The genetic stocks included DAS15 (the original durum source of *Sr47*), plus stem rust susceptible genotypes Rusty, LMPG-6, and Line E. Cultivars included ten durums originating from North Dakota, and

Table 2 STARP markers and their SNP source, primer sequences, and *T*m values

Marker	SNP or PLUG Source	Primers			
		Name	Type[a]	Sequence (5′–3′)[b]	*T*m (°C)
Xrwgsnp1	*Xiwa5145*	Xrwgsnp1FB	Forward primer 1	[Tail 1]GGCTTGTGGGTAAGTACCAC	55.83
		Xrwgsnp1FS	Forward primer 2	[Tail 2]GGCTTGTGGGTAAGACTCAG	56.11
		Xrwgsnp1R	Reverse primer	ATGTTCCATATGGAAGCGACATG	59.18
Xrwgsnp2	*Xiwa5051*	Xrwgsnp2FB	Forward primer 1	[Tail 2]TATACAATTGGTGTGCAACAT	56.76
		Xrwgsnp2FS	Forward primer 2	[Tail 1]TATACAATTGGTGCGCAAACA	59.50
		Xrwgsnp3R	Reverse primer	ACCGATGTAGTGGATGGAATGT	59.23
Xrwgsnp3	*Xtnac3119*	Xrwgsnp3FB	Forward primer 1	[Tail 2]ATGTGGTCAATCATTTCATCTCT	55.30
		Xrwgsnp3FS	Forward primer 2	[Tail 1]ATGTGGTCAATCATTTCATCCAC	56.15
		Xrwgsnp3R	Reverse primer	CACAAGCTGGCGATGCACTA	61.02
Xrwgsnp4	*Xtnac3119*	Xrwgsnp4FB	Forward primer 1	[Tail 2]ATTGCTACTACCTAATACCTA	58.84
		Xrwgsnp4FS	Forward primer 2	[Tail 1]ATTGCTACTACCTAATAGGCT	61.18
		Xrwgsnp4R	Reverse primer	AGCATGAATTCTTTAGTGCATCGC	60.50
Xrwgsnp5	*Xtnac3119*	Xrwgsnp5FB	Forward primer 1	[Tail 1]GAACTCGGCAAGAGATATAGTACT	57.44
		Xrwgsnp5FS	Forward primer 2	[Tail 2]GAACTCGGCAAGAGATATATACTA	55.20
		Xrwgsnp5R	Reverse primer	GTCCACGTAATGAACATACCC	61.83

[a]Forward primers 1 and 2 are asymmetrically modified allele-specific primers (AMAS-primers) originated from sequences of wheat B-genome and *Ae. speltoides*, respectively

[b]Tail 1 and Tail 2 are the universal sequences GCAACAGGAACCAGCTATGAC-3′ and GACGCAAGTGAGCAGTATGAC-3′, respectively (Long et al. 2017)

38 common wheats originating from China, North Dakota, South Dakota, Minnesota, Illinois, Kansas, and Idaho. Only markers we judged to be potentially useful in MAS were tested with the validation set. The validation test suggested that *Xwmc41* may amplify multiple alleles. To test this possibility, we used two populations developed for the purpose of marker-assisted backcrossing of *Sr47* to durum cultivar Carpio (Elias et al. 2015) and common wheat cultivar Faller (Mergoum et al. 2008). RWG37 was the source of *Sr47* for both populations. The Carpio and Faller populations consisted of 46 BC_5F_2 (Carpio*6/RWG37) and 32 BC_3F_2 (Faller*4/RWG37) plants, respectively. *Xwmc41* and *Xmag1729* were used to test each population and each plant was classified as homozygous resistant (HR), segregating (S), or homozygous susceptible (HS) based on *Xmag1729*.

Results

Markers were initially evaluated on a panel of 11 entries. An example of this evaluation is *Xgpw4396* (Fig. 2). Rusty had three major amplicons between 152 and 165 bp. These amplicons were absent in Rusty 2D(2B), indicating that the amplicons were produced by a locus in chromosome 2B. The amplicons were also absent in DAS15, indicating that the locus lies in the wheat segment that has been exchanged with *Ae. speltoides* chromatin. In Joppa, the amplicons showed a size polymorphism of 168 and 185 bp. The major amplicons were absent in homozygous plants from the RWG35 and RWG36 populations, but the Joppa amplicons were present in heterozygous plants from the RWG35 and RWG36 populations. In the RWG37 population, both homozygous and heterozygous plants had the Rusty amplicons. The conclusions from this test were that *Xgpw4396* was a dominant repulsion-phase marker that mapped to bin 2. RWG37 carried Rusty chromatin in the bin 2 region; and therefore, recombination between Joppa and Rusty chromatin is possible in this region. Although the Rusty amplicons appear to be dominant in the heterozygous plants from the RWG37 population (Fig. 2), this is likely a result of PCR conditions, as subsequent data in this paper will show that the Rusty and Joppa amplicons are co-dominantly inherited.

Factors affecting marker analysis

Not all of the analyses were as straightforward as *Xgpw4396*. There were factors such as additional amplicons, electrophoretic effects, and PCR parameters, which complicated the analysis of some markers. Before presenting the results of other markers, we first describe some of those factors. In evaluating *Xrwgs38*, we observed additional polymorphic fragments not described by Yu et al.

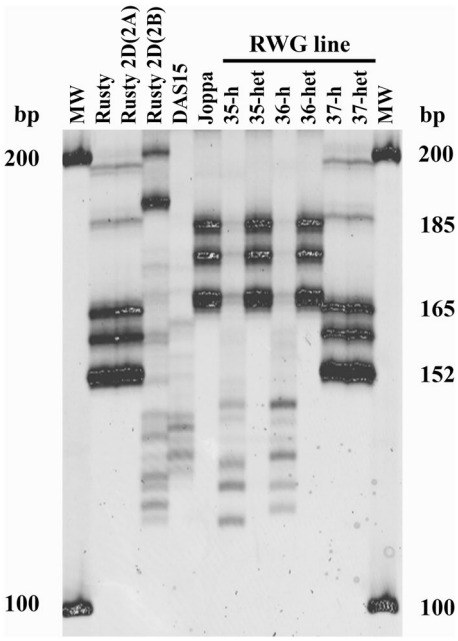

Fig. 2 Initial selection test for polymorphism of marker *Xgpw4396* using 11 genotypes. Amplicons from Joppa were absent in homozygous (h) RWG35 and RWG36, but were present in the heterozygous (het) plants of those populations, indicating that the *Xgpw4396* amplicons had dominant repulsion-phase inheritance. In the RWG37 population, both the homozygous and heterozygous plants had amplicons derived from Rusty, indicating that the *Xgpw4396* locus lies in bin 2. The presence and absence of amplicons between 152 and 165 bp in Rusty 2D(2A) and Rusty 2D(2B), respectively, indicate that these amplicons originate from loci on chromosome 2B of Rusty. *MW* molecular weight marker, *bp* base pair

(2015). We assigned the symbol *Xrwgs38a* to the original *Xrwgs38* fragments (175 and 187 bp). Co-dominant amplicons of 987 and 1024 bp and 1029 and 1080 bp, originated from *Ae. speltoides* and Joppa, respectively (supplemental Fig. S1), and these amplicons were assigned the marker symbol *Xrwgs38b*. Several markers produced amplicons that originated from Rusty, including a 433 bp amplicon by *Xmag1729* and 1146 and 1279 bp amplicons by *Xrwgs38b* (supplemental Fig. S2).

Electrophoretic conditions (buffer concentration and current) affected marker migration, most notably for *Xrwgs38b* (supplemental Fig. S2B-D). Best separation of *Xrwgs38b* amplicons was achieved using 0.5×TBS tank buffer and applying 70 W of current (supplemental Fig. S2B). In contrast, the best separation of *Xrwgs38a* amplicons, and most other markers, was achieved using lower current of 40 W.

PCR results can be affected by numerous factors, and in the case of *Xwmc41*, the number of PCR cycles was a factor. The diagnostic amplicon for *Xwmc41* was 172 bp. However, when the number of PCR cycles exceeded 30, the 172 bp amplicon was observed in all plants, with a

235/241 bp doublet becoming diagnostic (supplemental Fig. S3). The results indicated that the number of PCR cycles should not exceed 30 to allow for scoring of the 172 bp amplicon.

Genotyping of populations by rust assays and check markers

Individual BC_2F_2 families were assayed for reaction to race TTKSK and their BC_2F_2 plants were also analyzed with a set of check markers to verify that rust reaction and molecular marker analysis produced congruent results. A few families were excluded from the stem rust test due to insufficient seed samples; but, heterogeneity for *Sr47*, as determined by marker analysis, was the main reason for excluding families from rust testing. Heterogeneous families were excluded, because it was thought that it would be difficult to distinguish heterogeneous families from homogenous resistant families of *Sr47* due to the presence of *Sr13* in Joppa. *Sr13* is effective against race TTKSK (Jin et al. 2007). However, results of the stem rust analysis indicated that all families could be genotyped for *Sr47*, and partially for *Sr13*, based on rust tests (Table 3). In the 26 homozygous *Sr47Sr47* families, no plant had an infection type higher than 0;1, while in the 20 homozygous *sr47sr47* families, no plant had an infection type lower than ;2^-. The presence of *Sr13* was determined based on a combination of infection types (IT ;2 or higher) produced by *Sr13*. In the Joppa*3/RWG36-*sr47sr47* population, five families had plants with high infection types of 3^+, indicating segregation for *Sr13* within those families. Even in the single family heterogeneous for *Sr47* (Joppa*3/RWG36 population of Table 3), heterozygosity for *Sr13* was detected by the presence of a single plant having infection type 3+.

Results from markers *Xgpw4043, Xrwgs38a, Xgwm501, XcsSrAes1t*, and *Xwmc175* indicated that all five check markers produced results on RWG36 that were congruent with the stem rust analysis (Fig. 3; supplemental Table S2). However, only *Xwmc175* was able to genotype plants in all three RWG populations. This was attributed to the other four markers being located in either bin 2 or 4, while *Xwmc175* was located with *Sr47* in bin 3. Since bin 3 is the only bin present in all three RWG lines, any marker-locus present in bin 3 will co-segregate with *Sr47* in all three RWG lines, but any marker-locus located in bins 1, 2, or 4 will be associated with only one or two RWG lines. Unfortunately, *Xwmc175* had the disadvantage that it was a dominant repulsion-phase marker, making it impossible to distinguish heterozygotes from non-carriers of *Sr47*. The other four markers fell into two groups. Markers *Xgwm501* and *Xrwgs38a* correctly genotyped RWG35 and RWG36, but were null or incorrect on RWG37. The opposite was true for markers *XcsSrAes1t* and *Xgpw4043*, which correctly genotyped RWG36 and RWG37, but were null or incorrect on RWG35. This confirmed that these four marker loci are not located in bin 3 with *Sr47*. Instead, *Xgwm501* and *Xrwgs38a* loci are proximal to *Sr47* in bin 2, while the *XcsSrAes1t* and *Xgpw4043* loci are distal to *Sr47* in bin 4.

Taking into account the bin location of the markers, and with the exception of marker data of *Xgwm501* and

Table 3 Segregation observed in three Joppa backcross populations when BC_2F_2 plants were classified based on molecular marker analysis and when BC_2F_2 families were classified for reaction to race TTKSK of *Puccinia graminis* f. sp. *tritici*

Pedigree	Marker analysis		Seedling reaction to race TTKSK						Postulated BC_2F_2 genotype[b]
	Genotype[a]	BC_2F_2 Plants	BC_2F_2 Families	Plants with infection types					
				0	0;1	2^-	2^-	3^+	
Joppa*3/RWG35	*Sr47Sr47*	4	4	54	19				*Sr47Sr47*[c]
Joppa*3/RWG35	*Sr47sr47*	7	0						
Joppa*3/RWG35	*sr47sr47*	11	10			116	71		*sr47sr47Sr13Sr13*
Joppa*3/RWG36	*Sr47Sr47*	18	18	328					*Sr47Sr47*[c]
Joppa*3/RWG36	*Sr47sr47*	20	1		16	3		1	*Sr47sr47Sr13sr13*
Joppa*3/RWG36	*sr47sr47*	3	2			19	19		*sr47sr47Sr13Sr13*
Joppa*3/RWG36	*sr47sr47*	3	3			14	26	14	*sr47sr47Sr13sr13*
Joppa*3/RWG36	*sr47sr47*	1	1					19	*sr47sr47sr13sr13*
Joppa*3/RWG37	*Sr47Sr47*	5	4	15	55				*Sr47Sr47*[c]
Joppa*3/RWG37	*Sr47sr47*	12	0						
Joppa*3/RWG37	*sr47sr47*	5	4			36	36		*sr47sr47Sr13Sr13*
Joppa	*sr47sr47*		2			25			*sr47sr47Sr13Sr13*
LMPG6	*sr47sr47*		2					25	*sr47sr47sr13sr13*

[a]Genotype determined from check markers *Xgwm501, XcsSrAes1t, Xrwgs38a, Xgpw4043*, and *Xwmc175*

[b]Combines the *Sr47* marker genotype with postulations for *Sr13* based on the rust data

[c]*Sr47* phenotypically masks *Sr13*. These families may be either heterozygous or homozygous for *Sr13*

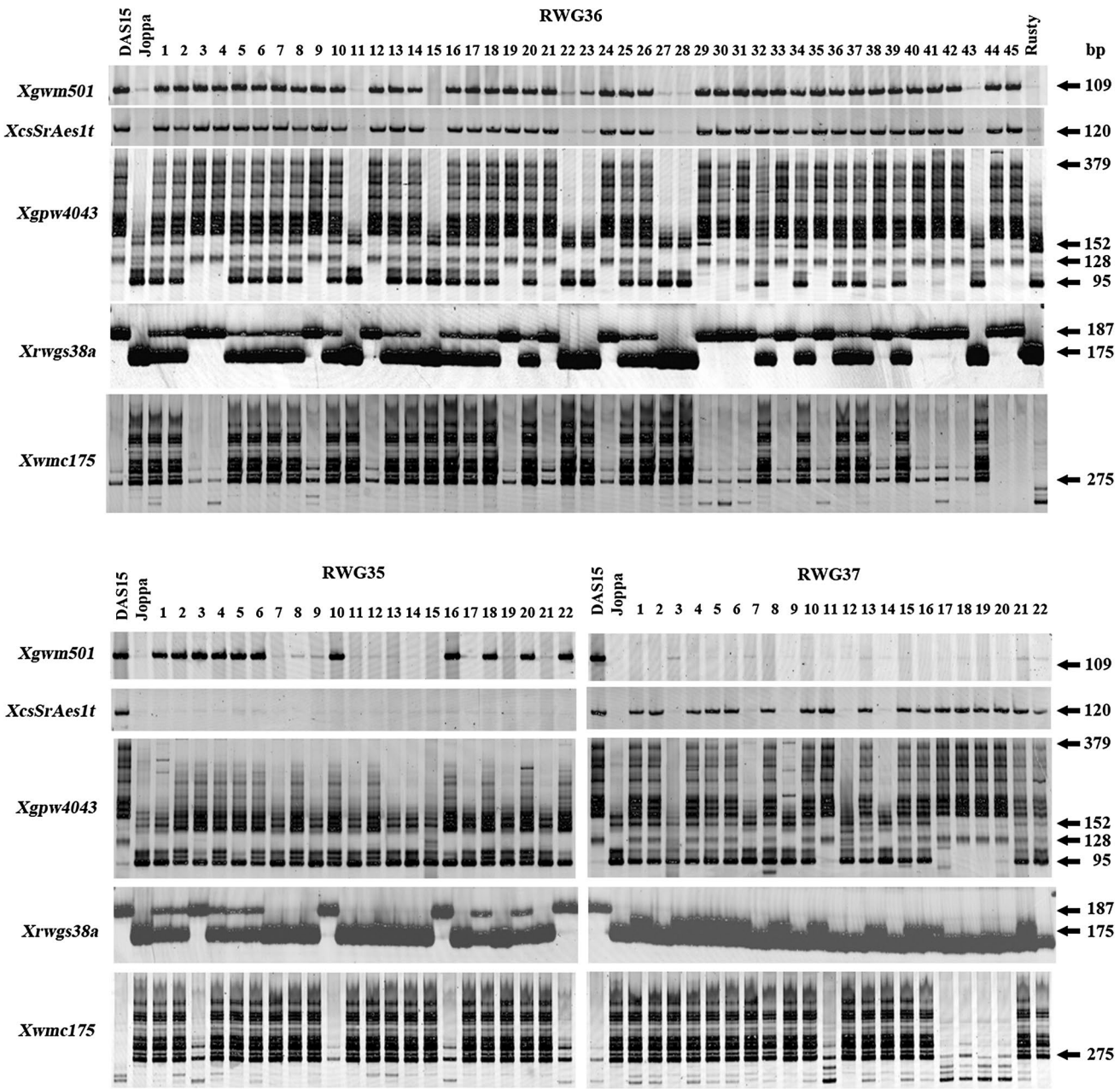

Fig. 3 Electrophoretograms of five check markers tested on the RWG35, RWG36, and RWG37 populations. Full populations are shown. When markers produced multiple polymorphic amplicons, such as *Xwmc175*, only the size of the smallest amplicon is reported. Dominant coupling-phase markers *Xgwm501* and *XcsSrAes1t* produced null amplifications in RWG37 and RWG35, respectively, indicating that their locus locates to bin 2 and bin 4, respectively. Because *Xwmc175* amplified bands in both RWG35 and RWG37, this dominant repulsion-phase marker located to bin 3. Co-dominant markers *Xgpw4043* and *Xrwgs38a* were located to bins 4 and 2, respectively, based on the absence of the 379 bp amplicon of *Xgpw4043* in RWG35, and the absence of the 187 bp amplicon of *Xrwgs38a* in RWG37

Xrwgs38a on RWG37 and *XcsSrAes1t* and *Xgpw4043* on RWG35, we concluded that the five check markers could accurately classify plants for the presence of the *Ae. speltoides* segment, and hence *Sr47*. The results of the rust analysis and the marker analysis were congruent, and based on the marker analysis, the postulated *Sr47* genotypes were determined for all BC_2F_2 plants.

Identification and physical mapping of markers for *Sr47*

A total of 49 markers from published sources and 17 new SNP-associated SSR markers were evaluated on the BC_2F_2 populations for assignment of the markers to the bins. The 17 new SSRs were developed from survey sequences in which five SNP markers were anchored. Five of these markers, derived from sequences near the SNP markers BS00009989 and BS00003585, were either monomorphic or were unreliable. Among 12 polymorphic markers, those derived from sequences near BS00110209 and BS00022064 mapped to bin 4, and those derived from sequences near BS00009461 mapped to bin 2. Ten of the 12 polymorphic markers were dominant repulsion-phase markers that were of little interest. We, therefore, only designated four markers as *Xrwgs39* through *Xrwgs42* (Table 1). Marker *Xrwgs39* (Fig. 4) is a dominant coupling-phase marker that mapped to bin 4. Marker *Xrwgs40* (Fig. 4) is a co-dominant marker that also mapped to bin 4, but which lacked robustness that probably limits its use. Markers *Xrwgs41* and *Xrwgs42* (supplemental Fig. S4) are dominant repulsion-phase markers that mapped to bins 4 and 2, respectively. In the case of *Xrwgs42*, when testing with RWG37, we observed recombination between Rusty and Joppa chromatin, as indicated by co-dominant 158 and 153 bp amplicons, respectively.

Excluding the STARP markers, 26 of the 53 (49 from published sources plus 4 newly assigned) markers included in the study (supplemental Table S1) were polymorphic between Joppa and one or more RWG lines; and, amplicon sizes for the polymorphic markers are reported in Table 4. Twenty-three of the 26 markers were polymorphic between Joppa and the *Ae. speltoides* segments, and, this included 13 dominant repulsion-phase, 5 dominant coupling-phase (*Xgwm501, Xwmc41, Xgpw1184*, *XcsSrAes1t*, and *Xrwgs39*), and 5 co-dominant (*Xrwgs38a, Xtnac3119, Xmag1729, Xgpw4043*, and *Xrwgs40*) markers. Along with their appropriate checks, co-dominant and dominant coupling-phase markers are illustrated in Fig. 4, and, dominant repulsion-phase markers are illustrated in supplemental Fig. S4. Similar to the checks, some markers were either null or detected Rusty chromatin for either RWG35 or RWG37, and based on these results, markers were assigned to bin 2 or bin 4 as appropriate (Table 4). Three of the 26 polymorphic markers, *Xgwm120, Xgwm191*, and *Xgwm388*, detected co-dominant polymorphisms between Joppa and Rusty chromatin in all three RWG populations, and therefore, these loci mapped proximal to bin 1 (Table 4; supplemental Fig. S5).

The newly developed STARP markers were initially screened on the set of 11 genotypes used to screen all SSR primers. Based on that result, we designated five polymorphic STARP markers as *Xrwgsnp1* through *Xrwgsnp5* (Table 2). These five markers were tested on the RWG35, RWG36, and RWG37 populations and results from acrylamide gel-electrophoresis compared to results from co-dominant marker *Xtnac3119* (Fig. 5) and other markers locating to bins 3 and 4 (supplemental Table S2). Based on the testing results, the *Xrwgsnp2* locus was mapped to bin 4 and the *Xrwgsnp1, 3, 4*, and *5* loci were located in bin 3 (Fig. 5). The four STARP markers in bin 3 were used to genotype the populations using the CFX384 Touch™ Real-Time PCR Detection System. For each marker, parental and heterozygous genotypes were plotted into three distinct groups. Genotyping results by the fluorescence signals corresponded to those by size separation.

Crossing-over between S- and B-genome chromatin would have been highly unusual and was not observed in this study. However, in the regions linked to the introgressed *Ae. speltoides* segments in the RWG lines, recombination is possible between Rusty and Joppa chromatin. Markers showing recombination can be exploited during MAS to eliminate as much Rusty chromatin as possible in advanced breeding lines. For ten markers, recombination between Joppa and Rusty chromatin was identified (Fig. 4; supplemental Figs. S4, S5; supplemental Table S2). As expected, none of the markers that mapped to bin 3 exhibited recombination. In addition, recombination was not observed for nine markers monomorphic between Rusty and Joppa (footnoted in supplemental Table S2). Markers, *Xgwm120, Xgwm191*, and *Xgwm388*, which are located proximal to the *Ae. speltoides* segment were the only markers that identified recombinants in all three RWG populations. Bin 2 markers *Xrwgs38b, Xwmc339, Xrwgs42*, and *Xgpw4396* exhibited recombination only in the RWG37 population (supplemental Table S2). Bin 4 markers *Xgpw4043, Xmag1729*, and *Xrwgs40* exhibited recombination only in the RWG35 population.

The physical mapping of markers and *Sr47* to bins in this study, and from Klindworth et al. (2012), was combined with mapping of *Sr9* and *Sr28* (Rouse et al. 2014), and the genetic maps of Sourdille et al. (2010) and Somers et al. (2004) to produce a composite genetic map (Fig. 6, left column). However, not all of the markers in the present study could be incorporated into the genetic map. Markers where no recombination was observed were physically mapped to bins (Fig. 6, right column), but their position within the bins could not be determined. The positions of markers where recombination was observed (Table 5), or whose distance from *Xwmc175* has been reported (*Xtnac3119* and *Xtnac9215*) (Chao et al. 2016), were assigned to regions within bins (Fig. 6, middle column).

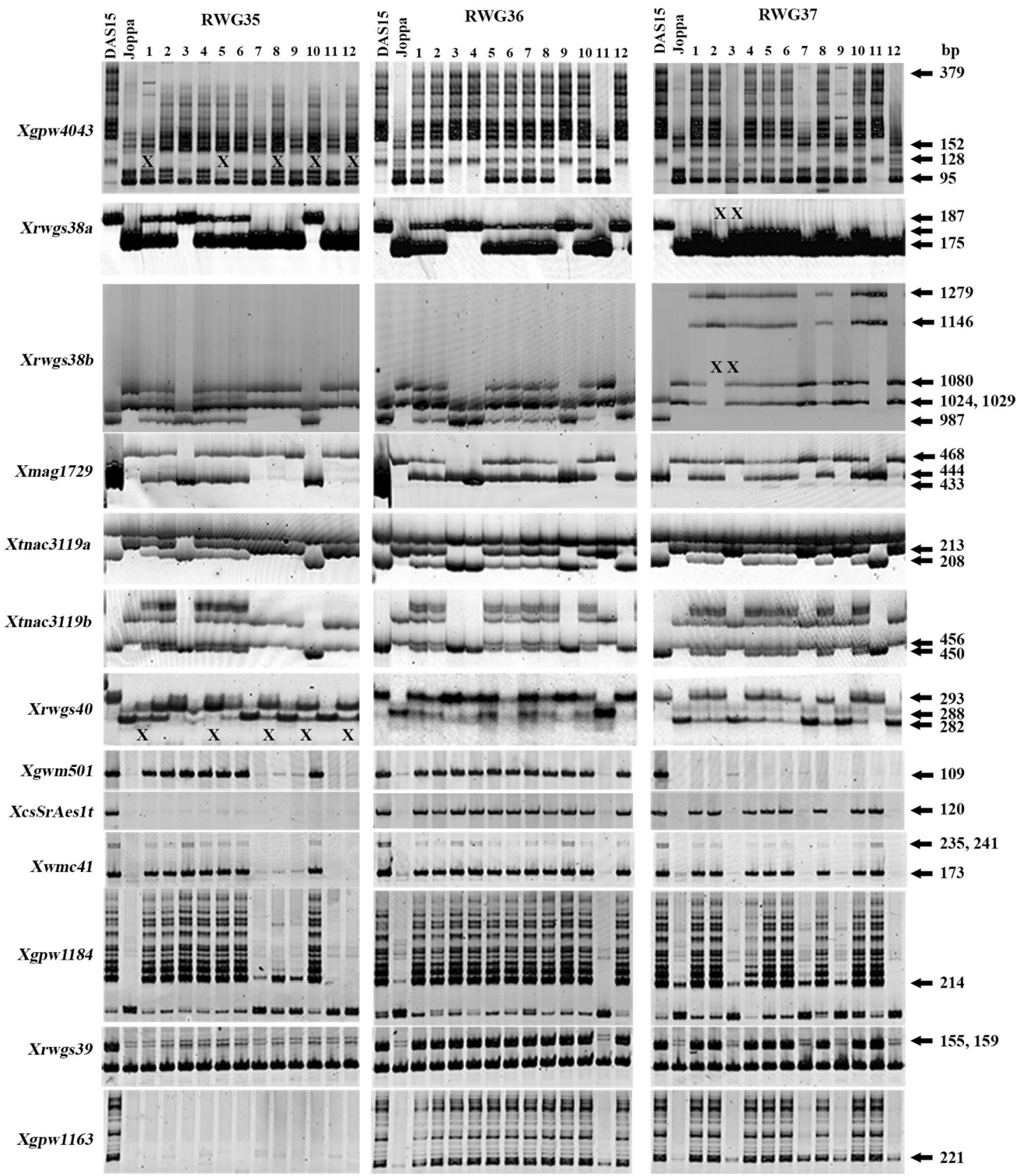

Fig. 4 Electrophoretograms of five co-dominant and six dominant coupling-phase markers tested on the RWG35, RWG36, and RWG37 populations. Only the first 12 plants of each population are shown. When markers produced multiple polymorphic amplicons, only the size of the smallest amplicon is reported. For an explanation of assigning markers to bins, see Fig. 3. X = a plant exhibiting recombination between Joppa and Rusty chromatin

Table 4 Inheritance of molecular markers linked to *Sr47* and amplicon size of PCR products

Bin/marker	Inheritance/phase	Amplicon size (bp)		
		Ae. speltoides segment	Durum cv. Joppa	Durum Rusty
Proximal markers				
Xgwm120	Co-dominant	94, 123	164, 181	136, 153
Xgwm191	Co-dominant	–	154, 194, 206	152, 191
Xgwm388	Co-dominant	–	173[b]	176[b]
Bin 2				
Xrwgs38a	Co-dominant	187	175	175
Xrwgs38b	Co-dominant	987, 1024[c]	1029, 1080[c]	1146, 1279
Xgwm501	Dominant/coupling	109	Null	Null
Xhbg391	Dominant/repulsion	Null	297[b]	297[b]
Xgpw4112[a]	Dominant/repulsion	Null	Untested	240[b]
Xgpw4396	Dominant/repulsion[d]	134, 261[b]	168, 185[b]	152, 165[b]
Xwmc339	Dominant/repulsion	240, null	253, 276[b]	243, 276, 467, 476
Xrwgs42	Dominant/repulsion[d]	Null	153	158
Bin 3				
Xwmc41	Co-dominant or dominant/coupling[e]	172, 235, 242	159, 167[e]	159, 172[e]
Xtnac3119a	Co-dominant	208, 220	213, 220	213, 220
Xtnac3119b	Co-dominant	450, 456	456, 480	450,456, 480
Xgpw1184	Dominant/coupling	214[b]	167	167
Xwmc175	Dominant/repulsion	Null	275[b]	Null
Xtnac9215	Dominant/repulsion	Null	272, 528	272, 528
Xgwm47[a]	Dominant/repulsion	Null	165[b]	165[b]
Xgpw4165[a]	Dominant/repulsion	Null	Untested	120
Xgpw7620	Dominant/repulsion	Null	391	Null
Xgpw7620	Recessive	249	Null	Null
Xmag3425	Dominant/repulsion	Null	498[b]	498[b]
Xrwgsnp1	Co-dominant	169	165	165
Xrwgsnp3	Co-dominant	135	139	139
Xrwgsnp4	Co-dominant	127	123	123
Xrwgsnp5	Co-dominant	97	100	100
Bin 4				
Xgpw4043	Co-dominant	128, 379[b]	95, 152[b]	95, 297[b]
Xmag1729	Co-dominant	444	468	433
Xrwgs40	Co-dominant	293	282	288
XcsSrAes1t	Dominant/coupling	120	Null	Null
Xrwgs39	Dominant/coupling	155, 159	Null	Null
Xgpw1163	Dominant/coupling	221[b]	Null	Null
Xwmc332[a]	Dominant/repulsion	Null	Untested	199[b]
Xwmc360	Dominant/repulsion	Null	153, 168	153, 168
Xwmc627[a]	Dominant/repulsion	Null	untested	120[b]
Xmag3512	Dominant/repulsion	Null	343, 362	343, 362
Xmag4281	Dominant/repulsion	Null	291	291
Xrwgs41	Dominant/repulsion	Null	183, 187, 284	183, 187, 284
Xrwgsnp2	Co-dominant	90	93	93

[a]Five markers, *Xgpw4112, Xgwm47, Xgpw4165, Xwmc332*, and *Xwmc627*, were not included in this study, but are reported here to complete the list of markers that are linked to *Sr47*

[b]Additional diagnostic bands were present

[c]Electrophoretic conditions strongly influenced migration of *Xrwgs38b* amplicons

[d]Co-dominant inheritance when detecting Joppa/Rusty alleles

[e]Three known alleles, including a null allele that results in dominant coupling-phase inheritance. Null if PCR cycles do not exceed 30

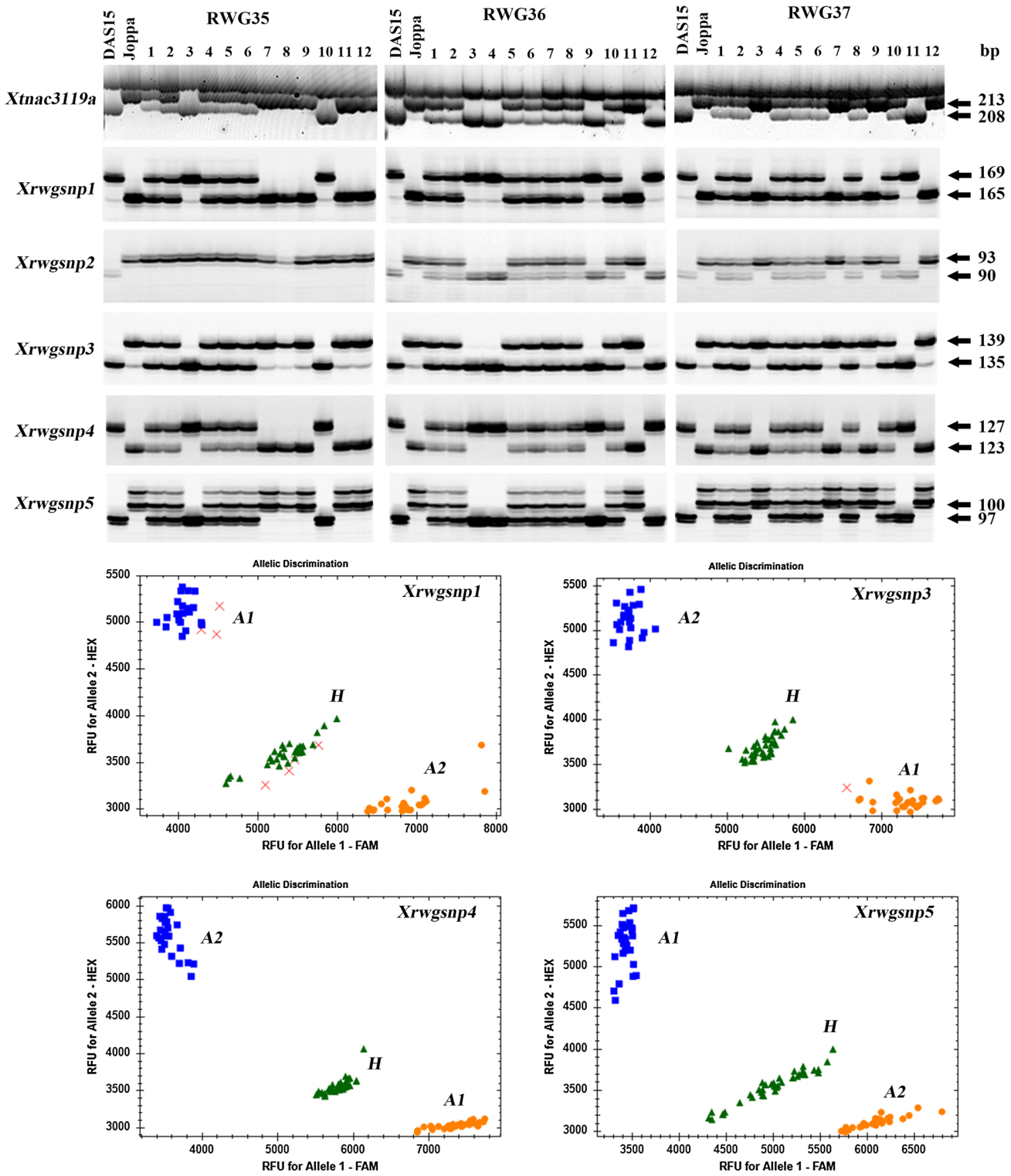

Fig. 5 Analysis of five STARP markers (*Xrwgsnp1* through *Xrwgsnp5*) on three BC_2F_2 populations derived from the backcrosses of durum cultivar Joppa with durum lines RWG35, RWG36, and RWG37. The gel images show the five STARP markers with comparisons to check marker *Xtnac3119a* analyzed by polyacrylamide gel-electrophoresis. The four plots show the genotype clusters of four STARP markers (*Xrwgsnp1, 3, 4*, and *5*) that map to bin 3 and analyzed using the CFX384 Touch™ Real-Time PCR Detection System. In each plot, A1 and A2 clusters represent the homozygous *Ae. speltoides* and wheat alleles, respectively, and H represents heterozygotes. Genotypes marked as an X in *Xrwgsnp1* and *Xrwgsnp3* plots were manually called. RFU is relative fluorescence unit

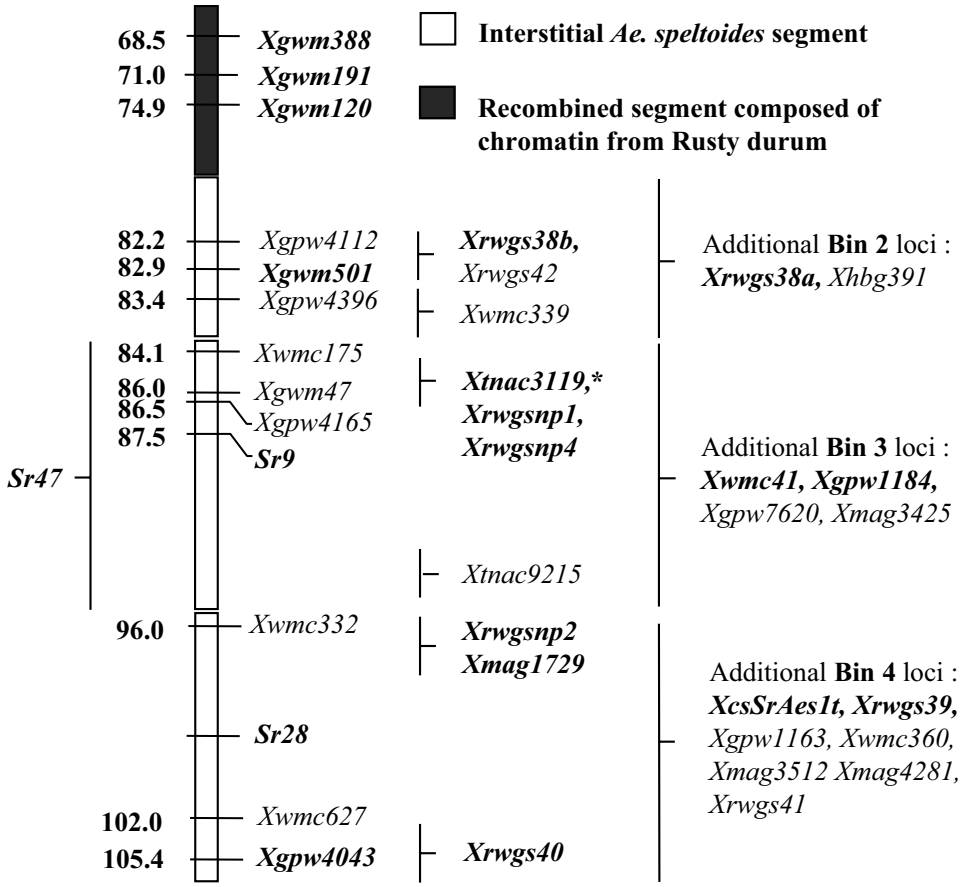

Fig. 6 Map of 2S region of the 2B/2S chromosome in durum wheat RWG36. Because no markers mapped to Bin 1, RWG36 was used to construct the map. The genetic map shown at left includes published markers whose map positions (cM) are derived from the Génoplante SSR map (Sourdille et al. 2010). Map positions of *Sr9* and *Sr28* are estimated from the study of Rouse et al. (2014). Markers mapped during the course of this study are shown in the middle and right columns. The *middle column* shows markers whose approximate position was determined based on recombination observed in this study or, for *Xtnac3119* and *Xtnac9215*, based on distance from *Xwmc175* reported in Chao et al. (2016). The *right column shows* markers physically mapped to a bin, but whose position within the bin could not be determined. Markers in *bold* are co-dominant or dominant coupling-phase, and all others are dominant repulsion-phase

Validation of markers and co-dominance of *Xwmc41*

Four STARP (*Xrwgsnp1, 3, 4* and *5*), two SSR (*Xmag1729* and *Xwmc41*), and one PLUG (*Xtnac3119*) markers were tested on nine durum and 28 common wheat cultivars. For *Xrwgsnp3* and *Xrwgsnp5*, three and five common wheat cultivars, respectively, carried the same allele as found in DAS15. For *Xrwgsnp1*, all common wheat and durum cultivars carried an allele that is different from DAS15 allele (Table 6). For *Xrwgsnp4*, DAS15 produced a 127 bp amplicon, while 11 durum, and most common wheat, cultivars produced a 123 bp amplicon. *Xrwgsnp4* also amplified a 126 bp amplicon in five common wheat cultivars. The absence of the 126 bp amplicon in durum suggests that this amplicon may originate from a D-genome chromosome. This result indicated that *Xrwgsnp1* and *Xrwgsnp4* should be useful on a wide number of cultivars, and should be the preferred STARP markers for MAS of *Sr47*.

The markers *Xmag1729, Xtnac3119*, and *Xwmc41* appear to be potentially useful for MAS of *Sr47*. In the validation set, only four of the ten durum cultivars had the $Xmag1729_{468}$ allele, with the remainder having $Xmag1729_{433}$ (Table 6). The small amplicon size difference between $Xmag1729_{433}$ and $Xmag1729_{444}$-*Sr47* makes reliable scoring more difficult, but is feasible in a long gel format (Fig. 7). In common wheat, 26 of 36 cultivars carried the $Xmag1729_{468}$ allele (Table 6); therefore, *Xmag1729* may be useful for a broader set of common wheat than durum wheat. Marker *Xtnac3119a* (and *Xtnac3119b*) mapped to bin 3 and should, therefore, be useful on all RWG populations. The validation test indicated that 31 of 40 hexaploid genotypes, and all 11 tetraploid genotypes in the test, carried the $Xtnac3119_{213}$ allele. Therefore, *Xtnac3119* should be compatible with most cultivars for MAS of *Sr47*. However, the data (Table 4; Fig. 4) also indicated that the small difference in amplicon size (208 vs. 213 bp for *Xtnac3119a*; 450 vs. 456 bp for *Xtnac3119b*) and presence of an additional non-diagnostic band (220 bp in *Xtnac3119a*) probably exclude use of *Xtnac3119* in agarose gels or mini-acrylamide gels.

Results for *Xwmc41* indicated that six durum cultivars carried the $Xwmc41_{null}$ allele. In addition, 4 durum and 13

Table 5 Classification of ten SSR markers on recombinant BC_2F_2 plants in three backcross populations having the pedigree Joppa*3//Rusty/RWG line

RWG line	Plant no.	Genotype[a]	Markers mapped to									
			Proximal to *Ae. speltoides* segment			Bin 2				Bin 4		
			Xgwm388	*Xgwm120*	*Xgwm191*	*Xrwgs38b*	*Xrwgs42*	*Xwmc339*	*Xgpw4396*	*Xmag1729*	*Xgpw4043*	*Xrwgs40*
RWG35	67-01	nd				SJ				RJ	**JJ**	**JJ**
RWG35	67-02	nd	**RR**			SJ				RJ		
RWG35	67-03	HR	**RJ**			SS				RR		
RWG35	67-04	nd	**JJ**	**JJ**	**JJ**	SJ				RJ		
RWG35	67-05	nd				SJ				RJ	**RR**	**RR**
RWG35	67-07	HS	**RJ**			JJ				JJ		
RWG35	67-08	HS				JJ				JJ	**RJ**	**RJ**
RWG35	67-09	HS	**RJ**			JJ				JJ		
RWG35	67-10	HR				SS				RR	**RJ**	**RJ**
RWG35	67-12	HS				JJ				JJ	**RJ**	**RJ**
RWG35	67-16	HR	**RJ**			SS				RR		
RWG35	67-18	nd	**RR**			SJ				RJ		
RWG35	67-19	HS	**RJ**			JJ				JJ		
RWG35	67-20	nd	**RR**			SJ				RJ		
RWG35	67-21	HS	**RJ**			JJ				JJ		
Recombinants/total plants			10/22	1/22	1/22	0/22	0/22	0/22	0/22	1/39[b]	5/22	5/22
			0.455	0.045	0.045	0	0	0	0	0.026	0.277	0.277
RWG36	68-03	HR	**RJ**			SS				SS		
RWG36	68-05	Seg	**RR**			SJ				SJ		
WG36	68-10	nd	**JJ**			SJ				SJ		
RWG36	68-24	HR	**RJ**	**RJ**	**RJ**	SS				SS		
RWG36	68-29	HR			**RJ**	SS				SS		
RWG36	68-41	HR	**RJ**			SS				SS		
RWG36	68-42	HR	**RJ**			SS				SS		
RWG36	68-43	HS	**RJ**	**RJ**	**RJ**	JJ				JJ		
Recombinants/total plants			7/45	2/45	3/45	0/45	0/45	0/45	0/45	0/45	0/45	0/45
			0.156	0.044	0.067	0	0	0	0	0	0	0
RWG37	69-02	nd	**RR**	**RR**	**RR**	**RR**	**RR**	**RR**	**RR**	SJ		
RWG37	69-03	HS	**RJ**	**RJ**	**RJ**	**RJ**	**RJ**	**RJ**	**RJ**	JJ		
RWG37	69-06	nd	**JJ**	**JJ**	**JJ**	RJ				SJ		
RWG37	69-15	nd	**RR**			RJ				SJ		
RWG37	69-16	nd	**RR**	**RR**	**RR**	RJ				SJ		
RWG37	69-18	HR	**RJ**			RR				SS		

Table 5 (continued)

RWG line	Plant no.	Genotype[a]	Markers mapped to										
			Proximal to *Ae. speltoides* segment			Bin 2				Bin 4			
			Xgwm388	*Xgwm120*	*Xgwm191*	*Xrwgs38b*	*Xrwgs42*	*Xwmc339*	*Xgpw4396*	*Xmag1729*	*Xgpw4043*	*Xrwgs40*
RWG37	69-19	HR	**RJ**			RR				SS		
RWG37	69-22	nd	**RR**	**RR**	**RR**	**RR**	**RR**			SJ		
Recombinants/total plants			8/22	5/22	5/22	3/22	3/22	2/22	2/22	0/22	0/22	0/22
			0.364	0.227	0.227	0.136	0.136	0.091	0.091	0	0	0

Classifications were S = *Ae. speltoides* chromatin, R = Rusty chromatin, J = Joppa chromatin. Recombinants are in bold. Blank indicates plants that did not differ in classification from the check markers, *Xrwgs38b* and *Xmag1729*

[a]Genotypes were determined based on stem rust infection types: *HR* homozygous resistant, *Seg* segregating, *HS* homozygous susceptible. *nd* no rust data

[b]Additional plants were tested to determine if the linkage of the *Xmag1729* locus and the bin 3\4 breakpoint could be easily broken

common wheat cultivars carried a 162 bp amplicon, 25 common wheat cultivars carried 159 and 168 bp amplicons, and two common wheat genotypes (Newton and LMPG-6) carried 159, 168, and 164 bp amplicons (Table 6). Since the presence of the $Xwmc41_{159-168}$ and $Xwmc41_{162}$ amplicons was mutually exclusive, we considered the possibility that the $Xwmc41_{null}$, $Xwmc41_{159-168}$, $Xwmc41_{162}$, $Xwmc41_{164}$, and $Xwmc41_{172}$ amplicons may be alleles. Two breeding populations, composed of backcrosses to Carpio ($Xwmc41_{162}$) and Faller ($Xwmc41_{159-168}$), allowed us to test all of these amplicons except $Xwmc41_{164}$ (Fig. 7). Results of the Faller BC_3F_2 population indicated that backcrossing had incorporated the $Xwmc41_{159-168}$ amplicons in all plants, and therefore, these amplicons were not allelic to the $Xwmc41_{172}$ amplicon. When the Carpio population was tested with *Xmag1729*, there were 6 *Sr47Sr47*, 25 *Sr47sr47*, and 15 *sr47sr47* plants. When the Carpio population was tested with *Xwmc41*, classifying the plants for the $Xwmc41_{162}$ and $Xwmc41_{172}$-*Sr47* amplicons produced results identical to the *Xmag1729* results. Therefore, the $Xwmc41_{162}$ and $Xwmc41_{172}$-*Sr47* amplicons were alleles that were co-dominantly inherited. The results indicated that *Xwmc41* worked well as a *Sr47* marker, though when the $Xwmc41_{159-168}$ amplicons are present, a long gel format will be preferable to achieve good separation of the 168 and 172 bp amplicons. Since *Xwmc41* is located in bin 3, it will be usable on all three RWG lines.

Discussion

Including markers reported by Klindworth et al. (2012), 28 markers have been assigned to three bins (Table 4). No markers have been assigned to bin 1. This may reflect the small size of bin 1, whose fraction length (FL) was calculated as 0.011 (0.819 − 0.808, Fig. 1). However, bin 2 also had a calculated FL of 0.011, and seven markers were mapped within bin 2. Therefore, a second possible explanation for the lack of markers in bin 1 may be that either the RWG35 or RWG36 breakpoint was not accurately mapped. If so, bin 1 may not exist, and the higher recombination rate for *Xgwm388* in the RWG35 vs. RWG36 population supports this theory (Table 5). A third possible explanation for the lack of markers in bin 1 is the uneven distribution of markers along a chromosome which could be a result of varying frequencies of polymorphism across chromosomal regions. This is supported by our observation of 9 markers located to bin 3 with an FL of 0.048, while 12 markers were located to bin 4 with an FL of 0.014 (Fig. 1; Table 4).

As a class, SSRs are generally co-dominant markers. Excluding the STARP markers, we found that dominant markers, and particularly repulsion phase, were common when analyzing durum/*Ae. speltoides* hybrids. The

Table 6 Amplicon size (bp) from DAS15, Rusty, 10 durum, and 40 common wheat cultivars or lines for five markers linked to stem rust resistance gene *Sr47*

Cultivar/line	Origin[a]	Type	Fragment size (bp) for marker[b]				
			Xwmc41	*Xmag1729*	*Xtnac3119*	*Xrwgsnp1*	*Xrwgsnp4*
DAS15 (check)	ND	Durum wheat	172, 235, 242	444	208	169	127
Rusty (check)	USDA	Durum wheat	Null	433	213	165	123
Divide	ND	Durum wheat	Null	433	213	165	123
Ben	ND	Durum wheat	Null	468	213	165	123
Tioga	ND	Durum wheat	Null	433	213	165	123
Grenora	ND	Durum wheat	Null	468	213	165	123
Lebsock	ND	Durum wheat	162	433	213	165	123
Monroe	ND	Durum wheat	Null	468	213	165	123
Alkabo	ND	Durum wheat	162	433	213	165	123
Mountrail	ND	Durum wheat	162	433	213	165	123
Carpio	ND	Durum wheat	162	433	213	165	123
Joppa	ND	Durum wheat	Null	468	213	165	123
Jimai 22	China	Common wheat	162	Null	213	165	123
Yangmai 16	China	Common wheat	159,168	468	208	165	123, 126
Shanrong 1	China	Common wheat	159,168	468	213	165	123
Shanrong 3	China	Common wheat	159,168	468	213	165	123
Jinan 17	China	Common wheat	162	468	208	165	123
Jinan 177	China	Common wheat	162	–	208	165	123
Zhengmai 9023	China	Common wheat	162	468	208	165	123, 126
Amidon	ND	Common wheat	162	468	213	165	123
Howard	ND	Common wheat	159,168	433	213	165	123
Alsen	ND	Common wheat	159,168	433	213	165	123
Grandin	ND	Common wheat	162	468	213	165	123
Glenn	ND	Common wheat	159,168	433	213	165	123
Faller	ND	Common wheat	159,168	468	213	165	123
Glupro	ND	Common wheat	162	468	213	165	123
Ernest	ND	Common wheat	162	–	213	165	123
Steele-ND	ND	Common wheat	159,168	433	213	165	123
Reeder	ND	Common wheat	159,168	433	213	165	123
Mott	ND	Common wheat	159,168	468	213	165	123
Kulm	ND	Common wheat	159,168	468	213	165	123
Parshall	SD	Common wheat	159,168	433	213	165	123
Granger	SD	Common wheat	159,168	468	208	165	123,126
Brick	SD	Common wheat	162	433	213	165	123
Russ	SD	Common wheat	159,168	468	213	165	123
Briggs	SD	Common wheat	159,168	468	213	165	123
Traverse	SD	Common wheat	162	468	213	165	123
Sabin	MN	Common wheat	159,168	468	213	165	123
Oklee	MN	Common wheat	162	468	208	165	123, 126
Ulen	MN	Common wheat	162	468	213	165	123
Ada	MN	Common wheat	159,168	433, 468	208	165	123
Tom	MN	Common wheat	159,168	468	213	165	123
Newton	KS	Common wheat	159,168, 164	468	213	165	123
IL06-14262	IL	Common wheat	162	468	213	165	123
Linkert	MN	Common wheat	159,168	468	208	165	123, 126
Stoa	ND	Common wheat	159,168	468	213	165	123
ID10856	ID	Common wheat	159,168	468	213	165	123

Table 6 (continued)

Cultivar/line	Origin[a]	Type	Fragment size (bp) for marker[b]				
			Xwmc41	*Xmag1729*	*Xtnac3119*	*Xrwgsnp1*	*Xrwgsnp4*
ID10889	ID	Common wheat	159,168	468	213	165	123
ID10930	ID	Common wheat	159,168	433	213	165	123
ID12422	ID	Common wheat	159,168	468	213	165	123
LMPG-6	Canada	Common wheat	159,168, 164	–	213	165	123
Line E	Australia	Common wheat	159,168	–	208	165	123

[a]Origin: CO, Colorado; KS, Kansas; ND, North Dakota; SD, South Dakota; MN, Minnesota; IL, Illinois; ID, Idaho

[b]Dash (–) indicates not tested

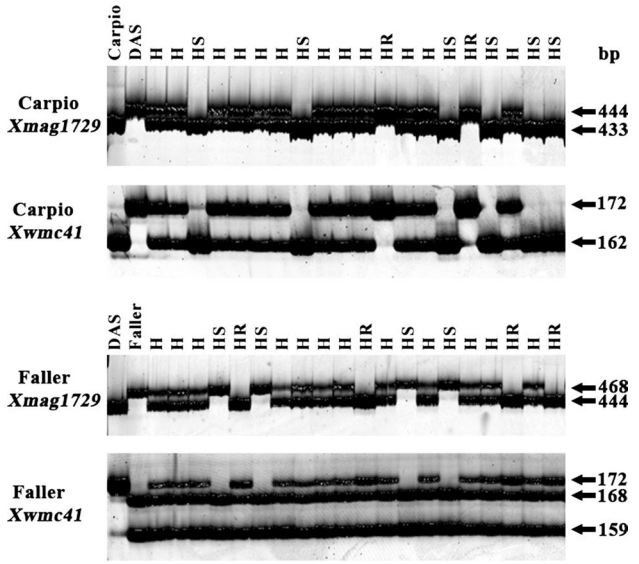

Fig. 7 Tests of Carpio and Faller progeny (with pedigrees Carpio*6/RWG37 and Faller*4/RWG37, respectively) with *Xmag1729* and *Xwmc41*. *Xmag1729* was used as the check to genotype each plant for the *Ae. speltoides* segment carrying *Sr47*. In the Faller population, all plants carried the $Xwmc41_{159-168}$ amplicons, indicating that $Xwmc41_{159-168}$ and $Xwmc41_{172}$ loci were not allelic. In the Carpio population, all plants could be classified as homozygous susceptible (HS), heterozygous (H), or homozygous resistant (HR) with either *Xmag1729* or *Xwmc41*; therefore, the $Xwmc41_{172}$ and $Xwmc41_{162}$ loci were allelic and *Xwmc41* was co-dominantly inherited in this cross

predominance of repulsion phase markers suggests that the primers which were designed from wheat sequences preferentially annealed or amplified wheat chromatin. Preferential amplification of wheat chromatin would explain why *Xrwgs42* and *Xgpw4396* were dominant repulsion-phase markers in the RWG35 and RWG36 populations (Joppa×*Ae. speltoides*), but were co-dominant in the RWG37 population (Joppa×Rusty). Preferential amplification also appeared to be a problem with dominant coupling-phase markers. In our breeding studies, similar to *Xwmc41*, there were instances where markers such as *Xgwm501* or *XcsSrAes1t* would amplify the target fragment in all plants, regardless of genotype (data not shown). In those instances, PCR conditions such as DNA concentration, primer concentration, annealing or denaturing conditions, buffers, and *Taq* polymerase could influence annealing, resulting in preferential amplification.

All of the markers reported in prior studies (Klindworth et al. 2012; Yu et al. 2015) for use in MAS of *Sr47* have some limitations. While *Xrwgs38a* is a robust co-dominant marker and is arguably the best of the markers, its location in bin 2 makes it unable to detect *Sr47* in RWG37-derived populations. Therefore, one of our objectives was to find additional co-dominant markers that map to bin 3. In addition to *Xrwgs38a*, markers *Xwmc41, Xmag1729*, and *Xtnac3119* will be useful for gel-based MAS; but, like *Xrwgs38a*, each has its own limitations. The validation test indicated that in durum wheat, the usefulness of *Xwmc41* is limited due to the presence of the $Xwmc41_{null}$ allele, which results in dominant coupling-phase inheritance. Co-dominant marker *Xtnac3119* mapped to bin 3, but is incompatible with gel-free marker systems. Co-dominant marker *Xmag1729* located to bin 4, making it unable to detect *Sr47* in RWG35-derived lines, and the validation test indicated that *Xmag1729* was incompatible with many durum and some common wheat genotypes. Despite the limitations of *Xrwgs38a, Xwmc41, Xtnac3119*, and *Xmag1729*, they should provide adequate coverage to detect *Sr47* in any cross.

Two co-dominant STARP markers, *Xrwgsnp1* and *Xrwgsnp4*, were mapped to bin 3 and the validation test indicated that they would detect *Sr47* across all or most cultivars, respectively. Thus, these two markers should be most useful in MAS of *Sr47*. Loci for STARP markers *Xrwgsnp3* and *Xrwgsnp5* also mapped to bin 3, but in the validation test, they could not detect *Sr47* in some genetic backgrounds, making them less reliable for breeding. The *Xrwgsnp2* locus mapped to bin 4, and therefore, this marker could not detect *Sr47* in populations derived from RWG35, and we cannot recommend *Xrwgsnp2* for use in detecting *Sr47*. The STARP markers will be particularly useful, because they can be used in high-throughput marker

analyses either in a gel-based or a gel-free platform as an alternative to KASP markers.

Wheat chromosome 2B harbors several TTKSK-resistance genes such as *Sr39* (Niu et al. 2011) and *Sr40* (Wu et al. 2009) on the short arm and *Sr9h* (Rouse et al. 2014), *Sr28* (Rouse et al. 2012), and a new *Sr* gene (Saini et al. 2016) on the long arm. It is recognized that pyramiding several TTKSK-effective *Sr* genes together is the best strategy for attaining durable resistance in commercial cultivars (Singh et al. 2006, 2011; Yu et al. 2014). The physical mapping indicated that bin 4 in RWG36 and RWG37 is comprised of *Ae. speltoides* chromatin, while bin 4 of RWG35 is wheat chromatin. Gene *Sr28* also maps to bin 4; therefore, it is possible to combine *Sr47* with *Sr28* using RWG35, but it is impossible to pyramid the two genes using either RWG36 or RWG37. The physical mapping of the molecular markers in this study will facilitate pyramiding of *Sr47* with other *Sr* genes on chromosome 2B into a single linkage block.

Author contribution statement DLK and SSX conceived the study. DLK and JS conducted the study and determined published markers for testing. YL and SSX developed and tested STARP markers. JS, JDF, and SSX developed new SNP-associated SSR markers. MNR and YJ conducted the stem rust tests. All authors contributed to writing the manuscript, with DLK and JS serving as lead authors.

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Compliance with ethical standards

Conflict of interest All authors have no conflict of interest.

Ethical standards The experiments were performed in compliment with the current laws of United States of America.

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