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Identification of single nucleotide polymorphism (SNP) markers closely linked with powdery mildew resistance gene *Pm5e* in wheat

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Wheat (*Triticum aestivum* L.) is one of the most important food crops worldwide. Powdery mildew (Pm), caused by *Blumeria graminis* f. sp. *tritici* is a severe disease in wheat production. Gene *Pm5e*, from a Chinese wheat cultivar Fuzhuang 30 has proven to be a valuable resistance source for Pm in breeding. To further map this gene and develop Kompetitive allele-specific PCR (KASP) assays for marker-assisted selection (MAS), a F_2 population containing 395 individuals was first phenotyped for Pm resistance, a bulked segregant analysis (BSA) was used to identify polymorphic SNPs using the 35K wheat SNPs chip. 27 polymorphic SNPs between bulks in the *Pm5e* region were identified and were converted into KASP assays to map *Pm5e*. A genetic linkage map of *Pm5e* was constructed with 2 SNP and 2 SSR molecular markers. *Pm5e* was mapped to a 9.5 cM interval and the two SNP markers AX-95000860 and AX-94638908 were the two closet flanking markers, which delimited *Pm5e* into a 14 Mb region. Identification of the molecular markers and development of the two KASP assays laid a solid base for MAS of gene *Pm5e* in breeding.

Key words: Linkage map, marker assisted selection, SNP marker, wheat

INTRODUCTION

Wheat is an adaptable and widely distributed world food crop, which provide about 21% of food calories and 20% of protein for the human (He et al., 2018). Powdery mildew (Pm) is a disease caused by the biotrophic fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*), which often occurs in wheat production areas with cool and humid climates (Cowger et al., 2012). In China, this foliar disease is endangering most regions of winter wheat and spring wheat productions (Liu et al., 2016).

Use of Pm resistance genes to develop resistant cultivars is the most effective way to control the epidemics of Pm and reduce the economic losses (Hulbert et al., 2001) however, the development of Pm-resistant wheat cultivars requires resistance genes. To date, seventy-eight designated and many other temporarily designated Pm resistance genes or alleles have been identified in wheat. Some of these genes have single alleles, while some of them have multiple alleles (e.g., *Pm1*, *Pm2*, *Pm3*, *Pm4*, *Pm5*, and *Pm54* loci) (Wu et al., 2018; Zhang et al., 2016).

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Pm5 was a recessive Pm resistance gene located on the long arm of 7B (Lebsock and Briggle, 1974). It is widely contributed in the cultivars and landraces of China and Europe (Huang et al., 1997; Zeller et al., 1998). Five alleles at the *Pm5* locus have been reported (Huang et al., 2000; Hsam et al., 2001). *Pm5e*, from Fuzhuang 30, a cultivar developed from the cross of two Chinese landraces, has proven to be a valuable Pm resistance source for breeding (Huang et al., 1997, 2003). The resistance gene in Fuzhuang 30 was first mapped to 7B (Huang et al., 2000), and Wang et al. (2000) designated the gene as *Pm5e*. Huang et al. (2003) mapped this gene to the distal end of 7BL by SSR markers.

Bulked segregant analysis (BSA) has been widely used to identify polymorphic molecular markers in genetic mapping by traditional molecular markers, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) (Tsilo et al., 2009; Xu et al., 2018). However, these markers cannot meet the demand for fine mapping of a gene as well as MAS due to inadequate density. The crop genomics landscape has been revolutionized due to the next generation sequencing (NGS) technologies, which provides a large amount of sequencing information with great improvements in coverage, time, and costs (Bevan and Uauy, 2013; Rasheed et al., 2017). These technologies facilitate the development of chip-based marker platforms for genotyping in an ultra-high-throughput fashion. In wheat, the 9K, 90K, 660K, 820K, and 35K wheat genotyping chip have been developed and widely used in genetic study (Manish et al., 2017; Rasheed et al., 2017; Windju et al., 2016; Xu et al., 2018).

Kompetitive allele-specific PCR (KASP) is a proprietary technology that can distinguish alleles at variant loci. KASP is a cost-effective single-step genotyping technology, cheaper than SSRs and more flexible than genotyping-by-sequencing (GBS) or chip-based genotyping, and thus has been widely used in linkage mapping, QTL mapping, and MAS (Liu et al., 2014; Patil et al., 2017; Semagn et al., 2014; Steele et al., 2018).

In a former study, using a F_2 population derived by crossing Fuzhuang 30 with Chancellor, we mapped *Pm5e* to 7BL, and identified two flanking SSR markers, *Xwmc364* and *Xbarc065* (Zhu et al. 2008). The objective of this study is to: (1) identify SNP markers closely linked with *Pm5e*, and (2) develop KASP assays that can be widely used in MAS of *Pm5e* to improve Pm resistance in wheat.

MATERIALS AND METHODS

Plant materials

A population of 395 F_2 was derived from a cross between the Pm-resistant parent Fuzhuang 30 and a Pm-susceptible parent Chancellor. Seeds from 212 randomly selected F_2 individuals were harvested to produce 212 F_3 families. Two susceptible cultivars Huixianhong and Mingxian 169 were used as the susceptible controls.

Pm inoculation and resistance identification

E09, a dominant local isolate of *Blumeria graminis* f. sp. *tritici* in China was used to identify the resistance at the seedling stage under artificial climate chamber conditions at 22°C day/18°C night with 60% relative humidity and a 12-h light/12-h dark photoperiod. Fuzhuang 30, Chancellor, Huixianhong, Mingxian 169, 395 F2 plants, and at least 15 plants from each F_3 family were tested for Pm resistance. Inoculation and resistance identification followed the methods described by Liu et al. (1999). Seedlings at one leaf stage were inoculated with E09 by dusting conidiospores that were multiplied on the susceptible plants of Huixianhong. Infection types (ITs) of all plants were scored on a 0–4 scale 15 days after inoculation. The inoculated plants with ITs 0-2 were divided into a resistant group with those of 0-4 to a susceptible group (Liu et al., 1999). The genotype of each F_2 individual for *Pm5e* was determined by the phenotype of the corresponding F_3 family.

DNA extraction and BSA analysis

Leaf tissue was harvested at the three-leaf stage of each F_2, dried in a SCIENTZ-18 freezer dryer (Ningbo Scientz, China) for 3 days, and ground to powder in a G200 mixer mill (Coyote Bio, China) for 3 min with the aid of a metal bead in each tube. DNA was isolated using a modified CTAB method (Liu et al., 2014; Saghai-Maroof et al., 1984). Bulked segregant analysis (BSA) was used to screen potential polymorphic single nucleotide polymorphism (SNP) markers associated with Pm resistance. Each of the two bulks consisted of 25 highly Pm-resistant and 25 highly Pm-sensitive F_2 individuals, respectively, from the F_2 population of Fuzhuang 30/Chancellor and screened by the 35K Axiom® Wheat Breeder Genotyping Array (Allen et al., 2017).

SSR, KASP analysis and map construction

Two SSR markers *Xwmc364* and *Xbarc065*, which have been identified to be linked with *Pm5e* were also screened in this population following the method described by Zhu et al. (2008). Polymorphic SNPs identified by 35K Axiom® Wheat Breeder Genotyping Array between the two bulks were converted into KASP assays and run across the F_2 population for linkage mapping.

KASP assay followed the method described by Liu et al. (2014). In brief, a 6 µL reaction was used for KASP assay, which includes 3 µL of 2× reaction mix, 0.106 µL of assay mix (LGC Genomics, Beverly, MA) and 3 µL of genomic DNA at 15 ng/µL. PCR and fluorescent endpoint readings were carried out using an ABI Quant StudioTM 12K Flex Real-Time PCR System (Life Technology, Grand Island, NY). PCR thermal cycling profile followed the manufacturer's manual (http://www.kbioscience.co.uk/ reagents/ KASP_manual.pdf).

Linkage mapping was performed using JoinMap 3.0 software (Van Ooijen and Voorrips 2001). Recombination fractions were converted into centiMorgans (cM) using the Kosambi function (Kosambi, 1944). Map construction followed the methods described by Liu et al. (2014).

BLAST analysis

Sequences containing SNPs linked with *Pm5e* were used as

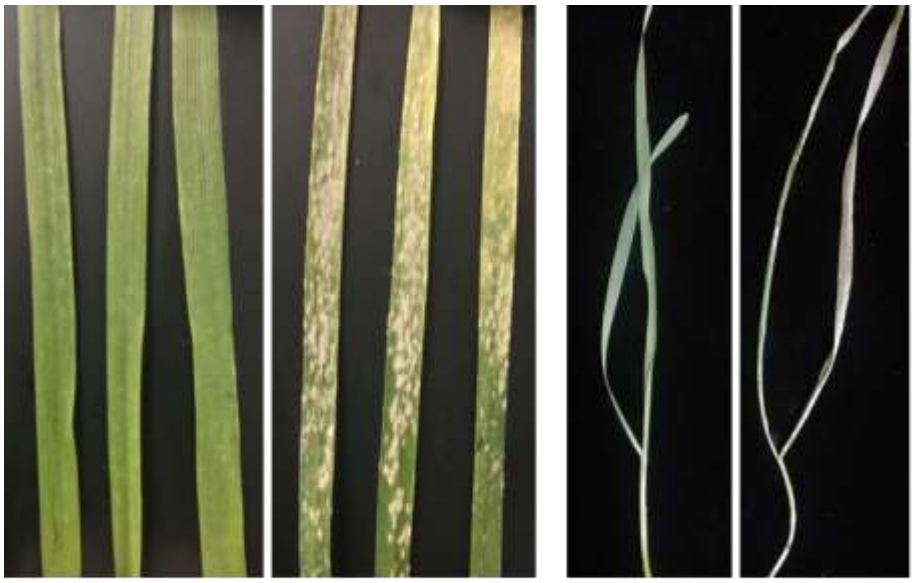

Figure 1. The powdery mildew infection phenotype of Fuzhuang30 (RP), Chancellor (SP) and F_2 resistant (R) and (S) susceptible individuals.

queries to search in the Chinese Spring genome sequence database released by the International Wheat Genome Sequencing Consortium (IWGSC) RefSeq1.0 (Rudi et al., 2018, https://urgi.versailles.inra.fr/) using BLAST to identify the physical positions of the SNPs on 7B. A significant match was declared when at least 98% nucleotide identity was identified with an e-value lower than e^{-20} (Liu et al., 2014).

RESULTS

Pm resistance evaluation

Fuzhuang 30 was immune to Pm, while Chancellor was highly susceptible (Figure 1). Among the 395 F_2 individuals, 108 were resistant and the remaining 287 were susceptible. The chi-square test showed that the segregation of resistant and susceptible F_2 plants fit to a 1:3 ratio (χ^2=2.256 <$\chi_{0.05}{}^2$=3.841). Among the F_3 families, 47 were resistant, 125 were segregating, 50 were susceptible and fit to a 1:2:1 ratio (χ^2=3.466<$\chi_{0.05(2)}{}^2$=5.991), indicating that Fuzhuang 30 carries a single recessive gene for Pm resistance to *Bgt* isolate E09.

Polymorphic SNP detected by 35K chip

After screening of the two bulks using the 35K Axiom® Wheat Breeder Genotyping Array, 1548 probes were identified as polymorphic between the two bulks. Blasting of these probes in the IWGSC wheat genome sequence database RefVer1.0 showed that these polymorphic probes were located on all wheat chromosomes (74 in average); however, the probes located on 7B (191) was significantly more than other chromosomes (Figure 2), indicating *Pm5e* was probably located on 7B.

Among the 191 probes on 7B, 98 were on 7BL, and only 27 were located between two wheat ESTs CJ729392 and CJ584170 (*Pm5e* region) (Table 1). According to the IWGSC wheat genome sequence database RefVer1.0, this region was a 41 Mb interval from 687 Mb to 728 Mb on 7BL.

KASP and SSR marker analysis

The 27 polymorphic SNPs were converted into KASP assays and run in two parents and randomly selected 40 F_2 individuals, only 2 assays AX-95000860 and AX-94638908 detected polymorphism between the parents and can separate the F_2 plants clearly. The primers for AX-95000860 KASP assay includes CAGGATTGGACTCGGCTGGAAAC as the AX-95000860-FAM forward primer, CAGGATTGGACTCGGCTGGAAAT as the AX-95000860-HEX forward primer, and ATGTCAGGTCACCACGATGC as the common reverse primer. The primers for AX-94638908 KASP assay include ATGATAACATGCTGCGCATGAC as the AX-94638908-FAM forward primer, ATGATAACATGCTGCGCATGAT as the AX-94638908-HEX forward primer, and TACACAAACTAGGTGGAGGTACAAC as the common reverse primer. The two assays were further run across

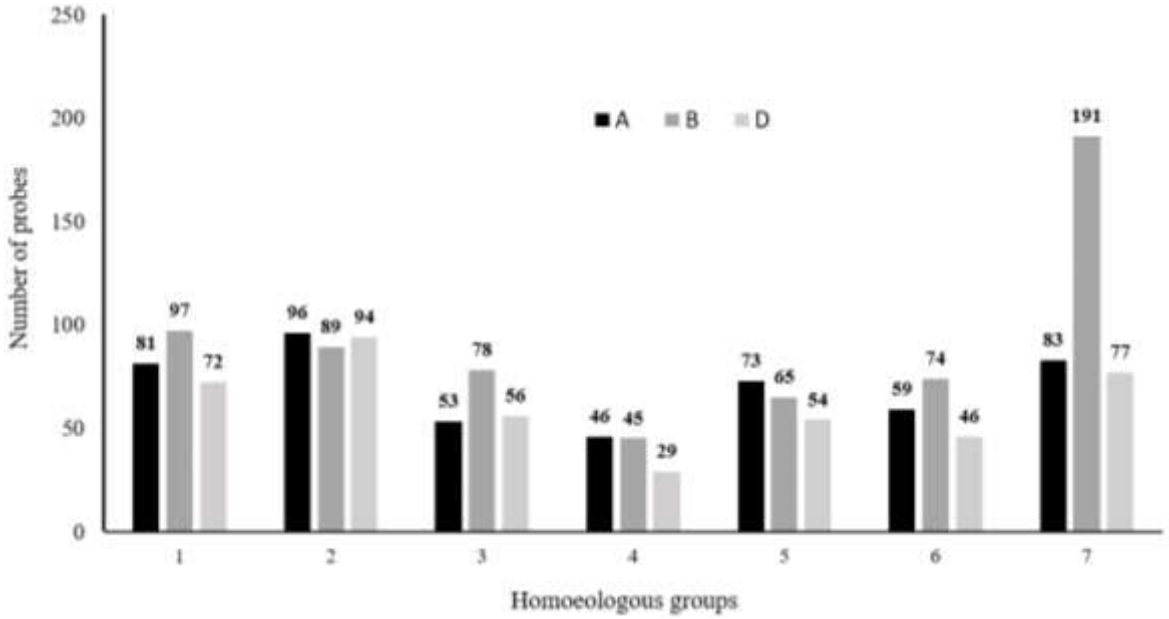

Figure 2. Distribution of polymorphic probes on wheat chromosomes.

Table 1. Polymorphic probes between the resistant and susceptible bulks on 7BL in the interval 687-728 Mb on 7BL.

Probe name	Resistant bulk	Susceptible bulk	Polymorphic SNP
AX-94418014	C/C	C/G	C/G
AX-94459856	C/G	C/C	G/C
AX-94463979	C/C	T/C	C/T
AX-94472687	C/C	C/G	C/G
AX-94535041	T/T	T/C	T/C
AX-94584717	T/T	T/C	T/C
AX-94596410	T/T	T/C	T/C
AX-94614297	C/C	A/C	C/A
AX-94628121	G/G	A/G	G/A
AX-94638908	C/C	T/C	C/T
AX-94667120	C/C	T/C	C/T
AX-94677860	G/G	C/G	G/C
AX-94677963	A/A	A/C	A/C
AX-94750259	C/G	C/C	G/C
AX-94826552	A/A	A/G	A/G
AX-94831799	T/T	T/C	T/C
AX-94878591	G/G	A/G	G/A
AX-94931476	T/C	T/T	C/T
AX-94960851	G/G	A/G	G/A
AX-94977792	T/T	T/C	T/C
AX-94999423	A/G	A/A	G/A
AX-95000860	C/C	T/C	C/T
AX-95140096	C/C	T/C	C/T
AX-95163625	C/C	T/C	C/T
AX-95186295	T/C	T/T	C/T
AX-95188770	T/G	T/T	G/T
AX-95652788	A/G	A/A	G/A

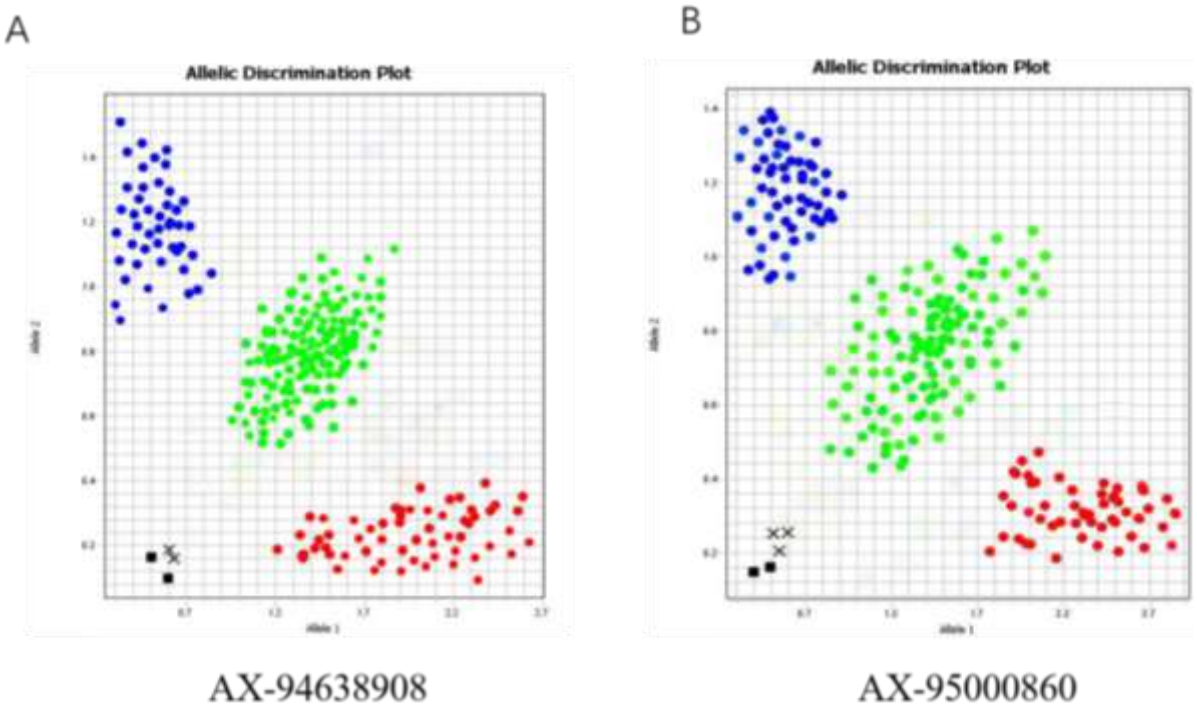

Figure 3. KASP assay of single nucleotide polymorphism (SNP) AX-94638908 (A) and AX-95000860 (B) in F_2 population. Allele X (KASPHEX, red) shows the T (A) and T (B) nucleotide, and allele Y (KASPFAM, blue) shows the C (A) and C (B) nucleotide. The green color dots indicate heterozygotes. The black squares and X in the left bottom are water and blank controls, respectively.

the F_2 population and can clearly separate the population into three groups with homozygous resistant, heterozygous and homozygous susceptible (Figure 3). The two SSR markers, *Xwmc364* and *Xbarc065*, which have been identified to be linked with *Pm5e* were also run across the F_2 population and can separate the genotypes of the F_2 individuals clearly (Figure 4).

Map construction

A linkage map including *Pm5e*, two SNP, and two SSR markers was obtained with a total genetic distance of 20.2 cM (Figure 5). Among them, the SNP markers were the two closet flanking markers of *Pm5e* with genetic distances of 4.2 cM and 5.3 cM apart from *Pm5e*, respectively. *Xwmc364* and *Xbarc065* were farther apart with *Pm5e*. Based on the IWGSC RefVer1.0 sequence, the two flanking SNP markers AX-95000860 and AX-94638908 delimited *Pm5e* to a 14 Mb interval from 707 Mb to 721 Mb on 7BL.

DISCUSSION

Since gene *Pm5e* gene was successfully excavated, the predecessors have successfully developed some molecular markers linked to it, including SSR markers and EST markers (Huang et al., 2003; Zhu et al., 2008; Xie, 2016). However, the mapped markers are far apart from *Pm5e*. Closely linked flanking markers are urgently needed for effective marker-assisted transfer of *Pm5e* to new wheat cultivars by MAS.

SNPs are the most abundant DNA sequence polymorphisms in a genome. In the last decade, next-generation sequencing (NGS) technologies have advanced rapidly and have become the cheapest and fastest technology for identification of genome-wide SNPs (Manish et al., 2017). SNP arrays have been developed and used for a variety of genetic and breeding applications including genome-wide association analysis and genomic selection in many crops (Manish et al., 2017; Allen et al., 2017).

In this study, BSA and wheat SNP chip was used to identify SNP markers closely linked with *Pm5e*, segregation of Pm resistance of resistant and susceptible individuals in the F_2 population showed a 1:3 ratio, indicating that Fuzhuang 30 carries a single recessive gene for Pm resistance to *Bgt* isolate E09, which is consistent with the former study (Huang et al., 2003). Based on the Pm resistance identification, a resistant and susceptible bulk was made and a 35K wheat SNP chip was used to screen the resistant and susceptible bulks to identify polymorphic SNPs between the two bulks, and

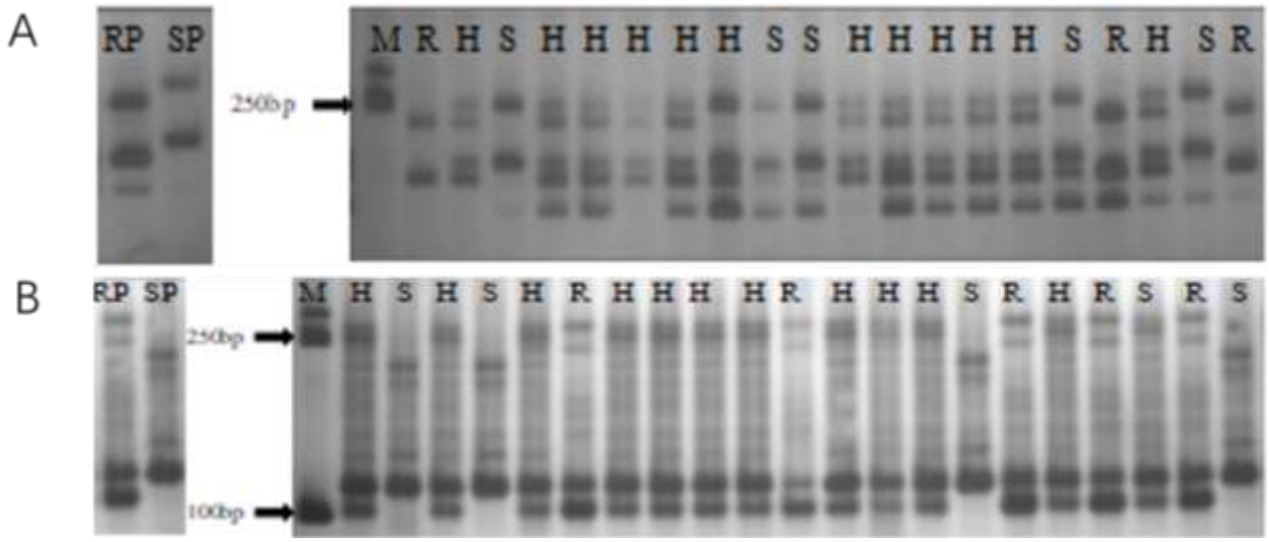

Figure 4. Segregation of SSR marker *Xwmc364* (A) and *Barc065* (B) in the Fuzhuang 30/Chancellor F_2 population. R, S and H indicate resistant, susceptible and heterozygous genotype and the marker locus. RP and SP indicate resistant and susceptible parent. M, marker ladder.

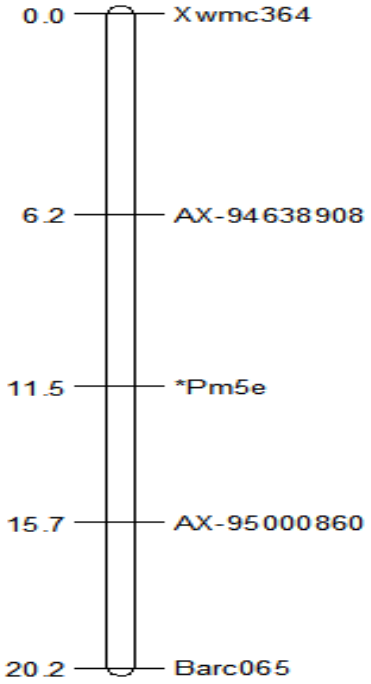

Figure 5. Linkage map of gene *Pm5e*.

1548 polymorphic SNPs were identified. Because *Pm5e* has been mapped on 7BL and two flanking wheat ESTs, CJ729392 and CJ584170, have been identified, we can easily delimit the physical interval of *Pm5e* according to the wheat reference genome sequence. Only 27 polymorphic SNP between the two flanking ESTs were used to develop KASP assays to fine map *Pm5e.* Two KASP markers, AX-95000860 and AX-94638908, were identified closely linked to *Pm5e* with genetic distance of 4.2 cM and 5.3 cM apart from *Pm5e*, which were much closer than the formally mapped markers, and the interval of the *Pm5e* region was decreased from 41 Mb to 14 Mb (Zhu et al., 2008; Xie, 2016), which indicated that using SNP chip to identify SNP markers for a specific gene was very effective.

Wheat SNP chip may not be cost effective for breeding selection due to high cost per sample if only a few SNPs are interested. KASP assay, however, is a time saving and cost-effective genotyping method for single SNP screening and has been successfully used in wheat genetic and breeding studies (Semagn et al., 2014; Rasheed et al., 2017). In this study, the identified polymorphic SNPs between the two bulks were converted into KASP assays to map *Pm5e* and two KASP assays closely linked with *Pm5e* were mapped, which can be used in MAS of *Pm5e*.

Conclusions

i) Twenty-seven polymorphic SNPs between the resistant and susceptible bulks in *Pm5e* genomic region were identified using the 35K wheat SNPs chips.
ii) KASP assays of the polymorphic SNPs were developed and a genetic linkage map of *Pm5e* was constructed together with 2 SNP and 2 SSR markers.
iii) *Pm5e* was mapped to a 9.5 cM interval and the two KASP markers AX-95000860 and AX-94638908 were the two closet flanking markers, which delimited *Pm5e* into a 14 Mb region and laid a solid base for MAS of *Pm5e* in breeding.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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