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ORIGINAL PAPER

Development of a multiple bulked segregant analysis (MBSA) method used to locate a new stem rust resistance gene (*Sr54*) in the winter wheat cultivar Norin 40

Habibollah Ghazvini · Colin W. Hiebert · Julian B. Thomas · Thomas Fetch

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Abstract An important aspect of studying putative new genes in wheat is determining their position on the wheat genetic map. The primary difficulty in mapping genes is determining which chromosome carries the gene of interest. Several approaches have been developed to address this problem, each with advantages and disadvantages. Here we describe a new approach called multiple bulked segregant analysis (MBSA). A set of 423 simple sequence repeat (SSR) markers were selected based on profile simplicity, frequency of polymorphism, and distribution across the wheat genome. SSR primers were preloaded in 384-well PCR plates with each primer occupying 16 wells. In practice, 14 wells are reserved for "mini-bulks" that are equivalent to four gametes (e.g. two F_2 individuals) comprised of individuals from a segregated population that have a known homozygous genotype for the gene of interest. The remaining two wells are reserved for the parents of the population. Each well containing a mini-bulk can have one of three allele compositions for each SSR: only the allele from one parent, only the allele from the other parent, or both alleles. Simulation experiments were performed to determine the pattern of mini-bulk allele composition that would indicate putative linkage between the SSR in question and the gene of interest. As a test case, MBSA was employed to locate an unidentified stem rust resistance (Sr) gene in the winter wheat cultivar Norin 40. A doubled haploid (DH) population ($n = 267$) was produced from hybrids of the cross LMPG-6S/Norin 40. The DH population segregated for a single gene ($\chi^2_{1:1} = 0.093$, $p = 0.76$) for resistance to *Puccinia graminis* f.sp. *tritici* race LCBN. Four resistant DH lines were included in each of the 14 mini-bulks for screening. The Sr gene was successfully located to the long arm of chromosome 2D using MBSA. Further mapping confirmed the chromosome location and revealed that the Sr gene was located in a linkage block that may represent an alien translocation. The new Sr gene was designated as *Sr54*.

Introduction

Wheat is a primary food crop grown worldwide on about 215 million hectares and providing about 20 % of the calories to 4.5 billion people (Singh et al. 2011). Long-term breeding goals include improved yield, quality, and pest resistance. Concomitant with this research, geneticists have worked to identify genes responsible for these traits, map their genetic location, and develop DNA markers for characterizing germplasm and marker-assisted breeding.

The principal difficulty in genetically mapping novel qualitative traits in wheat is identifying the chromosome carrying gene(s) involved. Once the chromosome location has been established, obtaining a genetic map position becomes a matter of routine. Cytology-based methods, which include monosomic analysis (Sears 1953), telocentric mapping (Sears 1962), and haploid-deficiency mapping (Thomas et al. 2001), have been used to locate genes.

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H. Ghazvini
Cereal Research Department, Seed and Plant Improvement Institute, AREEO, Ministry of Jahad Agriculture, Karaj, Iran

C. W. Hiebert (✉) · J. B. Thomas · T. Fetch
Agriculture and Agri-Food Canada, Cereal Research Centre, 195 Dafoe Rd. Winnipeg, Manitoba R3T 2M9, Canada
e-mail: colin.hiebert@agr.gc.ca

More recently, a variety of genetic strategies based on DNA markers have been implemented, such as whole genome mapping (WGM) and bulked segregant analysis (BSA; Michelmore et al. 1991).

While DNA marker technology offers several advantages, there are pros and cons to be considered for a given strategy. Both WGM and BSA require a segregated population, and WGM is labour intensive while BSA may identify only closely linked markers. In practice, the window of detection for a given marker in BSA is less than the theoretical limit. For example, in a recent study BSA was used in an attempt to locate the gene *SrCad*. However, BSA was unsuccessful and the location was finally determined by WGM (Hiebert et al. 2011), which is more time consuming and expensive compared to BSA. The simple sequence repeat (SSR) marker cfd49 that is linked to *SrCad* was used in the BSA screening, but did not indicate association with the resistance despite being located approximately 7 cM from the gene (Hiebert et al. 2011). Thus, there is a need for a method that has less labour and cost than WGM and increased sensitivity than BSA.

Another way that putative gene locations can be identified is by comparing near-isogenic lines (NILs) to the recurrent parent using markers that have known chromosome locations and provide good coverage of the genome (Hiebert et al. 2010a). Introgressions can be easily identified and mapping studies can focus on these regions. However, NILs take time to develop and introgressions need to be sufficiently large to be detected using a reasonable number of markers.

Hiebert et al. (2012) proposed a novel procedure for determining the chromosome location of qualitative genes that employs several small bulks of DNA from few individuals that are screened with a preselected set of SSR markers that are robust, polymorphic, and provide coverage of the wheat genome. This procedure, called multiple bulked segregant analysis (MBSA), combines the sensitivity of of WGM and the operational efficiency of BSA.

Stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn. (*Pgt*), is a disease of wheat that can cause devastating grain yield losses. Deploying cultivars with effective stem rust resistance (Sr) genes has been a sustainable method of disease control. In North America, the last wheat stem rust epidemic occurred in 1953 to 1955 (Peturson 1958). Since then, resistant cultivars have controlled stem rust. However, the discovery of Ug99 (Pretorius et al. 2000; Jin et al. 2008) has renewed interest in discovering new Sr genes (e.g. Hiebert et al. 2010b), developing markers for previously identified genes (e.g. Mago et al. 2011), and improving the suitability of Sr genes from alien transfers (e.g. Klindworth et al. 2012).

The gene *Sr42*, found in Norin 40 (RA McIntosh unpublished 1983; McIntosh et al. 1995), confers resistance to Ug99 (Pretorius et al. 2000). During a study aimed at mapping *Sr42* (Ghazvini et al. 2012) we identified a second Sr gene in Norin 40 that does not confer resistance to Ug99. There has been no report of a second Sr gene in Norin 40 therefore nothing is known about the genetics of this resistance.

The objectives of this study are to fully describe MBSA, demonstrate the effectiveness of MBSA using the unknown Sr gene in Norin 40 as a test-case, and to genetically map the unknown Sr gene in Norin 40. Based on the data presented here, this new Sr gene has been designated *Sr54* by R.A. McIntosh, the coordinator of the wheat genetic map.

Materials and methods

Population development, stem rust testing, and DNA extraction

A cross was made between LMPG-6S (Little Club//Prelude*8/Marquis/3/Gabo; obtained from the late Dr. D. Knott) and Norin 40 (accession CN30674 obtained from the Germplasm Resource Centre, Saskatoon, Canada). Norin 40 is a winter wheat that carries *Sr42* and a second gene with unknown chromosome location (*Sr54*). LMPG is a spring wheat line developed to be susceptible to all races of stem rust. Ten F_1 plants from LMPG-6S/Norin 40 were used to make doubled haploid (DH) lines using the maize pollination method (Thomas et al. 1997), and those with a spring growth habit ($n = 267$) were retained.

The DH population and parental lines were inoculated with urediniospores of *Pgt* race LCBN (Roelfs and Martens 1988) when the first leaf was fully emerged, following the methods described in Hiebert et al. (2011). Two weeks after inoculation, seedlings were rated for their infection type (IT) using the 0 to 4 scale described by Stakman et al. (1962). Plants with ITs from 0 to 2^+ were classified as resistant while plants with ITs of 3 or greater were classified as susceptible. Leaf tissue was collected from uninfected leaves after seedlings were rated for stem rust IT. Tissue was lyophilized and DNA was extracted using an ammonium acetate method (Chao and Somers, http://maswheat.ucdavis.edu/PDF/DNA0003.pdf, accessed Dec. 2010) based on the procedures of Pallotta et al. (2003). Working solutions of DNA were diluted to 15 ng/μL in sterile water.

Description of the multiple bulked segregant analysis (MBSA)

Our goal was to design a technique that would evaluate a reasonable number of simple sequence repeat (SSR) markers across the genome that would reliably identify the location of a gene(s) while maximizing the operational

efficiency and the genetic interval of detection. The strategy developed can be summarized as follows: (1) a set of SSR markers that covers the genome was established; (2) each SSR primer pair was preloaded and air-dried in a column (16 wells) of a 384-well PCR plate; (3) PCR plates with dried primers were mass-produced to increase efficiency; (4) a set of 14 "mini-bulks" that were fixed for the locus of interest was established and each mini-bulk represented the equivalent of four gametes from a segregated population and (5) the 14 mini-bulks and two parental lines were tested with the set of SSR markers and the allele composition of the mini-bulks was assessed to identify SSR markers with putative linkage to the gene of interest. We refer to this technique as multiple bulked segregant analysis (MBSA).

A set of 423 SSR markers (Röder et al. 1998; Pestova et al. 2000; Somers et al. 2004; Sourdille et al. 2004; Song et al. 2005) was selected based on profile simplicity, degree of polymorphism, and distribution across the genome (Supplementary Table 1). Each SSR primer pair was pipetted (0.2 pmol of forward primer and 2.0 pmol of reverse primer) into one column (16 wells) of a 384-well PCR plate using a Tecan Evolutions liquid handling robot (Tecan Austria GmbH, Grödia, Austria). Thus, each plate contained 24 primer pairs. Plates were mass-produced to increase efficiency. Primers were air-dried, and the plates were stored at −20 °C until used.

An array of combinations of number of mini-bulks and the number of gametes represented per bulk were evaluated in a preliminary simulation. It was determined that 14 mini-bulks with four gametes each constituted the best balance between efficiency and resolution. Thus, each mini-bulk contained the DNA from a composite of four DH lines with a resistant phenotype. For each primer pair, 14 of the 16 wells were used to analyse the mini-bulk samples, for a total of 56 independent gametes. The remaining two wells were used for DNA from the parental lines. PCR was set up by making 16 PCR cocktails, one for each mini-bulk and parental line, that contained the DNA for either a mini-bulk (18.75 ng of DNA/DH line/reaction) or parental line (75 ng) plus the PCR reagents (1x PCR buffer, 0.8 mM dNTPs, 1.5 mM MgCl2, 1.9 pmol M13 primer, and 1U *Taq* DNA polymerase; 10 μL reaction volume). The PCR product was analyzed using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Streetsville, ON, Canada) which allowed four PCR plates to be pooled for analysis for a total of five pooled plates for the entire analysis. PCR conditions and analysis on the ABI 3130xl were performed as described by Somers et al. (2004).

Each mini-bulk could have one of three allele profiles for a given polymorphic SSR marker: (1) only the allele from Norin 40 (resistant parent); (2) only the allele from LMPG-6S (susceptible parent); or (3) both alleles. Linked markers were expected to show a different allele distribution compared to unlinked markers. Since mini-bulks were comprised of four resistant DH lines, markers linked to *Sr54* were expected to should show an increased number of mini-bulks that only amplified the SSR allele from the resistant parent while unlinked markers were expected to show a random distribution. A simulation was performed (below) to determine the criterion needed to classify a marker as putatively linked. The criterion was applied to the allele distribution for each marker.

Simulation of MBSA

A simulation was conducted using Microsoft Excel 2010 for a range of linkage intensities to investigate the power of MBSA to detect linkage between a gene and a candidate co-dominant marker. Simulated rates (0, 10, 15, 20, 30, 40, and 50 %) of recombination between the gene and the candidate marker were tested. Three possibilities exist for each well: (1) the PCR product of the bulk may contain only the allele from the resistant parent; (2) only the allele from the susceptible parent; or (3) both fragments may be present. In order to determine the expected distribution at each rate of recombination, each gamete was assigned a random number between zero and one. The simulated gamete was declared recombinant and assigned a value of one if the random number was less than the simulated recombination rate (e.g.< 0.1 for 10 % recombination). If the gamete was non-recombinant, a value of zero was assigned. To simulate the mini-bulks, 56 values were assembled into 14 groups of 4 and each group was summed. A sum of zero corresponded to a mini-bulk with only the resistant parent allele, while a sum of four represented mini-bulks with only the susceptible parent allele. A sum of one, two, or three represented mini-bulks that contained both alleles. The frequency of each allele pattern was then summarised for each group of fourteen. This procedure was then iterated 10,000 times. The population of 10,000 simulations was tabulated (cross-classified) for the number of mini-bulks with the resistant parent allele and both alleles. The entire simulation was then repeated for each of the recombination frequencies listed above.

Testing markers for linkage to *Sr54*

Markers that met the criteria for putative linkage to *Sr54* were selected for testing across 96 DH lines. If linkage was observed, markers were tested on the entire DH population and additional SSR markers from adjacent chromosome regions were tested. A genetic map of the region carrying *Sr54* was constructed using MapMaker 3.0 (Lander et al. 1987) using the Kosambi mapping function (Kosambi 1944).

Results

Stem rust phenotypes

Norin 40 showed infection types (ITs) of ;1^- to ;1 and the resistant DH lines showed ITs of ;1^- to 11^+, while LMPG-6S showed an IT of 3^+3 and the susceptible DH lines showed ITs from 33^+ to 4. In the DH population ($n = 267$), there were 136 resistant lines and 131 susceptible lines, which fitted the expected segregation ratio for a single gene ($\chi^2_{1:1} = 0.093$, $p = 0.76$).

Simulation of MBSA

The simulation suggested that an efficient cut-off criterion for accepting the presence of linkage was if four or more mini-bulks contained only the allele of the resistant parent (Fig. 1). Among markers with no linkage (50 % recombination), this cut-off yielded a false-positive rate of 0.95 per 100 markers (Table 1). For linked markers, this cut-off criterion yielded virtually no false negatives for all recombination rates up to 10 %. At 15 % recombination the false negative rate was 1.8 %, while at 20 % recombination the false negative rate was 11.2 %. Even at 30 % recombination, a linked marker was distinguished from no linkage 41.8 % of the time (Table 1). Thus, the above criterion minimizes false negative results when markers are closely or moderately linked to the gene, while also minimizing the frequency of a false positive result for unlinked markers.

Location and mapping of *Sr54*

There were 256 polymorphic markers (60.5 %) between Norin 40 and LMPG-6S of the 423 SSR primer pairs tested for MBSA. This gave an average of 12.2 polymorphic markers per chromosome. Chromosome 5A had the most polymorphic markers (26), while chromosome 3D had the fewest (five). The A genome had the highest number of polymorphic markers (15.4 markers/chromosome), while the D genome had the fewest number of polymorphic markers (9.3 markers/chromosome).

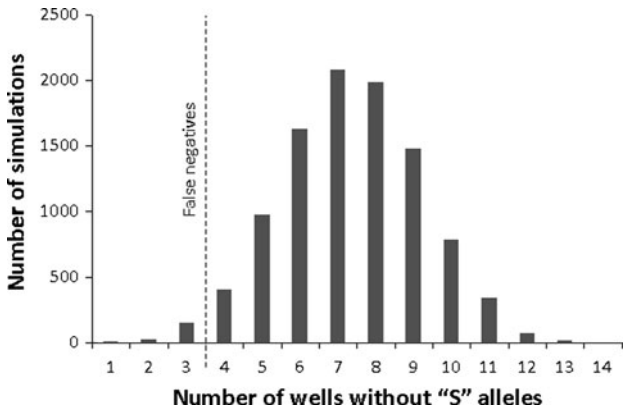

Fig. 1 Simulated distribution of a linked (15 % recombination) SSR marker using 14 mini-bulks composed from resistant DH lines

Table 1 Outcomes of multiple bulk segregant analysis (MBSA) using 10,000 simulations for seven recombination values between a marker and the gene of interest

Recombination rate	Expected results (%)[a]			
	Positive location	False negatives	False positive	Negative
0 %	100	0	–	–
10 %	99.9	0.1	–	–
15 %	98.2	1.8	–	–
20 %	88.8	11.2	–	–
30 %	41.8	58.2	–	–
40 %	10.3	89.7	–	–
50 %	–	–	0.95	99.05

[a] A criterion of four or more mini-bulks containing only the SSR allele corresponding to the parent showing the selected phenotype was used to indicate a positive hit for the marker

The mini-bulks produced marker profiles that contained the Norin 40 allele, the LMPG allele, or both alleles. The distribution of these profiles was an indicator of putative linkage between the gene of interest and the marker in question. The mini-bulks were comprised of four resistant DH lines. Using the criterion listed above in the MBSA simulation, a putative "hit" occurred when a marker had four or more wells with only the Norin 40 allele. There were two chromosomes (2D and 5D) that had at least one marker that met this criterion (Table 2; Fig. 2). The marker barc228 on chromosome 2D was a "perfect hit" (all 14 wells had only the Norin 40 parent allele), while the other markers on chromosome 2D were more distantly linked (Table 2). Marker gpw320 on chromosome 5D also met the cut-off criteria of four resistant mini-bulks (Table 2). All markers that met the cut-off criterion were tested across 96

Table 2 Distribution of SSR allele composition found in mini-bulks for markers that met the MBSA criterion for showing putative linkage to *Sr54*

Marker	Chr	Distance from *Sr54*	Number of mini-bulks		
			'R' allele only	Both alleles	'S' allele only
gpw320	5D	unlinked	4	9	1
wmc18	2D	44.7 cM	5	8	1
wmc601	2D	42.6 cM	5	8	1
wmc167	2D	15.8 cM	6	8	0
barc228	2D	0 cM	14	0	0

Chr. chromosome

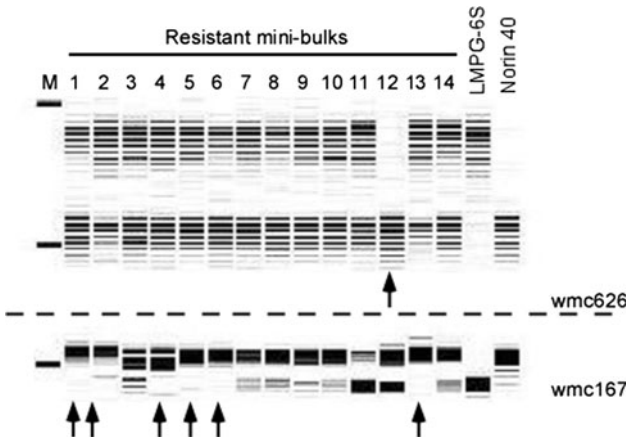

Fig. 2 A marker (wmc626) that did not meet the criterion for linkage to *Sr54* and one (wmc167) that did meet the criterion for linkage using multiple bulk segregant analysis (MBSA)

DH lines to determine which ones showed linkage to *Sr54*. Marker gpw320 on chromosome 5D was not linked, but several markers located on chromosome 2D showed linkage to *Sr54* (Fig. 3). Eighteen markers co-segregated with resistance. This region represents approximately 30 cM of the "normal" genetic map of chromosome 2D. Twelve of these co-segregating markers showed a null allele for Norin 40 (i.e. the null alleles were in coupling with *Sr54*).

Discussion

Identifying the chromosome that carries a gene of interest is an important step in genetically mapping new genes. Cytogenetic methods, such as monosomic analysis, reliably locate genes but are time consuming and labourious. In contrast, molecular methods such as BSA are rapid and can utilize populations developed for inheritance studies, which can also be used for further mapping. The chromosome location may not be detectable if polymorphic marker coverage is inadequate, but BSA reliably gives a positive result when linked markers are very close to the gene of interest. In instances where the genetic distance between the gene of interest and the nearest marker is large, a positive result relies on the failed detection of recombinant gametes that may be present in the bulks. This weakness may be exacerbated by the sensitivity of modern instruments and further limits the size of the genetic interval interrogated by each marker. For example, the marker cfd49 is located only 7 cM distal from the stem rust resistance gene *SrCad* (Hiebert et al. 2011), but gave a negative result in an initial screen to map the genetic location using BSA (Fig. 4). This indicates the weakness of using BSA even though the genetic map distance between cfd49 and *SrCad* was relatively close.

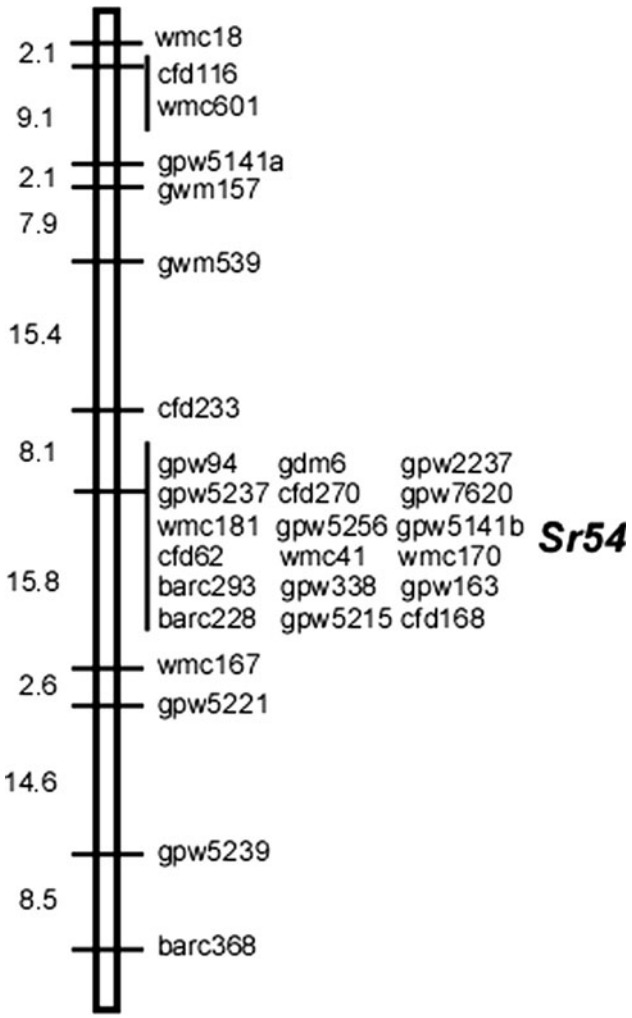

Fig. 3 Genetic map of chromosome 2D showing the position of *Sr54*

In contrast to BSA, MBSA is able to detect a larger genetic interval per marker. A recombination rate of 15 % represents a genetic interval of approximately 30 cM and showed a success rate of 98.2 % in the simulation experiments. A positive result is detected by skewed allele frequencies across several small bulks instead of relying on detecting two contrasting mono-allele bulks. The results of the simulation experiment (Table 1; Fig. 1) provide guidelines for an acceptable criterion for deciding which pattern across the mini-bulks constitutes a putative positive result. While a low rate of false positive markers is expected, this is acceptable as the actual location is easily determined once the candidate markers are either validated or eliminated by standard linkage analysis in the mapping population. In the event that no markers meet the criterion suggested by the simulation experiment, the stringency of criterion can be relaxed although this will undoubtedly introduce more false positive results. The net result of MBSA is a strategy that can quickly identify markers linked to genes in a relatively large genetic interval.

Fig. 4 Results of testing SSR marker cfd49 in an attempt to locate *SrCad* using bulk segregant analysis (BSA)

This was demonstrated in the present study and has also been our experience in several unpublished studies.

In the present study, segregation for a single gene (*Sr54*) was found in the DH population, and MBSA was used to locate the gene. Only markers on chromosomes 2D and 5D met the criteria for a positive result (Table 2). The marker on chromosome 5D (*gpw320*) was determined to be a false positive after further linkage analysis testing using a subset of the DH population. The observed number of false positives (one) was similar to the expected number based on a false positive frequency of 0.95 % predicted by simulation. Subsequent linkage analysis tests using markers on chromosome 2D mapped *Sr54* to chromosome 2DL. Since no other Sr genes have been mapped to the long arm of chromosome 2D, the gene designation *Sr54* was assigned by R.A. McIntosh, coordinator of the wheat genetic map. *Sr54* is known to be effective against only two *Pgt* races (LCBN and LBBC), which are relatively avirulent (data not shown). Norin 40 also carries *Sr42,* which is important to the wheat breeding and genetics community because it confers resistance to Ug99-type stem rust races (Pretorius et al. 2000; Ghazvini et al. 2012) but does not confer resistance to races LCBN and LBBB. Knowledge that *Sr54* is also present in Norin 40 is important in genetic studies targeting *Sr42*.

Interestingly, a large linkage block was observed in the region flanking *Sr54* (Fig. 3). This region represents approximately 30 cM of the normal genetic map of 2DL, and showed no recombination. This could indicate a large structural chromosome change such as an inversion. There are 18 SSR markers included in the linkage block that co-segregated with *Sr54* (Fig. 3), of which 12 were null in Norin 40. These null alleles may indicate that Norin 40 has a significant deletion on 2DL that is tightly linked to *Sr54*, but does not explain the lack of recombination with the six other markers in the linkage block. More likely, a large linkage block containing many null SSR alleles suggests that *Sr54* may be derived from a non-progenitor species of wheat (i.e. alien species). If *Sr54* is alien-derived, it is carried on an intercalary translocation. This would be difficult to prove because there is no information or indication to suggest the potential donor species. However, the SSR allele pattern is similar to that observed for other known alien transfers. For example, SSR alleles on an *Aegilops spletoides* translocation showed mostly null alleles and a few positive alleles during a study examining a leaf rust resistance gene carried on the translocation (CW Hiebert, unpublished data).

The true test of the sensitivity of MBSA is to examine the allele patterns observed for markers outside of the linkage block that are linked to *Sr54*. For example, MBSA indicated that a marker 15.8 cM from the linkage block, wmc167, was linked to *Sr54* (Table 2). Markers that were approximately 40 cM from the linkage block were also identified as putatively linked to *Sr54* by MBSA. It should be noted that the distances in shown in Table 2 are based on actual recombination values in the DH population used for MBSA and mapping, thus linkage implied by MBSA for markers located outside of the linkage block was not a product of the suppressed recombination in the region of the linkage block.

This study accomplished two goals: (1) the concept of MBSA was demonstrated using a gene with an unknown chromosome location, and (2) the genetics and location of an unknown gene (*Sr54)* in Norin 40 was resolved. The largest time investment in setting up MBSA is generating PCR plates with pre-loaded primers. This was simplified by diluting working stocks of primers in 96-well plates, which allowed mass-copies of 384-well PCR plates to be produced using a liquid handling robot. Much efficiency is gained by having pre-loaded plates on hand as the remainder of the protocol is rapid and employs routine procedures. The PCR setup is rapid because PCR cocktails are made and dispensed across several/all plates with a multi-channel pipette. The MBSA method described here provides a new strategy that can quickly predict the chromosome location of qualitative traits and should be useful in the identification of molecular markers that can be used in plant breeding.

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