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ORIGINAL ARTICLE

Fine mapping of the wheat powdery mildew resistance gene *Pm52* using comparative genomics analysis and the Chinese Spring reference genomic sequence

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Abstract

Key message **A high-resolution genetic linkage map was constructed using the comparative genomics analysis approach and the wheat reference genome, which placed wheat powdery mildew resistance gene *Pm52* in a 0.21-cM genetic interval on chromosome arm 2BL.**

Abstract The gene *Pm52* confers resistance to powdery mildew and has been previously mapped on chromosome arm 2BL in winter wheat cultivar Liangxing 99. Because of its effectiveness against the disease, this study was initiated to finely map *Pm52* using the comparative genomics analysis approach and the wheat reference genomic sequence. Based on the EST sequences that were located in the chromosome region flanking *Pm52*, four EST-SSR markers were developed, and another nine SSR markers were developed using the comparative genomics technology. These thirteen markers were integrated into a genetic linkage map using an $F_{2:3}$ subpopulation of the Liangxing 99 × Zhongzuo 9504 cross. *Pm52* was mapped within a 3.2-cM genetic interval in the subpopulation that corresponded to a ~40-Mb genomic interval on chromosome arm 2BL of the Chinese Spring reference genome. The *Pm52*-flanking markers *Xicsl163* and *Xicsl62* identified 344 recombinant individuals from 8820 F_2 plants. Nine SSR markers generated from the Chinese Spring genomic interval were incorporated into a high-resolution genetic linkage map, which placed *Pm52* in a 0.21-cM genetic interval corresponding to 5.6-Mb genomic region. The constructed high-resolution genetic linkage map will facilitate the map-based cloning of *Pm52* and its marker-assisted selection.

Introduction

Wheat powdery mildew, a foliar disease that is incited by the biotrophic fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*), frequently occurs in many of the temperate wheat (*Triticum aestivum* L.) producing regions of the world. Morgounov et al. (2012) reported that the average incidence and severity of the powdery mildew in 51 countries during 1969–2010

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were 54.5% and 7.5%, respectively. In China, this disease has been a serious yield constraint for wheat production since the 1970s, especially in the winter wheat-growing regions with high inputs of irrigation and fertilizers (Luo et al. 2009). The incidence of wheat powdery mildew was approximately 6 million hectares in 2018, and a similar scale of infected areas has occurred annually during the past decade (http://www.natesc.org.cn). In the future, powdery mildew is anticipated to cause greater damage to winter wheat production due to climate change, and the percentage of affected acreage has increased significantly by 8.5% per decade from 1970 to 2012 (Tang et al. 2017).

Host plant resistance and chemical control are the traditional approaches to mitigate the economic losses in wheat production caused by powdery mildew. The increase in grain yield through disease managements was estimated to be 15 million tons over the last decade (Liu et al. 2016). The discovery of resistance genes for developing resistant wheat cultivars is the preferred means to effectively and economically control wheat powdery mildew.

Various classes of molecular markers, in particular simple sequence repeat (SSR) markers, have been more efficient to identify powdery mildew (*Pm*) resistance genes in wheat cultivars compared to the conventional cytogenetic methods using the wheat aneuploids. However, it is difficult to develop a saturated genetic linkage map only based on SSR markers due to their limited number and scattered distribution on wheat chromosomes. The development of large numbers of chromosome-anchored wheat expressed sequence tags (ESTs) provides a valuable genomic resource (Lazo et al. 2004), which has been used to develop target gene-linked markers before the complete genome sequences of wheat become available. There exists a strong syntenic relationship among wheat and certain cereal crops and grass species that have complete genome sequences, such as rice (*Oryza sativa* L.) (International Rice Genome Sequencing Project, 2005), sorghum (*Sorghum bicolor* (L.) Moench) (Paterson et al. 2009), purple false brome (*Brachypodium distachyon* L.) (The International Brachypodium Initiative 2010), and barley (*Hordeum vulgare* L.) (The International Barley Genome Sequencing Consortium 2012). Therefore, comparative genomics analysis has been effectively applied to develop molecular markers for finely mapping *Pm* genes in wheat. Examples include the fine mapping of *MlHLT* (Wang et al. 2015), *MlIW172* (Ouyang et al. 2014), and *PmTm4* (Xie et al. 2017), as well as other *Pm* genes.

The release of the genome sequences of common wheat and its closely related species has greatly facilitated comparative genomics analysis and map-based cloning of disease resistance genes in wheat. Since the first whole-genome shotgun sequences of common wheat were available (Brenchley et al. 2012), a great effort has been made to improve the wheat genome sequence assembly from the scaffold level to the chromosome level (International Wheat Genome Sequencing Consortium 2014; Chapman et al. 2015; Zimin et al. 2017). Recently, the complete genome sequence of the wheat cultivar Chinese Spring RefSeq v.1.1 was released (The International Wheat Genome Sequencing Consortium (IWGSC) 2018). The genome sequences of the A and D genome donor species *T. urartu* Thumanjan Ex Gandilyan and *Aegilops tauschii* Coss., respectively, also have been updated (Luo et al. 2017; Ling et al. 2018). Meanwhile, the genome sequence of wild emmer wheat [*T. turgidum* ssp. *dicoccoides* (Körnicke ex Asch. & Graebn.) Thell.] (AABB genome) also was published (Avni et al. 2017). The improvement in next-generation sequencing (NGS) technologies and conversion of single nucleotide polymorphisms (SNPs) to Kompetitive allele specific PCR (KASP) markers have promoted the high-throughput discovery of DNA variants and detection of gene-linked markers. Taking the advantages of those genomic sequences, Wu et al. (2018b) confined *Yr26* to a 0.003-cM interval on wheat chromosome arm 1BL and Zou et al. (2018) cloned the powdery mildew resistance gene *Pm60* from *T. urartu* using a map-based cloning technique. It is expected that more and more genes for disease resistance and other agronomic traits will be identified and/or cloned in the future.

In general, there are two sources of *Pm* genes that confer resistance to powdery mildew, i.e., common wheat and its relatives. Some of the *Pm* genes identified in close or distant relatives may be linked with certain deleterious genes, so a pre-breeding step is required to improve their yield and agronomic performance. This impedes the direct application of such type of *Pm* genes in breeding programs. The resistance genes or alleles identified from commercial cultivars are preferred by breeders as those genes can be more easily incorporated for the improvement in disease resistance in wheat. Using the molecular marker-based mapping technology, several *Pm* genes had been identified in commercial Chinese wheat cultivars. For example, the resistance to powdery mildew in Yumai 66 and Zheng 9754 was controlled by *PmYm66* and *PmHNK54* on chromosome arm 2AL, respectively (Hu et al. 2008; Xu et al. 2011); Zhoumai 22 carried the *Pm* gene *PmHNK* on chromosome arm 3BL (Xu et al. 2010).

Liangxing 99 is a winter wheat cultivar and has been grown over 300 million hectares in northern China, since it was released in 2006 (http://202.127.42.178:4000/SpeciesQuery/Details/9917). It had also served as a control cultivar in the yield trials at the provincial (Shandong province) and the national levels, since 2010 and 2012, respectively. Liangxing 99 was effective against 81.3% of the 123 *Bgt* isolates collected from northern China and displayed an adult plant resistance in several years of field tests (Zou et al. 2017). It even had a greater percentage of resistance (94%) against another group of 49 Chinese *Bgt* isolates in a

separate study (Ma et al. 2018). Because of its superior agronomic performance and high level of resistance to the disease, Liangxing 99 has also been used as a parent to develop commercial wheat cultivars.

Resistance to powdery mildew in Liangxing 99 was conferred by a gene, designated *Pm52* (McIntosh et al. 2014). Previous study has located *Pm52* on chromosome arm 2BL (Zhao et al. 2013). A project was initiated to finely map *Pm52* to facilitate its efficient and accurate detection in the breeding efforts for improving disease resistance. The objectives of this study were (1) to develop molecular markers closely linked to *Pm52* using the comparative genomics analysis method taking the advantage of the existing genomic sequences of wheat and other cereal crops and (2) to construct a high-resolution linkage map.

Materials and methods

Plant materials

In 2006, Liangxing 99 (pedigree: Ji 91102/Lumai 14//PH85-16) was released by the Shandong Liangxing Seed Co. Ltd., Ningjin, Shandong province (http://www.ampcn.com/info/detail/12696.asp). To develop a saturated genetic linkage map for *Pm52*, the F_2 and $F_{2:3}$ mapping populations (consisting of 8820 members) produced by crossing Liangxing 99 to powdery mildew susceptible cultivar Zhongzuo 9504 were used throughout the study. A subpopulation consisting of 165 $F_{2:3}$ families were used to examine the polymorphisms of the molecular markers developed in the present study. Zhongzuo 9504 was also used to increase *Bgt* isolate E20, which was avirulent on the *Pm* genes 1a, 1c, 2, 12, 13, 16, 17, 20, 24, 2 + 6, and 5 + 6, but virulent on the *Pm* genes 1e, 3a, 3b, 3d, 3e, 3f, 3g, 4a, 4b, 4c, 5a, 5e, 6, 7, 8, 11, 19, Y39, PS5A, 33, DR147, H, Mlxbd, and 1 + 2 + 9 (Li et al. 2011), for phenotyping the mapping population and served as the susceptible control in all the powdery mildew tests. The cultivars derived from Liangxing 99, i.e., Zhongxinmai 99 (pedigree: Liangxing 99/222), DH51302 (pedigree: DH6388/Lankaoaizao 8//Liangxing 99), and Heng 10-5039 (pedigree: Heng 7228/Shangdong 93-5031//Liangxing 99), were used to confirm the *Pm52*-linked markers. Based on the reactions to 27 *Bgt* isolates, Zhongxinmai 99 and DH51302 were proposed to carry *Pm52*; and Heng 10-5039 did not carry *Pm52* (Zou et al. 2017).

Evaluation of powdery mildew resistance

The seedling reactions of the $F_{2:3}$ families that were derived from the F_2 plants of the subpopulation and the recombinant plants were tested using the *Bgt* isolate E20 to predict the genotypes of the corresponding F_2 plants. Fifteen seeds of each $F_{2:3}$ family were planted in plastic pots (6.5 cm × 6.5 cm × 6.5 cm in dimension) that were placed in plastic trays. Liangxing 99 and Zhongzuo 9504 were grown in each tray as the resistant and the susceptible controls, respectively. When the first leaves were unfolded, the plants were inoculated by dusting with *Bgt* conidiospores that were increased on Zhongzuo 9504 seedlings. After incubation in a dew chamber in the dark for 24 h, the inoculated plants were grown in a greenhouse under a daily cycle of 14 h of light at 22 ± 2 °C and 10 h of darkness at 18 ± 2 °C to promote the development of disease symptoms. Fifteen days after inoculation when the disease was fully developed on the susceptible control plants, the infection types (ITs) of all plants were determined individually on a 0–4 scale as previously described (Liu et al. 1999). Plants with ITs 0, 0;, 1, and 2 were classified in the resistant group, and ITs 3 and 4, in the susceptible group. Each $F_{2:3}$ family was tested at least twice to ensure consistency of the phenotypes.

Development of molecular markers

The EST sequences in the bin 2BL2-0.36-0.50 of chromosome arm 2BL were used to search against the barley genomic sequences (http://www.barleygenome.org.uk). Based on the sequences of the collinear region identified, EST-SSR markers were designed and subjected to polymorphism analysis between Liangxing 99 and Zhongzuo 9504, as well as the resistant and susceptible DNA bulks that were separately generated by mixing equal amounts of DNA from the representative plants of ten homozygous resistant and ten susceptible $F_{2:3}$ families from the subpopulation. The polymorphic EST-SSR markers were validated using the subpopulation to confirm their genetic linkage relationship with *Pm52*. The corresponding EST sequences of the polymorphic EST-SSR markers flanking the target resistance gene were used as queries to search the *Ae. tauschii* SNP database (http://probes.pw.usda.gov/WheatDMarker/), and the *Brachypodium* (http://mips.helmholtz-muenchen.de/plant/brachypodium/), rice (http://rice.plantbiology.msu.edu/), and sorghum (http://mips.helmholtz-muenchen.de/plant/sorghum/) genomic sequences with an *E*-value cutoff of 10e−10. The orthologous genomic regions were determined by comparative genomics analysis of the putatively conserved gene pairs in the genomes of *Ae. tauschii*, *Brachypodium*, rice, and sorghum. The resulting orthologous gene pairs were then used to search the Chinese Spring 454 contigs sequence (Brenchley et al. 2012) and the whole genomic assembly sequences for Chinese Spring wheat (IWGSC WGS v1.1, NRGene DeNovoMAGIC, Seq Repository of Wheat Portal on URGI, INRA, France) (The International Wheat Genome Sequencing Consortium (IWGSC) 2018) to find homologous contig or scaffold sequences that were used to develop SSR markers using BatchPrimer3 (You et al. 2008).

Marker analysis

Genomic DNA was isolated from leaf tissues of each F_2 plant and the 165 $F_{2:3}$ families of subpopulation and the cultivars derived from Liangxing 99 using the CTAB method (Saghai-Maroof et al. 1984). Each reaction mixture (10 µl) of DNA amplification was composed of 50–100 ng of template DNA, 0.4 µM the forward and reverse primers each, 0.2 U of *Taq* polymerase, 0.2 mM dNTPs, and 1 × PCR buffer with 2 mM Mg^{2+}. DNA amplification was started from one denaturation cycle at 94 °C for 5 min, and then 35 cycles at 94 °C for 45 s, 50–60 °C (depending on the specific primers) for 45 s, and 72 °C for 1 min. The reaction was terminated with an extension cycle at 72 °C for 10 min. The amplification products were separated on 8% non-denaturing polyacrylamide gels (39 acrylamide/1 bisacrylamide), and the banding patterns were visualized after silver staining.

Identification of recombinant individuals from the F_2 mapping population

The SSR markers developed were integrated into a linkage map using a subpopulation consisting of 165 $F_{2:3}$ families of Liangxing 99 × Zhongzuo 9504 cross. Markers *Xicsl62* and *Xicsl163* were used to genotype 8820 F_2 plants to identify recombinant plants between the two markers with the capillary electrophoresis technology (Tagliaro et al. 1998). Amplification of DNA was carried out in a 10 µl reaction mixture which included 50–100 ng of template DNA, 0.4 µM each of the forward primer added with 5-hexachloro-fluorescein (HEX) or 5-carboxy-fluorescein (FAM) joint and reverse primer, 0.2 U of *Taq* polymerase, 0.2 mM dNTPs, and 1 × PCR buffer with 2 mM Mg^{2+}. DNA amplification procedure was described previously (Wu et al. 2018a). The amplification products were analyzed on a capillary electrophoresis apparatus (SMYG2016-JL028-1, SoftGenetics LLC, State College, PA) equipped with the software GeneMarker.

Construction of a high-resolution linkage map of *Pm52*

The software Mapmaker/Exp Version 3.0b was used to establish the linkage relationship between markers and the target resistance gene (Lincoln et al. 1993). The Kosambi function was used to determine the genetic distances. The logarithm of the odd ratio (LOD) threshold of 3.0 was used, and the maximum distance between markers was 50.0 cM.

Physical mapping and gene annotation of the *Pm52* goal interval

The sequences of the markers *Xicssl326* and *Xicscl795* that flanked *Pm52* were used as queries to search against the Chinese Spring whole genomic assembly sequences (IWGSC WGS v1.1, NRGene DeNovoMAGIC, Seq Repository of Wheat Portal on URGI, INRA, France). The information on Chinese Spring genomic sequence was used to identify the genes that were included in the interval between the two *Pm52*-flanking markers. Then, those genes were used as queries to blast against the genomic sequences of *Ae. tauschii* (http://aegilops.wheat.ucdavis.edu/ATGSP/index.php), *T. turgidum* ssp. *dicoccoides* (Avni et al. 2017), and *T. urartu* (http://www.mbkbase.org/Tu/).

Results

Development of polymorphic EST-SSR markers linked to *Pm52*

Previously, *Pm52* was located in the bin 2BL2-0.36-0.50 of chromosome arm 2BL, so the EST sequences in this interval were aligned with the barley genomic sequence. It was determined that this interval was collinear with a 46.5-Mb region on barley chromosome 2. Based on the wheat EST sequences that felt in this collinear region, 83 pairs of SSR primers were designed (Table S1). Among them, four EST-SSR markers *Xicsl30*, *Xicsl34*, *Xicsl54*, and *Xicsl62* were linked to *Pm52*, but they were all located on the same side of the target gene when examined using a subpopulation consisting of 165 $F_{2:3}$ families from the Liangxing 99 × Zhongzuo 9504 cross (Fig. 1a).

Development of SSR markers based on comparative genomics analysis

The coding sequences of collinear barley genomic sequence were used as queries to search against the Chinese Spring 454 contigs (Brenchley et al. 2012). Based on sequences of the homologous contigs or scaffolds on chromosome arm 2BL, 87 pairs of SSR primers were designed (Table S2). *Pm52* was flanked by markers *Xicsl90* and *Xicsl163* as verified using the subpopulation, and the genetic distances between these markers and the target gene were 1.9 cM and 1.9 cM, respectively (Fig. 1a).

The sequences of markers *Xicsl90* and *Xicsl163* were used as queries to blast against the *Ae. tauschii* SNP database and the *Brachypodium*, rice, and sorghum genome sequences. This interval was collinear with *Ae. tauschii* chromosome 2 (AT2D1947-AT2D2020), *Brachypodium* chromosome 5 (Bradi5g16920–Bradi5g18910), rice chromosome 4 (Os04g45340–Os04g48416), and sorghum chromosome 6 (Sb06g023780–Sb06g025980). Based on the Chinese Spring 454 contigs or scaffold sequences of

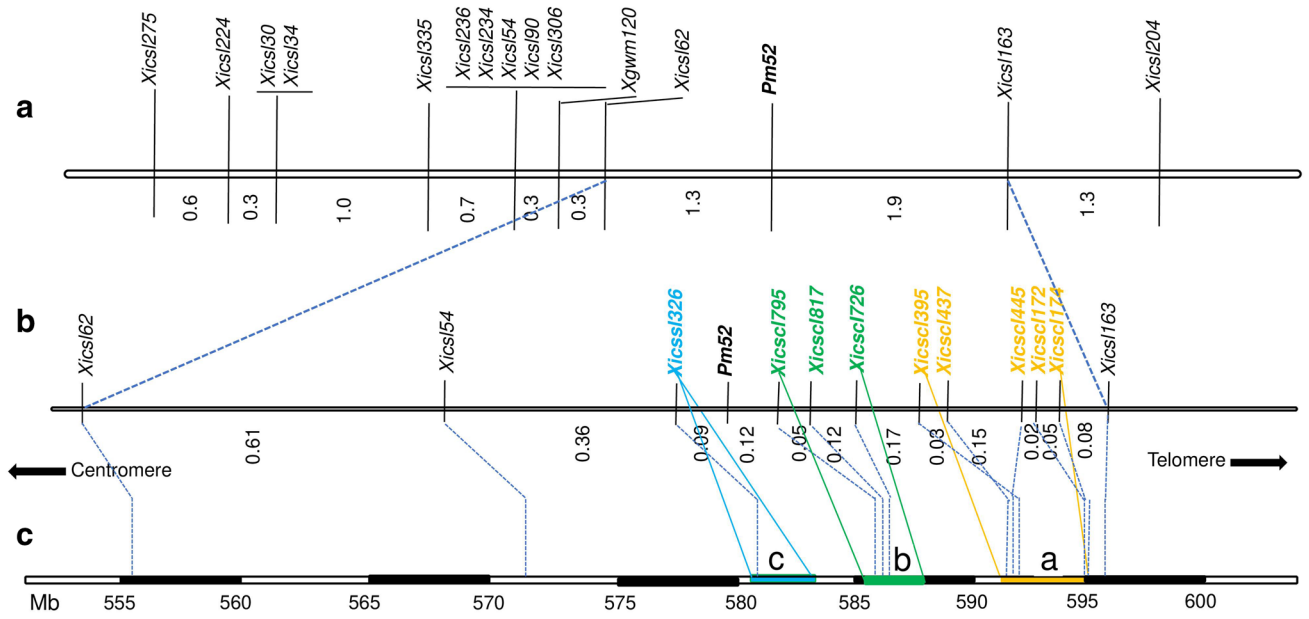

Fig. 1 Genetic linkage map and physical map of Chinese Spring genomic sequence flanking the *Pm52* locus. **a** Primary genetic linkage map of *Pm52* using the subpopulation. **b** The high-resolution genetic map of *Pm52*. **c** The corresponding physical location of the polymorphic linkage markers of *Pm52*, and diagram of selection physical interval to further design SSR primers. The blue oval frame shows the interval (581167165–583268161) that was used to design 176 primer pairs. The green oval frame shows the interval (586243207–588134752) that was used to design 178 primer pairs. The orange oval frame shows the interval (591820638–596417733) used to design 409 primer pairs

chromosome arm 2BL that corresponded with the orthologus genes, 178 SSR markers were developed (Table S3). Markers *Xicsl204*, *Xicsl224*, *Xicsl234*, *Xicsl236*, *Xicsl275*, *Xicsl306*, and *Xicsl335* were linked to *Pm52*, and *Pm52* was localized in the genetic region between markers *Xicsl62* and *Xicsl163* at genetic distances of 1.3 cM and 1.9 cM, respectively (Fig. 1a).

Identification and phenotyping of the recombinant F_2 plants from the cross Liangxing 99 × Zhongzuo 9504

The *Pm52*-flanking markers *Xicsl163* and *Xicsl62* were used to identify recombinants in the F_2 mapping population of the Liangxing 99 × Zhongzuo 9504 cross. Both markers were able to clearly differentiate different genotypes of the F_2 plants using the capillary electrophoresis method (Fig. 2). A total of 344 recombinant plants were identified from 8820 F_2 plants. The $F_{2:3}$ families produced by selfing the recombinant F_2 plants were phenotyped using *Bgt* isolate E20. Among them, 65 $F_{2:3}$ families were resistant with IT 0 or 1, 130 susceptible with IT 3 or 4, and the remaining 149 were heterozygous with both resistant and susceptible plants within a family. The DNA from the corresponding recombinant F_2 plants was used in the subsequent study for finely mapping *Pm52*.

Development of markers tightly linked to *Pm52* and construction of a high-resolution genetic linkage map

The sequences of *Xicsl62* and *Xicsl163* were blasted against the whole genomic assembly sequences of Chinese Spring (IWGSC WGS v1.1, NRGene DeNovoMAGIC, Seq Repository of Wheat Portal on URGI, INRA, France), and the two markers spanned a region of about 40 Mb (556678110–596417752) (Fig. 1c). Then, all the genomic sequences of Chinese Spring in this interval were used to design SSR markers, and 409 SSR primer pairs were designed based on the 5-Mb (591820638–596417733) sequence that extended from *Xicsl163* to *Pm52* (Fig. 1c(a) and Table S4). After examining the polymorphisms between the parental cultivars as well as the contrasting DNA bulks, five polymorphic markers *Xicscl395*, *Xicscl437*, *Xicscl445*, *Xicscl172*, and *Xicscl174* were located between *Xicsl163* and *Pm52* and were 0.46, 0.49, 0.64, 0.66, and 0.71 cM distal to *Pm52*, respectively (Fig. 1b).

Since *Pm52* was located between *Xicscl395* and *Xicsl54*, 163 SSR primer pairs were designed based on the 2-Mb (58401490–58632689) segment of the sequences that were 12 Mb away from marker *Xicsl54* in the direction toward *Pm52* (Table S5). In addition, 178 SSR primer pairs were developed based on the 2 Mb (586243207–588134752) of sequences that extended from marker *Xicscl395* toward

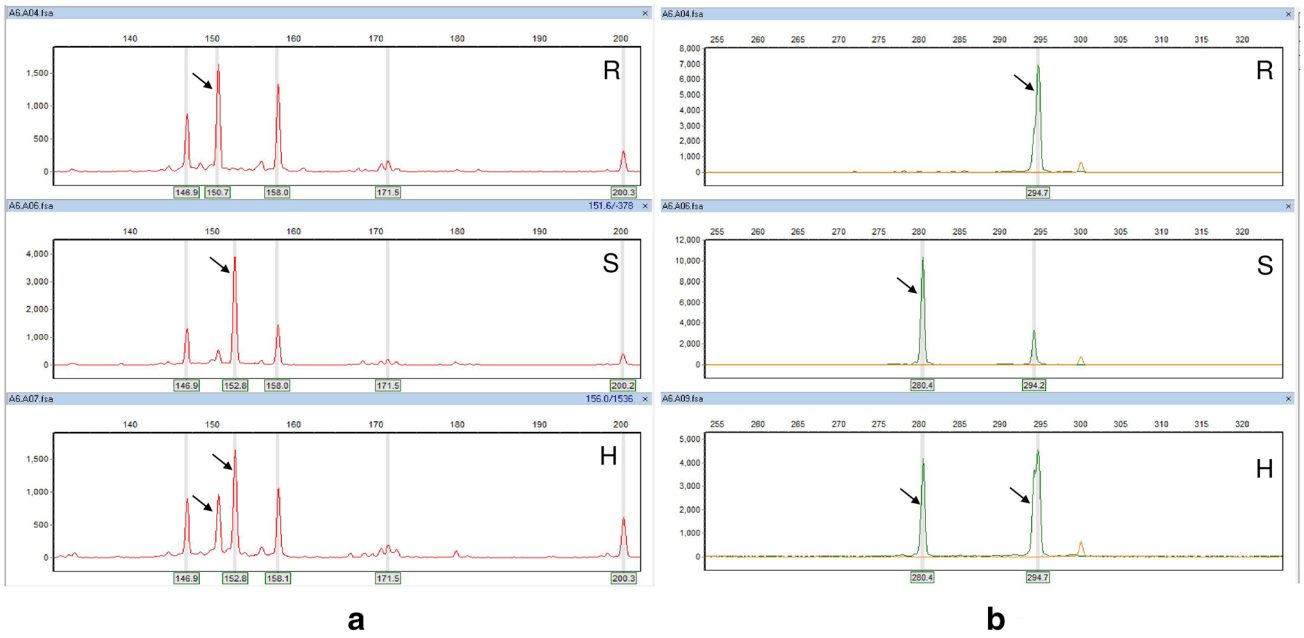

Fig. 2 Amplification banding types of markers *Xicsl62* (**a**) and *Xicsl163* (**b**) by the capillary electrophoresis apparatus. R: resistant; S: susceptible; H: heterozygous

Pm52 (Fig. 1c(b) and Table S6). Polymorphic markers *Xicscl795*, *Xicscl817,* and *Xicscl726* were placed 0.12, 0.17, and 0.29 cM distal to *Pm52*, respectively (Figs. 1b and 3a). *Pm52* was then flanked by *Xicscl795* and *Xicsl54* at distances of 0.12 and 0.45 cM, respectively. So, 176 SSR primer pairs were designed based on the sequences starting from a site 3 Mb distant to *Xicscl795*, which extended 2 Mb (581167165–583268161) toward gene *Pm52* (Fig. 1c(c) and Table S7). *Xicssl326* was closely linked to *Pm52* (Figs. 1b and 3b). Based on this high-resolution genetic linkage map, *Pm52* was flanked by markers *Xicssl326* and *Xicscl795*, at genetic distances of 0.09 and 0.12 cM, respectively (Fig. 1b). The genotypes of 130 recombinant plants in the genetic interval between markers *Xicsl54* and *Xicscl726* are shown in Table 1.

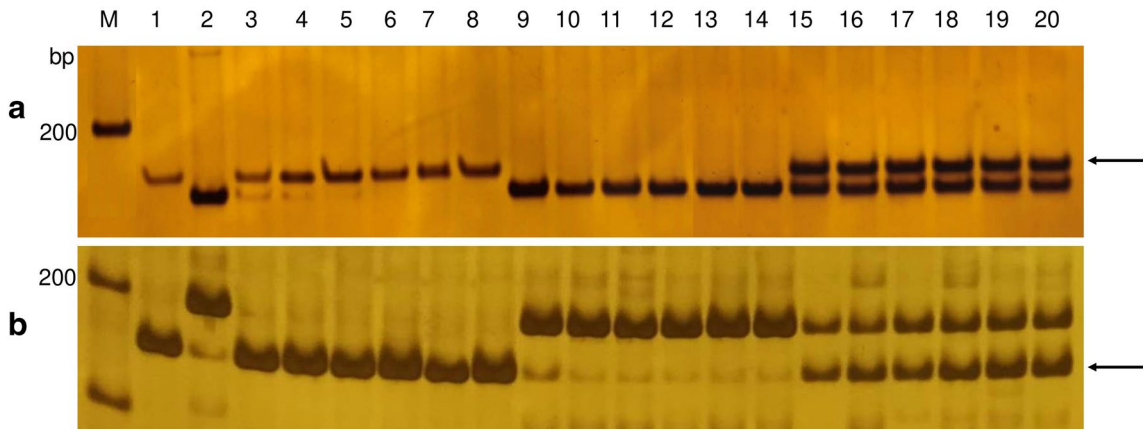

Fig. 3 Amplification patterns of *Pm52*-flanked SSR markers *Xicscl795* (**a**) and *Xicssl326* (**b**) developed from the Chinese Spring genomic sequence in the parents and selected $F_{2:3}$ families of Liangxing 99×Zhongzuo 9504 cross in 8% silver-stained non-denaturing polyacrylamide gels. Lane M: DL2000 (Tiangen Biotech Co., Beijing, China); lane 1: Liangxing 99; lane 2: Zhongzuo 9504; lanes 3–8: homozygous resistant $F_{2:3}$ families; lanes 9–14: homozygous susceptible $F_{2:3}$ families; and lanes 15–20: heterozygous $F_{2:3}$ families. Arrows indicate the polymorphic bands that are specific for *Pm52*

Table 1 Genotypes of the SSR markers *Xicscl726*, *Xicscl817*, *Xicscl795*, *Xicssl326,* and *Xicsl54* for the 130 F_2 recombinant individuals of the Liangxing 99 × Zhongzuo 9504 cross used to develop the markers closely linked to *Pm52*

SSR markers			Phenotype	SSR markers		Recombinant individuals totally 130 lines
Xicscl726	*Xicscl817*	*Xicscl795*		*Xicssl326*	*Xicsl54*	
H	H	H	H	H	A	8
H	H	H	H	H	B	14
A	A	A	A	A	H	15
B	B	B	B	B	H	24
H	H	H	H	A	A	3
H	H	H	H	B	B	7
A	A	A	A	H	H	1
B	B	B	B	H	H	7
A	A	A	H	H	H	10
B	B	B	H	H	H	7
H	H	H	A	A	A	2
H	H	H	B	B	B	2
A	A	H	H	H	H	1
B	B	H	H	H	H	4
H	H	A	A	A	A	1
H	H	B	B	B	B	3
A	H	H	H	H	H	3
B	H	H	H	H	H	9
H	B	B	B	B	B	4
H	A	A	A	A	A	5

The alleles are abbreviated according to their origin: A, resistant phenotype or genotype; B, susceptible phenotype or genotype, and H, heterozygous phenotype or genotype

Physical mapping, comparative genomics analysis and the gene annotation of *Pm52* goal interval

To determine the physical locations of the *Pm52*-linked markers, the sequences of all markers anchored in the high-resolution genetic map were used to blast against the Chinese Spring genomic sequence, and the relative physical positions of those markers were generally consistent with the genetic linkage map (Fig. 1). The genetic region of markers *Xicssl326* and *Xicscl795* corresponded to a 5.6-Mb (581242466–586768319) genomic interval in the Chinese Spring reference genome and contained 36 annotated protein-coding genes (Table S8, Fig. 4b).

The corresponding orthologous genomic sequences on *Ae. tauschii* 2DL, *T. turgidum* ssp. *dicoccoides* 2BL and *T. urartu* 2AL were annotated to detect orthologous gene pairs. This interval on Chinese Spring 2BL had a collinear relationship with a 6.5-Mb (491433391–497983654) genomic region on the chromosome 2DL of *Ae. tauschii* with 31 orthologous genes, a 5.6-Mb (579618841–584257015) region on chromosome 2BL of *T. turgidum* ssp. *dicoccoides* with 26 orthologous genes, and a 4.8-Mb (619434655–624213456) region on chromosome 2AL of *T. urartu* with 22 orthologous genes; however, the genomic region of *T. urartu* on

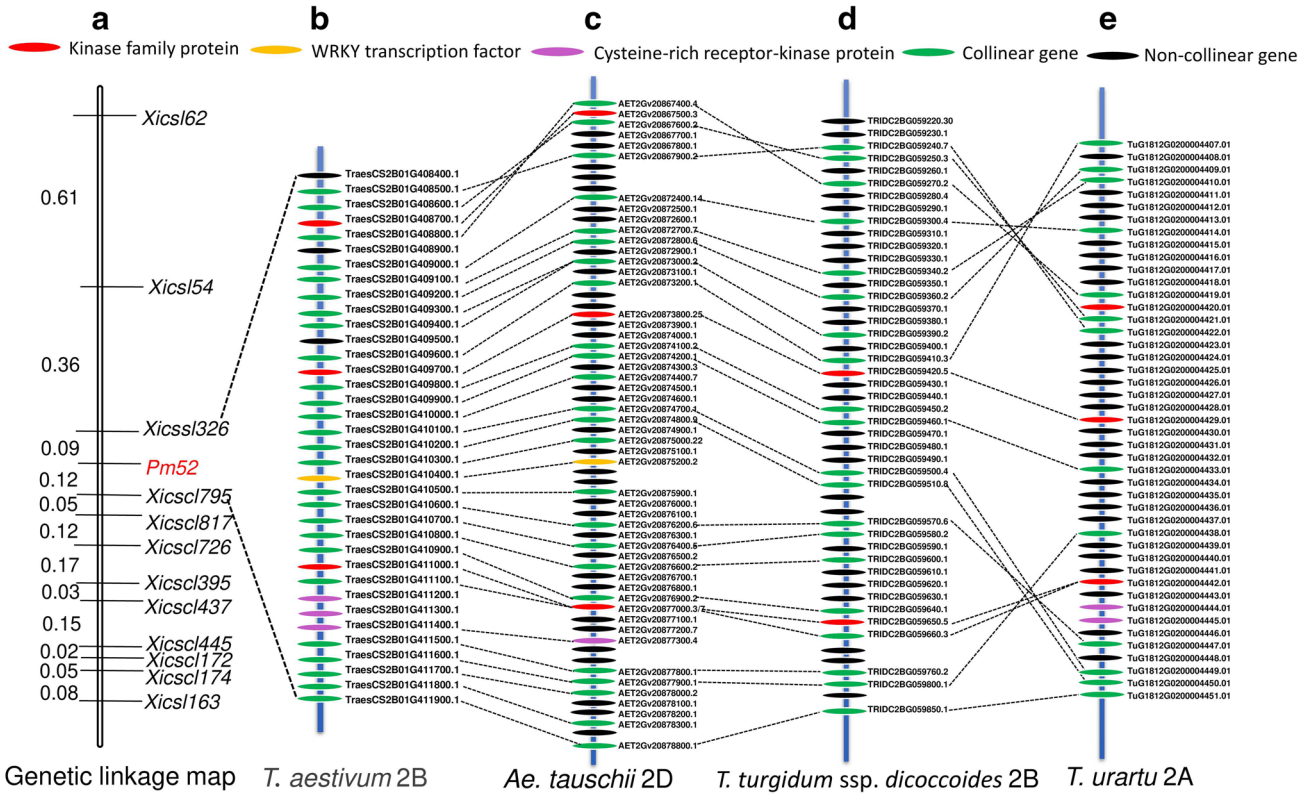

Fig. 4 Genetic linkage map of the powdery mildew resistance gene *Pm52* (**a**), and genes among Chinese Spring wheat (**b**), *Ae. tauschii* (**c**), *T. turgidum* ssp. *dicoccoides* (**d**) and *T. urartu* (**e**) in the corresponding genomic interval between the *Pm52*-flanking markers

2AL did not well collinear with *T. aestivum* 2BL with a large segment inversion (Table S8, Fig. 4).

Seven disease resistance-associated genes were identified in the collinear genomic regions (Table S8). Among them, three genes encode for kinase family proteins, one for the WRKY transcription factor, and the other three for cysteine-rich receptor-kinase-like proteins. The three kinase proteins (i.e., TraesCS2B02G408700.1, TraesCS2B02G409700.1, and TraesCS2B02G411000.1) on Chinese Spring 2BL have three orthologs in *Ae. tauschii* 2DL and *T. urartu* 2AL, but only two orthologs on *T. turgidum* ssp. *dicoccoides* 2BL. The WRKY transcription factor was only annotated on Chinese Spring 2BL and *Ae. tauschii* 2DL, but not on *T. turgidum* ssp. *dicoccoides* 2BL or *T. urartu* 2AL. However, the three cysteine-rich receptor-kinase-like proteins on Chinese Spring 2BL detected one and two orthologs on *Ae. tauschii* 2DL and one *T. urartu* 2AL, and none on *T. turgidum* ssp. *dicoccoides* 2BL.

Validation of the *Pm52*-linked markers in the cultivars derived from Liangxing 99

The two *Pm52*-linked markers *Xicsl234* and *Xicscl817* were used to analyze three wheat cultivars that were derived from Liangxing 99 and have been phenotyped previously. Zhongxinmai 99 and DH51302 produced identical banding patterns to Liangxing 99, indicating that they may carry *Pm52*. Heng 10-5039 amplified different banding patterns with these markers from Liangxing 99, indicating the absence of *Pm52*. This result is consistent with the phenotyping tests with 27 *Bgt* isolates in a previous study (Zou et al. 2017), and demonstrates that markers *Xicsl234* and *Xicscl817* are able to detect *Pm52* in the progeny cultivars of Liangxing 99.

Discussion

The fine mapping of *Pm52* involved the use of SSR mapping, comparative genomics analysis, and wheat reference genome sequencing. It was difficult to identify a sufficient number of polymorphic genomic SSRs linked to the target gene from the available SSR primer pairs, so four EST-SSR markers *Xicsl30*, *Xicsl34*, *Xicsl54*, and *Xicsl62* were developed that were linked to *Pm52* based on the EST sequences on the wheat chromosome arm 2BL. Then, the sequences of those markers were used as queries to blast against barley genomic sequence to find the collinear genomic region on chromosome arm 2HL. An additional nine polymorphic SSR markers linked to *Pm52* were developed based on the results of comparative genomics analysis. *Pm52* was placed in a 3.2-cM genetic interval using the subpopulation, which corresponded to a 40-Mb genomic interval (556678090–596417733) on the Chinese Spring 2BL genomic sequences. Based on those sequences, nine gene-linked SSR markers were developed and a new high-resolution linkage genetic map was constructed using the 344 recombinants from the mapping population of Liangxing 99 × Zhongzuo 9504 cross containing 8820 F_2 plants identified by the *Pm52*-flanking markers *Xicsl163* and *Xicsl62* with genetic distance of 1.85 cM. In the newly developed genetic linkage map, *Pm52* was re-localized into a 0.21-cM genetic interval between markers *Xicssl326* and *Xicscl795* at genetic distances of 0.09 and 0.12 cM, respectively.

Results of the present study demonstrated that the comparative genomics approach, which takes advantage of the genomic synteny among wheat and other cereal crops, is effective for developing molecular markers for the fine mapping of disease resistance genes in wheat. However, the genomic sequences of gramineous crops such as barley, rice, sorghum, as well as *B. distachyon*, were apparently distinct from that of hexaploid wheat. The efficiency of marker development would not be high if homologous sequences shared between these species were used as template sequences. During the saturation of the genetic linkage map of *Pm52*, only 13 pairs of primers were polymorphic out of 348 tested primer pairs. The release of the diploid, tetraploid, and hexaploid wheat genome sequences, in comparison with the barley, rice, sorghum, and *B. distachyon* genome sequences, improved comparative genomics analysis and marker development. During the marker development, the genomic region of *Pm52* on 2BL was shown to have good synteny with *Ae. tauschii* chromosome arm 2DL and *T. turgidum* ssp. *dicoccoides* chromosome arm 2BL. So, they were useful for the further encryption of the genetic map. As *Pm52* originates from common wheat cultivar Liangxing 99, it should be relatively easy to narrow the interval around *Pm52* based on the improvements of the wheat reference genome sequences.

The *Pm52* genomic interval was thus reduced to 5.6-Mb interval of the Chinese Spring 2BL containing 36 predicted protein-coding genes. Seven of the annotated genes associated with plant disease resistance, including kinase family proteins, WRKY transcription factor, and cysteine-rich receptor-kinase-like proteins, were observed in this genomic interval (Table S8). Zuo et al. (2014) reported the map-based cloning of *qHSR1* from maize (*Zea mays* L.) and found its encoding product spanned the plasma membrane, potentially serving as a wall-associated kinase to perceive and transduce extracellular signals. The rice WRKY transcription factor gene *OsWRKY11* was reported to regulate the defense-associated genes (Lee et al. 2018). A group of the LecRK-VI.2-responsive cysteine-rich receptor-like kinase genes CRK4, CRK6, and CRK36 enhanced a pattern-triggered immunity response that was associated with resistance of tomato (*Solanum lycopersicum* L.) to a virulent bacterial

strain of *Pseudomonas syringae* pv. tomato DC3000 (Yeh et al. 2015). Previously, only five genes for wheat powdery mildew resistance have been isolated, i.e., *Pm3b* (Yahiaoui et al. 2004), *Pm8* (Hurni et al. 2013), *Pm2* (Sánchez-Martín et al. 2016), *Pm60* (Zou et al. 2018), and *Pm21* (He et al. 2018; Xing et al. 2018). All of them were the nucleotide-binding site and leucine-rich repeat (NBS-LRR) type of resistance genes. In addition to the NBS-LRR proteins, other genes with different mechanisms that function in resistant responses to diseases in plants have been characterized (Kourelis and van der Hoom 2018; Klymiuk et al. 2018).

Pm52 may use a different regulation mechanism from the NBS-LRR proteins for powdery mildew resistance. The final cloning of *Pm52* is required to ensure a full understanding of its underlying mechanism. Another possibility is that the target gene *Pm52* was not present in the genomic sequence of the Chinese Spring 2BL corresponding region. In that case, gene editing technology that clusters regularly interspaced short palindromic repeats-associated system (CRISPR-Cas) can be used to verify whether the gene exists in the final candidate interval (Li et al. 2013). Alternatively, construction and sequencing of a bacterial artificial chromosome (BAC) library and sequencing of the target gene region of BAC physical map contigs also can further predict the candidate genes. A new technology, MutChromSeq on the basis of flow sorting and sequencing of mutant chromosomes, has been used to isolate *Pm2* (Sánchez-Martín et al. 2016). All these technologies will facilitate the map-based cloning of *Pm52*.

The utilization of a resistance gene in breeding and agriculture depends on not only its effectiveness against the pathogen, but also the genetic background of its host cultivar. Some genes, such as those that reside in lines with poor agronomic performance, need a pre-breeding step to break the linkages between the target genes and the deleterious ones to optimize their genetic backgrounds by hybridization, repeated backcrossing, and marker-assisted selection (MAS). A time-consuming procedure is required to overcome the linkage drag before a resistance gene can be used by breeders in developing disease-resistant wheat cultivars (Summers and Brown 2013). Other sources of resistance, such as those that are identified in commercial wheat cultivars, are associated with desirable traits with promising agronomic performance and quality property. This circumvents the steps for improving the genetic background of a resistance gene.

A number of cultivars have been commercialized in different provinces, for example, Shi 4366 (Liangxing 99/Shiyou 17, 2015), Zhongxinmai 99 (Liangxing 99/222, 2016), Ji 738 (Gao 9618/Liangxing 99, 2016), Wanfeng 126 (Liangxing 99/Yannong 21, 2018), and Xitu 555 (Heng 4164/Han 6172//Liangxing 99, 2018) in Hebei province, Liangxing 68 (Liangxing 872/Liangxing 99, 2018) and Xinruimai 29 (Liangxing 99/Yan 5072, 2018) in Shandong province, and Wankenmai 1221 (Liangxing 99/Hui 0208, 2016) in Anhui province. Some of the cultivars developed using Liangxing 99 as a parent, i.e., Zhongxinmai 99, may carry *Pm52* as revealed by molecular marker detection and tests for disease resistance (Zou et al. 2017). However, cultivars derived from Liangxing 99 do not necessarily carry *Pm52*. Zou et al. (2017) reported that four of the ten Liangxing 99-derived cultivars may carry *Pm52*, but the other six cultivars may not carry this gene. The identification of molecular markers will facilitate the identification of *Pm52* in the Liangxing 99-derived cultivars.

Author contribution statement HJL, PW, and JL conceived and designed the study. PW and JH conducted the experiments. PW, JL, and ZL analyzed the data. JZ, DQ, YQ, YL, TL, HZ, LY, and HWL performed the phenotypic tests and other researches involved in this study. PW and HJL wrote the manuscript with contributions from YZ, ZL, and ZZ.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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