Mol Breeding (2015) 35:96
DOI 10.1007/s11032-015-0292-7

Genetic mapping of a putative *Agropyron cristatum*-derived powdery mildew resistance gene by a combination of bulked segregant analysis and single nucleotide polymorphism array

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Received: 24 November 2014 / Accepted: 2 March 2015 / Published online: 13 March 2015

Abstract The wheat relative *Agropyron cristatum* (L.) Gaertn. ($2n = 4x = 28$; genomes PPPP) has often been used as a donor of useful genetic variation for wheat improvement, including enhanced disease resistance to powdery mildew caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*). In this report, resistance to powdery mildew was transferred from *A. cristatum* to common wheat, and the resulting introgression line PB3558 exhibited all-stage resistance. To identify the resistance gene, genetic analysis was conducted using F2, F2:3 and recombinant inbreed line populations derived from the cross of PB3558 and the susceptible cultivar Jing 4841. Segregation ratios from inoculation with *Bgt* isolate E09 indicated that the resistance was conferred by a single dominant gene, temporarily designated *PmPB3558*. Bulked segregant analysis (BSA) was applied to screen for molecular markers linked to *PmPB3558*, and five published markers were found. In order to increase the density of the genetic map, we developed ten novel single sequence repeat markers based on the single nucleotide polymorphism (SNP) loci with polymorphisms produced from a combination wheat 90 k SNP array and BSA. *PmPB3558* was located on wheat chromosome arm 5DS and flanked by markers *Xcfd81* and *Xbwm25*. Because there are other powdery mildew resistance genes located on 5DS, 21 *Bgt* isolates were used to compare the reaction differences. *PmPB3558* showed unique reactions, suggesting that it was most likely a novel allele. This is the first documentation on transferring an alien powdery mildew resistance gene from *A. cristatum*, and the germplasm acquired in this study will be useful for broadening the genetic basis for wheat breeding.

Keywords *Agropyron cristatum* · Powdery mildew · Bulked segregant analysis · Single nucleotide polymorphism

Introduction

Wheat production is threatened by various pathogens, and powdery mildew caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*) is one of the most devastating diseases. Epidemics of powdery mildew cause severe wheat yield losses in many wheat-growing regions of the world, especially in regions with cool and moist climates (Everts and Leath 1992; Cowger et al. 2012). Although fungicide application can be employed to

Electronic supplementary material The online version of this article (doi:10.1007/s11032-015-0292-7) contains supplementary material, which is available to authorized users.

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reduce the damage from disease, this can cause environmental problems and the acquisition of fungicide tolerance by pathogens. Therefore, development of resistant cultivars is the most economical and environmentally friendly strategy to control wheat powdery mildew (Huang et al. 2000; Huang and Roder 2004).

At present, more than 60 formally designated powdery mildew resistance (*Pm*) genes or alleles at 45 loci (*Pm1–Pm50*, *Pm18* = *Pm1c*, *Pm22* = *Pm1e*, *Pm23* = *Pm4c*, *Pm31* = *Pm21*) have been documented in bread wheat and located on wheat chromosomes (McIntosh et al. 2008, 2011b; Mohler et al. 2013; Herrera-Foessel et al. 2014; Ben-David et al. 2010). These *Pm* genes have been mapped with different molecular markers, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), sequence tagged site (STS), simple sequence repeat (SSR), cleaved amplified polymorphic sequence (CAPS), sequenced characterized amplified region (SCAR) and single nucleotide polymorphism (SNP) markers. PCR-based DNA markers are more attractive for mapping genes, due to the small amount of template required and efficient handling of large population sizes. High-density wheat SSR maps have greatly facilitated the identification and mapping of *Pm* genes to specific chromosomes or chromosome regions in wheat (Roder et al. 1998; Somers et al. 2004; Song et al. 2005; Huang and Roder 2004; Landjeva et al. 2007). However, these SSR markers are sometimes insufficient for mapping genes in chromosomal regions with low gene density or poor recombination. In this case, novel SSR markers or markers of other types need to be developed. The SNP marker is one of the preferred choices because of its high variation and density in genomes (Akhunov et al. 2009; Paux et al. 2012; Allen et al. 2013). High-throughput SNP genotyping platforms are now available with wheat 9K SNP and 90K SNP chips (Wang et al. 2014a; Lai et al. 2012; Cavanagh et al. 2013; Berard et al. 2009; Colasuonno et al. 2014; Avni et al. 2014). SNP chips greatly facilitate the identification of SNPs closely linked to the particular trait, but it is not economic to genotype every individual in a population. To overcome this problem, in this report we developed a procedure to discover novel SSR markers by a combination of bulked segregant analysis (BSA) and SNP array.

Introgression of powdery mildew resistance genes from wild wheat relatives has been an active area of research. Six *Pm* genes (*Pm2*, *Pm10*, *Pm15*, *Pm19*, *Pm34* and *Pm35*) were identified from *Aegilops tauschii* ($2n = 2x = 14$, genome DD) (Qiu et al. 2006; Miranda et al. 2007; Tosa et al. 1987; Tosa and Sakai 1991; Lutz et al. 1995; Miranda et al. 2006). Four *Pm* genes (*Pm1b*, *Pm4d*, *Mlm2033* and *Mlm80*) were identified from *Triticum monococcum* ($2n = 2x = 14$, genome AA) (Yao et al. 2007; Hsam et al. 1998; Schmolke et al. 2012). *Triticum dicoccoides* ($2n = 4x = 28$, genomes AABB) was the source of several *Pm* genes including *Pm3K*, *Pm16*, *Pm26*, *Pm30*, *Pm31*, *Pm36*, *Pm41*, *Pm42*, *PmG16*, *PmG25* and *MlZec1* (Chen et al. 2005; Rong et al. 2000; Hua et al. 2009; Liu et al. 2002; Xie et al. 2004; Blanco et al. 2008; Wang et al. 2014b; Yahiaoui et al. 2006; Ben-David et al. 2010; Alam et al. 2013; Mohler et al. 2005), while *Secale cereale* L. ($2n = 2x = 14$, genome RR) was the donor of *Pm7*, *Pm8*, *Pm17* and *Pm20* (Hsam and Zeller 1997; Mohler et al. 2001; McIntosh et al. 2011a; Hsam et al. 1995; Zeller and Hsam 1996; Friebe et al. 1994). *Pm 21* was identified from *Haynaldia villosa* (2n = 2x = 14, genome VV), a species related to wheat that is highly resistant to powdery mildew (Chen et al. 1995, 2013; Xie et al. 2012). Some *Pm* genes have been successfully used in commercial production and have prevented significant economic losses in wheat production, such as *Pm2*, *Pm6*, *Pm8* and *Pm21* (Huang et al. 1997; Huang and Roder 2004; Xie et al. 2012). Unfortunately, some *Pm* genes, such as *Pm8*, have been rendered ineffective to powdery mildew within a short period of use due to variation of the pathogenic virulence (Hurni et al. 2013; Hsam and Zeller 2002; McDonald and Linde 2002). Therefore, finding new resistant genes and alleles, especially with resistance to a broad spectrum of pathogen races, becomes an urgent task to prevent wheat from attack by the disease and secure the world's food supply.

Agropyron cristatum (L.) Gaertn. ($2n = 4x = 28$; genomes PPPP), a perennial species of the Triticeae, has long been considered as a useful genetic resource for wheat genetic improvement. It harbors numerous genes beneficial to cultivated wheat, such as stress tolerance and resistance to numerous diseases including powdery mildew resistance (Dewey 1984; Dong et al. 1992; Han et al. 2014). PB3558 (*T. aestivum*, $2n = 6x = 42$, genomes AABBDD), which is a

derivative produced from cross between *A. cristatum* and common wheat Fukuhokomugi (Fukuho), displays high resistance to powdery mildew at both seedling and adult stages. However, the gene underlying powdery mildew resistance is unknown. In this report, we (1) explored the genetic basis of the powdery mildew resistance gene from PB3558, (2) developed novel SNP-based SSR markers for constructing the genetic map of the resistance gene, and (3) determined the chromosome location of the resistance gene and its relationship with previously reported *Pm* resistance genes.

Materials and methods

Materials

PB3558 is a homogenenous F7 line derived from the cross of *A. cristatum* (Accession No. Z559) and Fukuho. Wheat cultivar Jing 4841, highly susceptible to powdery mildew, was chosen as one parent to cross with PB3558, and wheat cultivar Zhongzuo 9504 was used as the susceptible control in the powdery mildew assessment. Both Jing 4841 and Zhongzuo 9504 were provided by Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. Chinese Spring (CS), CS nullisomic–tetrasomic lines (N5DT5A, N5DT5A, N5AT5B, N5AT5D, N5BT5A and N5BT5D), CS ditelosomic lines (Dt5DL and Dt5DS) and CS deletion lines (5DS-1, 5DS-2 and 5DS-5), kindly provided by the Wheat Genetic and Genomic Resources Center, Kansas State University, were used in chromosome assignment of the molecular markers associated with the powdery mildew resistance gene from PB3558.

Disease assessments at the seedling stage

Evaluation of seedling reactions to different *Bgt* isolates was carried out in a separate greenhouse that was not exposed to any other isolates. The *Bgt* isolate E09, avirulent to PB3558 and virulent to Jing 4841, was selected to inoculate the two parents and genetic populations for mapping the powdery mildew resistant gene from PB3558. Twenty seedlings of each line in the F2:3 population were tested against *Bgt* isolate E09 to determine the genotypes of the F2 individuals, and another 20 seedlings of each line from RIL-F8 (RIL, recombinant inbred line) population to determine the genotypes of RIL-F8 individual plants. Seedlings at the one-leaf stage were dusted with fresh conidiospores from susceptible cultivar Zhongzuo 9504. The plants were grown in a high humidity environment at 18–20 °C with a photoperiod of 12 h of light per day after inoculation. Infection types (ITs) were scored on the first leaf of each plant using a 0–4 scale at about 15 days after inoculation, when susceptible control Zhongzuo 9504 displayed severe symptoms. Plants were classified into two groups according to IT score: plants with IT 0–2 were considered resistant, while plants with IT 3–4 were considered susceptible (Liu et al. 2002; Chen et al. 2005). Twenty-one *Bgt* isolates collected from different parts of China were used to compare the reactions of PB3558 and other lines to determine whether the resistance gene in PB3558 was different from the known powdery mildew resistance genes on chromosome arm 5DS. Evaluation for 21 *Bgt* isolates was carried out using detached leaf segments as described by Limpert et al. (1988). Leaves from six individual plants of each genotype were inoculated with each isolate separately and the experiment was repeated three times.

Genomic in situ hybridization analysis

Genomic in situ hybridization (GISH) was carried out as previously described (Han et al. 2003). *A. cristatum* genomic DNA (labeled with Dig-Nick-Translation Mix) and Fukuho genomic DNA were used as probe and blocker, respectively. Wheat and *A. cristatum* chromosomes were pseudo-colored as blue and red, respectively. All cytological images were taken under a Nikon Eclipse E600 fluorescence microscope and captured with a CCD camera.

DNA extraction and bulk segregant analysis

Leaves of young seedlings were harvested to isolate genomic DNA following the CTAB method (Allen et al. 2006). BSA was performed to screen for polymorphic markers among PB3558, Jing 4841, the resistant DNA bulk and the susceptible DNA bulks, respectively (Michelmore et al. 1991). The resistant DNA bulk was generated by equal quantities of DNA from 50 homozygous highly resistant (IT = 0) plants, while the susceptible DNA bulk was generated by equal quantities of DNA from 50 homozygous highly

susceptible (IT = 4) plants. All these 100 homozygous plants were from the individual lines of the PB3558 × Jing 4841 RIL-F8 population. Two parents and two DNA bulks were used to select published wheat SSR markers and newly developed SSR markers. The published wheat SSRs were chosen approximately every 10 cM along the chromosomes according to the reported consensus map (Roder et al. 1998; Somers et al. 2004; Song et al. 2005; Gao et al. 2003). Novel SSR markers were developed according to SNP loci with polymorphisms between the resistant and susceptible bulks as described below. All polymorphic SSR markers obtained were used to genotype the individuals of the RIL-F8 population for mapping the powdery mildew resistance gene in PB3558. PCR was performed with the reaction mixture (10 μL) containing 40 ng of template DNA, 0.2 μM of the forward and reverse primers, 1 U of *Taq* polymerase, 0.5 mM dNTPs and 1 μL 10× buffer. The amplification was programmed at 94 °C for 5 min, followed by 36 cycles of 94 °C for 40 s, 52–60 °C for 40 s and 72 °C for 1 min. The reaction was terminated after an extension at 72 °C for 10 min. The resulting PCR products were separated on 8 % nondenaturing polyacrylamide gel, and the bands were visualized by silver staining.

Development of novel SSR markers

The wheat 90 k SNP array was used to genotype two parents and two DNA bulks in BSA following Illumina's Infinium assay protocol (www.illumina.com). SNP clustering and genotype calling were performed using Illumina's GenomeStudio Polyploid Clustering v1.0 software following the procedure described previously (Wang et al. 2014a). SNP markers were removed from the dataset if they were either monomorphic, showed more than 20 % missing values or ambiguous SNP calling, or had a minor allele frequency below 5 %. The flanking sequences of SNPs with polymorphism were used as queries to search *Ae. tauschii* D genome sequeces and scaffolds (Jia et al. 2013). New SSR markers were designed in the vicinities of the above-mentioned SNPs using the software SSR Finder and polymorphic SSR markers were selected to construct the high-density genetic map. SSR markers were assigned identifiers prefixed with *Xbwm* for "Beijing wheat microsatellite".

Statistical analysis and linkage map construction

Chi squared (χ^2) tests for goodness of fit were performed to determine the deviations of observed segregation ratios from theoretically expected ratios. Linkage between markers and the powdery mildew resistance gene in PB3558 were established with the software Mapmaker 3.0, with an LOD score threshold of 3.0 (Lincoln et al. 1993).

Results

The origin and inheritance of the powdery mildew resistance gene in PB3558

PB3558 was a homogeneous F7 line derived from the cross of *A. cristatum* and Fukuho. PB3558 and *A. cristatum* were highly resistant to powdery mildew at both seedling and adult stages, while Fukuho was susceptible to powdery mildew at all stages. These results suggested that resistance to powdery mildew in PB3558 was derived from *A. cristatum*. We then tried to detect *A. cristatum* chromosomal fragments in PB3558. The somatic cells of PB3558 were blocked with Fukuho genomic DNA and probed by *A. cristatum* genomic DNA following the standard GISH procedure. As shown in Fig. S1, 21 pairs of wheat chromosomes were all present but no visible translocation signals were detected in PB3558, suggesting that the translocated *A. cristatum* chromosomal fragments might be too small to be detected by GISH (Fig. 1).

To investigate the inheritance of the powdery mildew resistance, PB3558 was crossed to Jing 4841, a wheat cultivar highly susceptible to powdery mildew, to produce F2, F2:3 and RIL-F8 populations for genetic analysis of the powdery mildew resistance gene. When challenged with the popular *Bgt* isolate E09 in China, PB3558 and Jing 4841 displayed high resistance (IT = 0) and high susceptibility (IT = 4), respectively. Therefore, E09 was chosen to score infection types of the PB3558 × Jing 4841 populations. As shown in Table 1, 16 F1 plants produced from PB3558 × Jing 4841 cross exhibited similar reactions to the isolate E09 as the resistant parent PB3558 did. 280 F2 plants were studied, of which 214 resistant plants and 66 susceptible plants were observed. A Chi squared test indicated that these plants

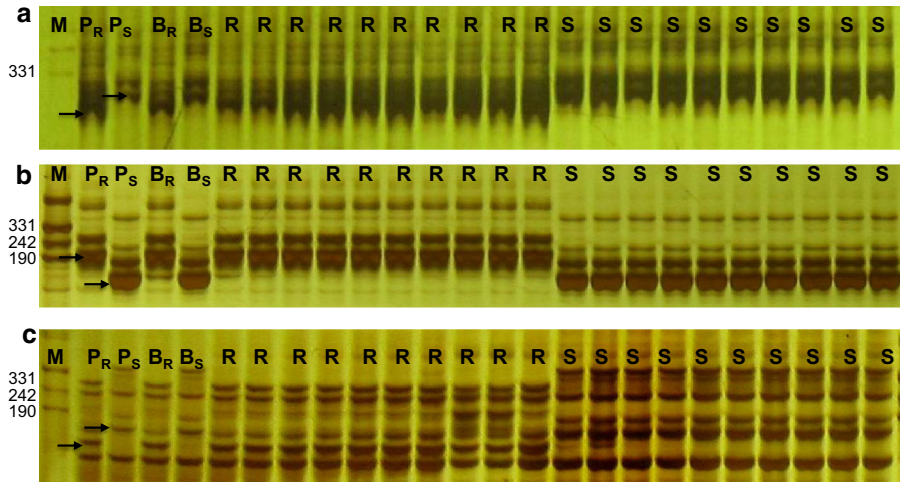

Fig. 1 Examples of amplification patterns of *PmPB3558*-linked polymorphic markers *Xcfd81* (**a**), *Xbwm25* (**b**) and *Xbwm21* (**c**) from two parents, two DNA bulks, and selected plants from PB3558 × Jing 4841 RIL-F8 population by 8 % silver-stained non-denaturing polyacrylamide gels. *M* DNA ladder, P_R resistant parent PB3558, P_S susceptible parent Jing 4841, B_R resistant DNA bulk, B_S susceptible bulk, *R* homozygous resistant RIL-F8 individual plant, *S* homozygous susceptible RIL-F8 individual plant. The *arrows* indicate DNA fragments' polymorphic bands

Table 1 Genetic analysis of powdery mildew resistance to the *Bgt* isolate E09 in PB3558 × Jing 4841 F1, F2, F2:3 and RIL-F8 populations

Parents and populations	No. of plants	Observed ratio			Expected ratio	χ^2	*P* value
		HR	HZ	HS			
PB3558	16	16		0			
Jing 4841	16	0		16			
PB3558 × Jing 4841 F1	16	16		0			
PB3558 × Jing 4841 F2	280	214		66	3:1	0.30	0.5809
PB3558 × Jing 4841 F2:3	254	65	126	61	1:2:1	0.13	0.9385
PB3558 × Jing 4841 RIL-F8	233	104	0	127	1:1	2.29	0.1302

HR homozygous resistant line, *HZ* heterozygous resistant line, *HS* homozygous susceptible line

segregated in a ratio of 3:1 ($\chi^2 = 0.30$, $P = 0.5809$). F2 plants were selfed to generate the F2:3 lines. From these 254 F2:3 lines generated, we found 65 homozygous resistant lines, 126 segregating lines and 61 homozygous susceptible lines, fitting to the ratio of 1:2:1 ($\chi^2 = 0.13$, $P = 0.9385$). PB3558 × Jing 4841 F2:3 lines were further used to establish a PB3558 × Jing 4841 RIL-F8 population by single seed descent, from which 104 resistant lines and 127 susceptible lines were observed. All progenies of both resistant and susceptible lines showed no segregation for powdery mildew resistance in the next generation, indicating that they were all homozygous lines. The ratio between 104 homozygous resistant lines and 127 homozygous susceptible lines fitted to the ratio of 1:1 ($\chi^2 = 2.29$, $P = 0.1302$). Taken together, we concluded that a single dominant gene conferred the powdery mildew resistance in PB3558, and it was tentatively designated as *PmPB3558*.

Molecular mapping of the powdery mildew resistance gene *PmPB3558*

Among 954 published wheat SSR markers distributed throughout all the wheat chromosomes, 374 markers (39.2 %) were found to display polymorphism

between PB3558 and Jing 4841. These markers were then used to perform BSA, and four markers (*Xgwm205*, *Xcfd78*, *Xcfd81* and *Xgpw302*) on chromosome arm 5DS were polymorphic between the contrasting DNA bulks, indicating linkage with the powdery mildew resistance gene *PmPB3558*. We also found one SCAR marker *Xscar112* linked to *PmPB3558* on chromosome arm 5DS (Fig. 2). Since these markers were not adequate for mapping *PmPB3558*, we set out to develop more novel SSR markers by a combination of BSA and SNP array. When two parents and two DNA bulks were genotyped with the wheat 90 k SNP chip, 10,306 SNP loci showed polymorphisms between two parents, of which 131 SNP markers showed polymorphisms between the two DNA bulks. By searching the previous report, 28 SNP markers were found on wheat chromosome arm 5DS, much more than on any other chromosome arm (Wang et al. 2014a) (Table S1). Therefore, SNP markers on wheat chromsome arm 5DS were mostly likely linked with *PmPB3558*, and this was consistent with the results acquired using SSR and SCAR markers. The flanking sequences of the 28 SNP markers were then used as queries to search the D genome sequeces from *Ae. tauschii.* Scaffolds with highest similarities were acquired, and the corresponding information is shown in Table S1. Twenty-five new SSR markers were designed in the vicinities of the SNP markers (Table S2). Of them, ten SSR markers (*Xbwm16*, *Xbwm8*, *Xbwm9*, *Xbwm11 Xbwm3*, *Xbwm2*, *Xbwm14 Xbwm25*, *Xbwm21* and *Xbwm20*) were polymorphic between the two DNA bulks and were also found to associate with *PmPB3558* (Table S2 and Fig. 2). Based on the linkage analysis, a linkage map spanning chromosome arm 5DS (64.7 cM in length) was constructed, and *PmPB3558* was flanked by markers *Xcfd81* and *Xbwm25* at genetic distances of 5.5 and 3.9 cM, respectively (Fig. 2).

Chromosomal localization of *PmPB3558*

Four SSR markers *(Xgwm205*, *Xcfd78*, *Xcfd81* and *Xgpw302*) and one SCAR marker *Xscar112* linked to *PmPB3558* were previously found on chromosome arm 5DS. Additionally, ten newly developed SSR markers were also found on chromosome arm 5DS, and were verified using the CS, CS nullisomic–tetrasomic lines (N5DT5A, N5DT5A, N5AT5B, N5AT5D, N5BT5A and N5BT5D), CS ditelosomic lines (Dt5DL and Dt5DS) and CS deletion lines (5DS-1, 5DS-2 and 5DS-5). Amplification patterns of three SSR markers (*Xcfd81*, *Xbwm25* and *Xbwm21*) were shown in Fig. 3 as examples. These results further indicated that all these markers closely linked to *PmPB3558* were located on chromosome arm 5DS, more precisely on the deletion bin C-5DS1-0-0.63.

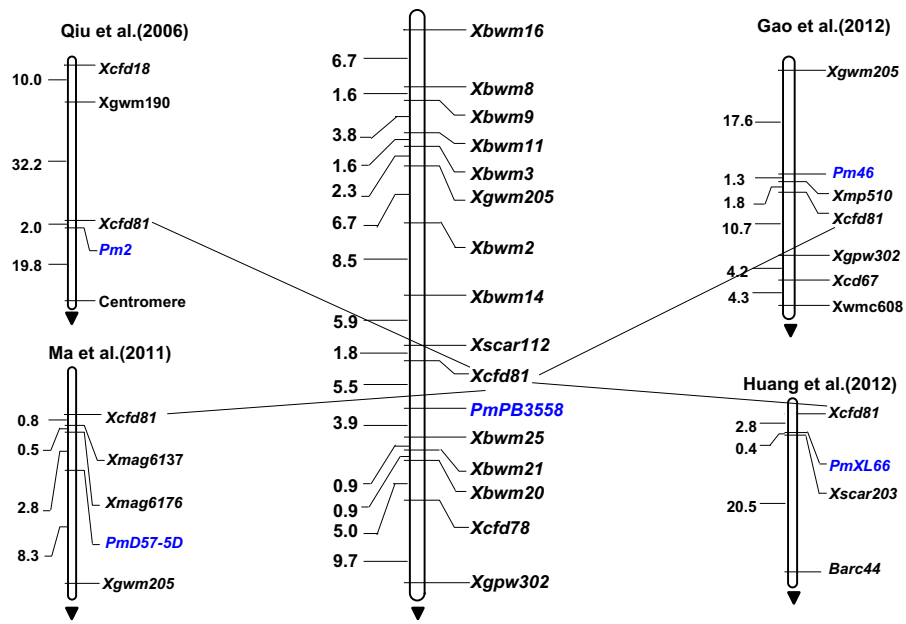

Fig. 2 Linkage map of *PmPB3558* and comparison with the known *Pm* genes on wheat chromosome arm 5DS. Genetic distances are shown to the *left* in cM. *Black arrow* points to the centromere

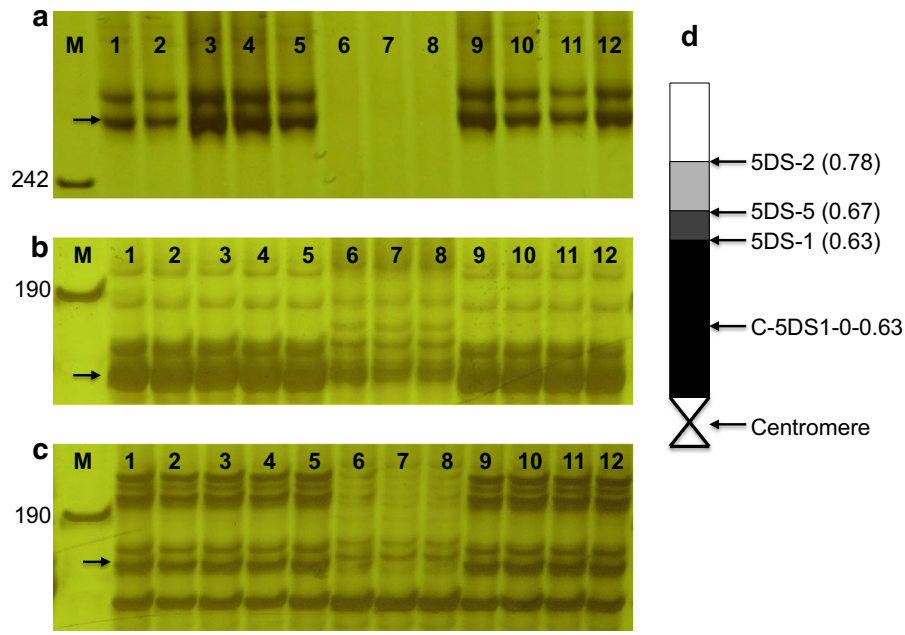

Fig. 3 Amplification patterns of SSR markers *Xcfd81* (**a**), *Xbwm25* (**b**) and *Xbwm21* (**c**) in Chinese Spring (CS), CS nullisomic–tetrasomic lines (N5DT5A, N5DT5A, N5AT5B, N5AT5D, N5BT5A and N5BT5D), CS ditelosomic lines (Dt5DL and Dt5DS) and CS deletion lines (5DS-1, 5DS-2 and 5DS-5). The deletion map of 5DS is shown in (**d**). *M* DNA ladder, *1* CS, *2* N5AT5B, *3* N5AT5D, *4* N5BT5A, *5* N5BT5D, *6* N5DT5A, *7* N5DT5B, *8* DT5DL, *9* DT5DS, *10* 5DS-1, *11* 5DS-2, *12* 5DS-5

Comparative reactions to 21 *Bgt* isolates between PB3558 and other lines with known powdery mildew resistance genes on wheat chromosome arm 5DS

According to the previous reports, there were several *Pm* genes located on wheat chromosome arm 5DS, such as *Pm2*, *Pm46* and *PmLX66*. In order to distinguish the disease reaction differences between them, 21 *Bgt* isolates were used. PB3558 (*PmPB3558*), Ulka/8*Cc (*Pm2*), Tabacco (*Pm46*), Liangxing 66 (*PmLX66*) and Jing 4841 as well as the susceptible control Zhongzuo 9504 were inoculated with 21 *Bgt* isolates at the one-leaf stage. These isolates showed different virulence patterns. PB3558 was resistant to 17 of the 21 *Bgt* isolates tested, and the reaction patterns of *PmPB3558* were different from *Pm2*, *Pm46* or *PmLX66*. *PmPB3558* differed from *Pm2* in its reactions to four *Bgt* isolates (E18, E20, E21 and Bg79-1), from *Pm46* in its reactions to six *Bgt* isolates (E11, E18, E20, Bg44-4, Bg79-1 and Bg84-3), and from *PmLX66* in its reactions to six *Bgt* isolates (E18, E22, Bg44-4, Bg77-2, Bg79-1 and Bg86-2). *A. cristatum* was found resistant to all 21 *Bgt* isolates tested, and thus it displayed a broader spectrum of disease resistance than PB3558 (Table S3). The disease reactions of PB3558, Ulka/8*Cc, Tabacco, Liangxing 66, Jing 4841 and Zhongzuo 9504 to six different *Bgt* isolates are shown in Fig. 4. Therefore, the resistance spectrum of PB3558 is different from all the wheat cultivars tested above.

Discussion

The origin of *PmPB3558*

The discovery of novel powdery mildew resistance genes is the most effective method of controlling powdery mildew in wheat, and alien chromosomal translocation is a classic method of transferring genes from wild relatives to common wheat. *A. cristatum* is an important perennial Triticeae species and a valuable source of resistance to powdery mildew (Dewey 1984; Dong et al. 1992; Han et al. 2014). In this report, the introgression line PB3558, highly resistant to powdery mildew, was obtained. The resistance gene *PmPB3558* came from *A. cristatum*, since the only common wheat parent of PB3558, Fukuho, was highly susceptible to powdery mildew. One obstacle to the application of alien translocations in practical breeding is that the large transferred chromosome segments often carry additional genes conferring undesirable traits or do not adequately compensate for the wheat genes they replace in non-homoeologous regions, resulting in 'linkage drag' (Friebe et al. 1996). In this sense, the smaller the translocation chromosome fragment, the

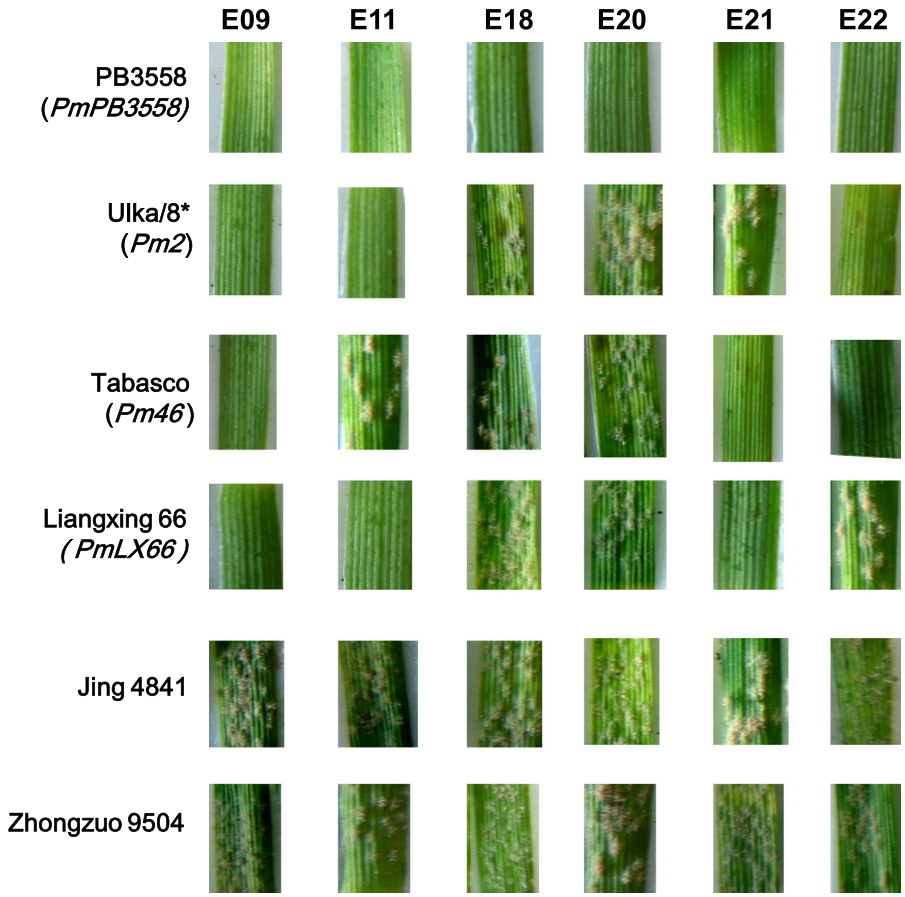

Fig. 4 Reactions of PB3558 (*PmPB3558*), Ulka/8*Cc (*Pm2*), Tabacco (*Pm46*), Liangxing 66 (*PmLX66*), Jing 4841 and Zhongzuo 9504 to six different *Bgt* isolates

better the material is in breeding. Indeed, some translocation lines with desirable traits have been occasionally transferred to recipient genotypes by cryptic translocation without detectable cytological or genetic changes (Kuraparthy et al. 2007). A GISH signal in PB3558 was not detected using *A. cristatum* genomic DNA as a probe, suggesting that PB3558 does not possess a large alien chromosomal segment and may instead contain a cryptic translocation. Alien chromosomal segments resulting from such small translocations cannot be easily detected by standard cytogenetic methods other than high-resolution GISH.

Comparison between *PmPB3558* and other *Pm* genes on wheat chromosome arm 5DS

PmPB3558 was assigned to wheat chromosome arm 5DS, flanked by the SSR marker *Xcfd81* and *Xbwm25* at genetic distances of 5.5 cM and 3.9 cM, respectively. To date, six *Pm* genes have been reported on chromosome 5D: *Pm34*, *Pm35*, *Pm2*, *PmD57-5D*, *PmLX66* and *Pm46* (Miranda et al. 2006, 2007; Qiu et al. 2006; Ma et al. 2011; Gao et al. 2012). *Pm34*, *Pm35* and *Pm2* were originally from *Ae. tauschii*, *PmD57-5D* from the common wheat line D57, *Pm46* from German wheat cultivar Tabasco, and *PmLX66* from Chinese wheat cultivar Liangxing 66. *Pm34* and *Pm35* were mapped on wheat chromosome arm 5DL, while the other four mapped on wheat chromosome 5DS. *Pm34* and *Pm35* were located on the distal part of 5DL, 2.6 cM away from *Xbarc144*, and on the proximal end of 5DL, 11.9 cM away from *Xcfd26*, respectively (Miranda et al. 2006, 2007). *Xbarc144* and *Xcfd26* were not linked to *PmPB3558*, suggesting that *PmPB3558* was different from *Pm34* and *Pm35*. *Pm2* was firstly physically mapped on wheat chromosome arm 5DS and flanked by marker *Xcfd81* at a genetic distance of 2.0 cM (Qiu et al. 2006), and

PmD57-5D was considered to be most likely *Pm2* (Ma et al. 2011). In this study, *PmPB3558* was flanked by marker *Xcfd81* at the genetic distance of 5.5 cM, so *PmPB3558* was 3.5 cM away from *Pm2* (Fig. 2). To distinguish between *PmPB3558* and *Pm2*, 21 *Bgt* isolates were used to test the reactions; four *Bgt* isolates exhibited different reaction patterns (Table S3). Combining the results of molecular markers and disease tests, *PmPB3558* is most likely a new allele at the *Pm2* locus. However, an allelism test is required to further examine the allelic relationship between *PmPB3558* and *Pm2*. As shown in Fig. 2, *Pm46* is distal to *Xcfd81* at a genetic distance of 3.1 cM, and *PmLX66* is proximal to *Xcfd81* at a genetic distance of 2.8 cM (Gao et al. 2012; Huang et al. 2012). Therefore, *PmPB3558* was 8.6 and 2.7 cM away from *Pm46 and PmLX66*, respectively. In addition, compared with *Pm46* and *PmLX66*, *PmPB3558* showed different reactions with six of the 21 *Bgt* isolates, accounting for 28.5 % of the total isolates tested (Table S3). Moreover, *PmPB3558* was the only genotype immune to isolates E18 and Bg79-1. Therefore, these results indicated that *PmPB3558* was different from *Pm46* and *PmLX66*, which will be further verified by the allelism tests.

As shown in the previous reports, many powdery resistance genes have multiple alleles, due to the cluster feature of resistance genes. These powdery mildew resistance genes are non-randomly distributed in the genome, but form clusters in gene-rich regions. In addition to the five allelic genes for *Pm1* (1a–1e), *PmG16*, *MlIM72*, *Mlm2033*, *Mlm80*, *MIAG12* and *HSM1* are also likely allelic to this locus (Hsam et al. 1998; Zhang et al. 2012; Singrun et al. 2003; Yao et al. 2007; Ben-David et al. 2010; Ji et al. 2008; Maxwell et al. 2009; Li et al. 2014). *Pm3* is the best characterized wheat powdery mildew resistance gene locus, at which 15 resistance alleles have been identified (Yahiaoui et al. 2006; Hsam et al. 2015; McIntosh et al. 2011a; Bhullar et al. 2009; Srichumpa et al. 2005; Huang et al. 2004; Hartl et al. 1993; Yahiaoui et al. 2009). Four allele have been reported for *Pm4* (4a–4d) (Schmolke et al. 2012), and *PmPS5A* was also a member of the *Pm4* complex (Zhu et al. 2005). The presence of *Pm* gene clusters often confers quantitative and durable disease resistance when combined together by marker-assisted selection (MAS) (Paillard et al. 2000; Gupta et al. 2010). A number of commercially grown cultivars have been found to have *Pm* gene combinations, such as Normandie with *Pm1*, *Pm2* and *Pm9* (Schneider et al. 1991) and Kronjuvel with *Pm4b* and *Pm8* (Liu et al. 2000). Besides powdery mildew resistance genes, the same is true for leaf rust (Nocente et al. 2007), stem rust (Mago et al. 2011) and so on. In this study, *PmPB3558* may be located in resistance gene-rich regions and is potentially applicable in gene pyramiding.

Novel SSR markers increase the density of the genetic map of *PmPB3558*

Various populations are available for constructing genetic maps and mapping genes, such as F2, RIL, near-isogenic (NIL) and doubled haploid (DH) populations. Of them, the F2 population is the easiest to construct and most widely used. However, evaluation of traits of F2 individuals is sometimes not accurate. Therefore, alternative strategies can be used to improve the efficiency of genetic mapping, such as RILs, NILs or DHs, which are permanent populations that enable replicated phenotyping across different environments (Michelmore et al. 1991; Peng et al. 2014). In this study, we used the PB3558 × Jing 4841 RIL-F8 population instead of the F2 population to improve the accuracy of phenotyping the powdery mildew resistance at the seedling stage. It should be noted that PB3558 also shows adult plant resistance (APR) to powdery mildew in the field. Further investigation is needed as to whether *PmPB3558* also contributes to the observed APR. All reported *Pm* genes on wheat chromosome arm 5DS were mapped only with limited published SSR markers, which was due to the relative low level of DNA polymorphism and low recombination frequency on chromosome arm 5DS. In our efforts to tag *PmPB3558*, we developed novel SSR markers based on flanking sequences of polymorphic SNP markers. Three new SSR markers (*Xbwm25*, *Xbwm21* and *Xbwm20*) located between *Xcfd81* and *Xcfd78* were found more closely linked to disease resistance gene *PmPB3558*. Therefore, this study provides better marker coverage of the *Pm* gene on wheat chromosome arm 5DS than in the previous studies. In this study, PB3558 is an ideal germplasm for resistance to powdery mildew and the identified molecular markers closely linked to *PmPB3558* can simplify wheat breeding programs such as cultivar

development and pyramiding of additional resistance genes.

Acknowledgments This work was funded by the National Natural Science Foundation of China (No. 31271714) and the National Basic Research Program of China (the 973 program, No. 2011CB100104). We would like to thank Dr. Hongjie Li at the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences for help with the assessment of *Bgt* isolates.

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