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ORIGINAL ARTICLE

Fine mapping of wheat powdery mildew resistance gene *Pm6* using 2B/2G homoeologous recombinants induced by the *ph1b* mutant

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Abstract

Key message **Using the *ph1b* mutant, the recombination frequency between the homoeologous region of 2B and 2G was significantly increased. By this, we narrowed *Pm6* to a 0.9 Mb physical region.**

Abstract The powdery mildew (*Pm*) resistance gene *Pm6* from *Triticum timopheevii* (2*n* = 48, AAGG) was mapped to the long arm of chromosome 2G and introduced into common wheat in the form of 2B–2G introgressions. The introgression line IGV1-465 has the shortest 2G segment, which is estimated 37 Mb in size when referring to 2BL genome reference of Chinese Spring (CS). The further fine mapping of *Pm6* was impeded by the inhibition of allogeneic chromosome recombination between 2B and 2G in the *Pm6* region. In the present study, to overcome 2B/2G recombination suppression, a *ph1b*-based strategy was employed to produce introgressions with reduced 2G fragments for the fine mapping of *Pm6*. IGV1-465 was crossed and backcrossed to the CS*ph1b* mutant to produce plants with increased 2B/2G chromosome pairing frequency at the *Pm6* region. A total of 182 allogeneic recombinants were obtained through two-round screening, i.e., first round of screening of 820 $BC_1F_{2:3}$ progenies using the flanking markers *CIT02g-14*/*CIT02g-19* and second round of screening of 642 $BC_1F_{2:4}$ progenies using the flanking markers *CIT02g-13*/*CIT02g-18*, respectively. Through marker analysis using 30 chromosome 2G-specific markers located in the *Pm6* region, the identified recombinants were divided into 14 haplotypes. *Pm* resistance evaluation of these haplotypes enabled us to narrow *Pm6* to a 0.9 Mb physical region of 2BL, flanked by markers *CIT02g-20* and *CIT02g-18*. Six wheat varieties containing *Pm6* were identified from a natural population, and they showed increased *Pm* resistance. This implied *Pm6* is still effective, especially when used in combination with other *Pm* resistance genes.

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Wentao Wan and Jin Xiao have contributed equally to this work.

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Introduction

During the evolution of common wheat, the presence and balance of the *Ph* (*Pairing homoeologous*) system have ensured homologous chromosome pairing and recombination, and the disruption of this balance leads to increased homoeologous chromosome recombination (Riley and Chapman 1958). Among the *Ph* genes, *Ph1b* from hexaploid wheat (Sears 1977) and *Ph1c* from tetraploid wheat (Giorgi 1978) on chromosome arm 5BL exhibit the most dominant effects. The recombination frequency between homoeologous chromosomes can be improved by mutation or deletion of the *Ph1b* gene. By introducing the Chinese Spring *ph1b* mutant (CS *ph1b*) into specific background containing a single alien chromosome or whole-arm alien translocation, compensating wheat–alien translocations or introgressions have been developed by inducing homoeologous recombination between wheat and alien chromosomes (Qi et al. 2007). Employing CS *ph1b*, Zhao et al. (2013) generated

translocations of *Haynaldia villosa* 4VS with reduced fragment sizes and mapped the wheat yellow mosaic virus resistance locus to the terminal region. Through a similar approach, translocations or recombinants between wheat and *Thinopyrum*, *Lophopyrum elongatum*, *Leymus* and barley were developed by Xin et al. (2001), Qi et al. (2008), Mullan et al. (2009) and Rey et al.(2015), respectively.

Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), is a worldwide disease that occurs in major wheat-producing countries including China (Li et al. 2011). The disease is favored by intensive cultivation methods associated with modern agriculture such as the growing of semi-dwarf and high-yielding cultivars under conditions with high levels of nitrogen fertilization and irrigation (Bennett 1984). This biotrophic fungus appears on susceptible varieties from seedling to mature stages and causes a significant reduction in grain yield as well as kernel quality in severe epidemic regions. Compared with the application of fungicides, breeding for resistant varieties is widely accepted as the most economical and environmentally safe approach for disease control. To date, 82 formally designated powdery mildew (*Pm*) resistance genes associated with 59 loci have been reported (Li et al. 2019; McIntosh et al. 2013; Sun et al. 2018; Tan et al. 2018a, b; Zhang et al. 2018, 2019; Zou et al. 2018), several of which have been extensively used in breeding, such as *Pm8* (Purnhauser et al. 2011).

The cloning of *Pm* genes will be helpful for improving *Pm* resistance via a transgenic approach as well as the understanding of resistance mechanisms at the molecular level. To date, eight *Pm* genes have been cloned, among which six were derived from common wheat or its donor species, such as *Pm2* from *Aegilops squarrosa* (Sanchez-Martin et al. 2016), *Pm60* from *Triticum urartu* (Zou et al. 2018) and four genes (*Pm3*, *Pm38*, *Pm39* and *Pm46*) from hexaploid wheat (Herrera-Foessel et al. 2014; Lagudah et al. 2009; Lillemo et al. 2008; Yahiaoui et al. 2004), and the other two are from wild relatives of wheat, i.e., *Pm8* from rye (*Secale cereal*) (Hurni et al. 2013) and *Pm21* from *H. villosa* (Xing et al. 2018). *Pm3*, *Pm38*, *Pm39*, *Pm46* and *Pm60* were cloned via conventional map-based cloning. *Pm8* was isolated by homology-based cloning (Hurni et al. 2013). *Pm2* and *Pm21* were cloned through a mutant chromosome sequencing approach (Sanchez-Martin et al. 2016; Xing et al. 2018).

The tetraploid *T. timopheevii* ($2n = 4x = 28$, genome AAGG) is included in the second gene pool of wheat. Several *Pm* resistance genes have been transferred from *T. timopheevii* to hexaploid wheat, including *Pm6* (Jorgensen and Jensen 1973), *Pm27* (Peusha et al. 2000), *Pm37* (Perugini et al. 2008) and *MlAG12* (Maxwell et al. 2009). *Pm6* on the long arm of chromosome 2G has been widely used in wheat breeding for *Pm* resistance via the development of wheat–*T. timopheevii* 2B/2G introgression lines. Compared with other *Pm* resistance genes, the resistance conferred by *Pm6* exhibits a distinctly developmental stage-dependent manner, with moderate effectiveness at the seedling stage and high resistance from the fourth leaf stage onward (Bennett 1984). Although virulent isolates overcoming *Pm6* resistance have occurred, *Pm6* is still effective, especially when used in combination with *Pm2* (Costamilan and Trigo 2005; Purnhauser et al. 2011; Švec and Miklovičová 1998; Vechet 2006). The fine mapping and further cloning of *Pm6* will facilitate not only the better utilization of *Pm6* in wheat breeding, but also a better understanding of the molecular mechanism of developmental stage-dependent disease resistance in plants.

In a previous study, using RFLP and/or STS, along with SSR molecular markers, *Pm6* was mapped within a 0.8 cM genetic distance (Ji et al. 2006; Tao et al. 2000). With the help of comparative genomics analysis, Qin et al. (2011) allocated *Pm6* to a 614 Mb–753 Mb physical region of wheat chromosome 2B. Two *TaRLKs* (*TaRLK1* and *TaRLK2*) located in this region were cloned and proved to positively regulate wheat *Pm* resistance (Chen et al. 2016). However, we later excluded *TaRLKs* as candidates for *Pm6* because when we further narrowed the *Pm6* region, we found that the *TaRLKs* (~ 629 Mb region) were not included in the introgressed 2G fragment in the IGV1-465 line, which exhibits the shortest 2G fragment introgression. It is suspected that recombination between the introgressed 2G fragment and its corresponding wheat chromosome 2B may be repressed. Thus, increasing the 2B/2G recombination frequency is critical for the fine mapping and final cloning of *Pm6*.

To approach the *Pm6* region for target gene cloning, we constructed a population with an increased homoeologous chromosome recombination frequency via the introduction of the *ph1b* mutant gene. We succeed in narrowing the *Pm6* region from 37 to 0.9 Mb, which will greatly facilitate the discovery of the candidate gene for *Pm6*.

Materials and methods

Plant materials

Prins (AABBDD) is a Swedish Spring wheat variety that is susceptible to wheat powdery mildew. *T. timopheevii* (AAGG) is the tetraploid donor of *Pm6*. IGVI-438, IGVI-458, IGVI-463, IGVI-464, IGVI-465 and IGVI-466 are six *T. aestivum* (*c.v.* Prins)–*T. timopheevii* introgression lines with different 2G introgressed segments, all of which carry the *Pm6* gene. The above materials were kindly provided by Dr. J. MacKey, Swedish Agricultural University, Uppsala, Sweden.

Common wheat variety Chinese Spring nulli-tetrasomic lines N2AT2D, N2BT2D and N2DT2B were used for the validation of genome-specific markers. The Chinese Spring

ph1b mutant (CS *ph1b*) is a deletion line of 5BL, in which *Ph1b* is absent and can be used to induce meiotic recombination between the 2G introgression fragment and its counterpart 2B in common wheat. The above germplasms were kindly provided by the Wheat Genetics Resource Centre at Kansas State University, Manhattan, USA.

A natural population consisting of a panel of 387 wheat varieties was kindly provided by Professor Jizeng Jia, Chinese Academy of Agricultural Sciences, Beijing, China.

Mapping population and recombinant screening

To fine map *Pm6*, a strategy is proposed to improve the 2B/2G allogeneic chromosome recombination frequency in the *Pm6* region by employing the *Ph1* pairing control system. The workflow is delineated in Fig. 1. The introgression line IGV1-465, containing the shortest introgressed 2G segments, was crossed with CS *ph1b*, followed by backcrossing the F_1 individuals to CS *ph1b*. The derived BC_1F_1 progenies were screened using the 2G-specific codominant marker *CINAU141* and the *Ph1b*-specific marker *ABC302.3*. The BC_1F_1 plants that were heterozygous for *Pm6* and homozygous for *ph1b* were self-crossed to produce BC_1F_2. Through analysis using the *Pm6* flanking markers, the BC_1F_2 plants were divided into two groups: recombinant and nonrecombinant. The identified recombinants were phenotyped for *Pm* resistance and for the fine mapping of *Pm6*. Those nonrecombinants that were heterozygous for *Pm6* and homozygous for *ph1b* (residual heterozygous lines, RHL) were further self-pollinated to generate $BC_1F_{2:3}$ progenies (equivalent to BC_1F_2) for the second round of screening of new 2B/2G recombinants, and so forth for the following generations. The identified new recombinants were phenotyped for *Pm* resistance, and to further narrow the genetic region of *Pm6*. Recombinant frequency (%) = (no. of recombinants)/(no. of plants identified) × 100.

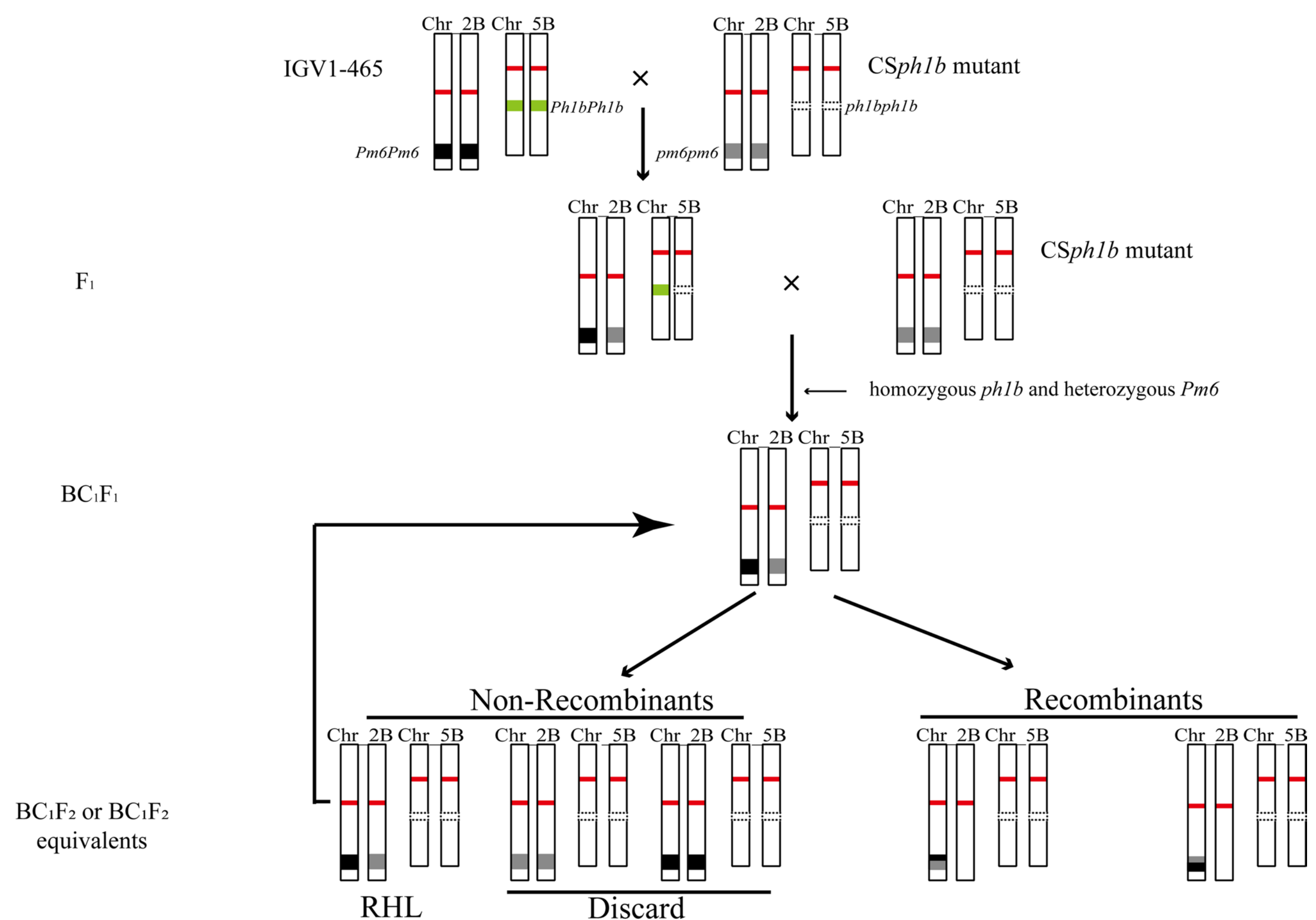


Fig. 1 Workflow for the identification of individuals heterozygous for *Pm6/pm6* and lacking *Ph1b* for the subsequent screening of recombinants between 2B and 2G chromatin in the *Pm6* region in their offspring. The black boxes in the chromosomes represent the *Pm6* region, and the gray boxes in the chromosomes represent the *pm6* region. The green boxes represent the *Ph1b* region, and the dotted boxes represent the *Ph1b* deletion. The red boxes represent the centromere. RHL: residual heterozygous lines (color figure online)

Evaluation of powdery mildew resistance

All the tested parents and recombinants were grown under controlled greenhouse conditions, and their *Pm* resistance was evaluated in vitro at the fifth leaf stage thereafter. The detached leaf segments of the samples were maintained on culture medium (0.5% agar and 20 mg/l 6-BA) in a Petri dish, inoculated with *Bgt* isolate E26 for 5–6 days in a pathogen-free environment. At 7 days postinoculation, when the susceptible variety CS *ph1b* became severely infected, photographs were taken. The plants were rated as resistant (R) or susceptible (S), with the former showing either no visible symptoms or necrotic flecks, while the latter exhibited no necrosis and showed high to full sporulation.

For the natural population, we determined *Pm* resistance using *Bgt* isolate E26 and the *Bgt* mixture. We evaluated *Pm* resistance in vivo. The inocula were increased on susceptible plants under spore-proof greenhouse conditions prior to setting up the disease evaluation experiment. Inoculation was accomplished by gently shaking conidia from the leaves of infected plants onto the foliage.

Bgt isolate E26 was kindly provided by Dr. Yilin Zhou (Institute of Plant Protection, Chinese Academy of Agricultural Sciences). The *Bgt* mixture was obtained from a native population of *Bgt* spores collected from Nanjing, Jiangsu Province, China. The evaluation of powdery mildew resistance was performed three times.

Marker development and marker analysis

Intron targeting (IT) markers were developed following the procedure of Wang et al. (2017) with some modifications. We extracted the coding sequences of genes located within the *Pm6* region of Ta_chr2B and performed a BLASTn search against the genomic reference of Chinese Spring and the survey sequence of flow-sorted 2G (data not shown). All genes matching the 2AL, 2BL, 2DL and 2G assemblies and possessing at least one predicted exon–exon junction were selected. The intron sizes of the corresponding genes were then calculated and compared against each other. Those homologous genes whose intron sizes were at least 10% different in 2G from those in 2AL, 2BL and 2DL were chosen to design primers for IT markers. The primers were designed in the exons flanking the targeted introns using the online software Primer 3 (version 4.1.0, http://frodo.wi.mit.edu/primer3/) according to the 2G sequences, assuming that exon regions and exon–intron structures of orthologous genes are highly conserved among the four genomes. The designed primers were expected to anneal the genomic DNA of 2AL, 2BL, 2DL and 2GL sequences but to amplify different fragment sizes from different genomes.

To increase the marker density, simple sequence repeat (SSR) and single-nucleotide polymorphism (SNP) markers were developed. The corresponding genomic sequence of the *Pm6* region of Ta_2B was extracted and SSR primer pairs were designed using the online software WebSat (http://wsmartins.net/websat/). For SNP marker development, IGV1-465 and CS*ph1b* were genotyped with a Wheat55K array (Affymetrix® Axiom® Wheat55). SNPs located in the *Pm6* region were employed for SNP primer design using the online software BatchPrimer3 v1.0 (https://wheat.pw.usda.gov/demos/BatchPrimer3/). The criteria for primer design were as follows: melting temperature of 55 °C and primer length of 18–25 bp (optimum: 20). All primers were synthesized by the TSINGTE Company (Nanjing, China).

Genome sequences

The reference genome sequences of Chinese Spring, *T. dicoccoides*, *T. urartu*, *Ae. tauschii* and *Hordeum vulgare* were downloaded from the EnsemblPlants Web site (http://plants.ensembl.org/index.html) and used for marker development and microcolinearity analysis.

Results

Enrichment of the marker density targeting the *Pm6* region

In a previous study, Ji et al. (2006) and Qin et al. (2011) mapped a total of 29 molecular markers to the *Pm6* locus. Referring to the reference genome of Chinese Spring (IWGSC 2018), these markers were assigned to wheat chromosome 2B. Seven of them were located in the introgressed 2G fragment in IGV1-465 ($2G_{465}$), flanking a physical distance of 28 Mb from 695 to 723 Mb (Fig. S1). Microcolinearity analysis indicated that the *Pm6* region in 2G was highly conserved with the corresponding region of other *Triticum* species, including 2A from CS, *T. dicoccoides* and *T. urartu*, 2B from CS and *T. dicoccoides*, 2D from CS and *Ae. tauschii* and 2H from *H. vulgare* (Fig. S2).

To enrich marker density in the *Pm6* region, three types of markers were developed, including IT, SNP and SSR markers. For primer design, we enlarged the physical distance of the *Pm6* region from 28 to 48 Mb including an additional 10 Mb of neighbor sequences in both directions. In total, 50 IT, 100 SSR and four SNP primer pairs were designed. Specific markers for the *Pm6* region of 2G were screened by amplification using Prins (AABBDD), *T. timopheevii* (AAGG) and six *T. aestivum*–*T. timopheevii* introgression lines. We found that 30 markers generated distinct PCR products only in *T. timopheevii* and all the six *T. aestivum*–*T. timopheevii* introgression lines. They were referred to as $2G_{465}$-specific markers, including 22 IT, two SNP and six SSR markers (Table 1). Amplification in Chinese Spring

Table 1 Molecular markers used for the fine mapping of *Pm6*

Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	Type
CIT02g-1	tgtcacctacccattcagct	ttctccaatgcttcgagtgc	Intron target
CIT02g-2	gagagcattcgtcggtttcc	attcgaccgcctcaaatcca	Intron target
CIT02g-3	gaccgtgccttccattgttg	tgttcacacaagcagcaagt	Intron target
CIT02g-4	tgaccctaaaacagtctcaaaga	tgttgtaaatgagaagtgcacct	Intron target
CIT02g-5	ggtcaccttcttcatagcgc	tcctcagcttcaccacttcc	Intron target
CIT02g-6	cggcatcgtccaggaaatg	tgctttggttcgagttggtg	Intron target
CIT02g-7	cctctcttcctgtcccttatgg	actaccgatgagagttccaga	Intron target
CIT02g-8	aagaaagcgcgcaccatg	gcagtccacgaaccgctc	Intron target
CIT02g-9	aaatcgaagccttgcaccaa	ggacaaagtgcgcgaagt	Intron target
CIT02g-10	tgggactggttagcacttga	cgatgaggaataagtgggca	Intron target
CIT02g-11	caaagcttgcaagatgggtg	ttccagcccctctagtgatc	Intron target
CIT02g-12	tggaacgtctagaccacagg	ccaagaccatcttgctttgg	Intron target
CIT02g-13	agagaagtggaggtgatggc	cacggaggctgggttcac	Intron target
CIT02g-14	tcttcctctcttcctgtccc	actaccgatgagagttccaga	Intron target
CIT02g-15	gagagcattcgtcggtttcc	gcttcctggatcatctgagc	Intron target
CIT02g-16	gcatcaataaatccctttctgca	tttcctccagttcatcgccc	Intron target
CIT02g-17	ctggatgaacttccccaaaa	tcaatcttgaacatctccctca	Intron target
CIT02g-18	ggccttagtggtgatgcagt	gcggcttgtcggtgtatag	Intron target
CIT02g-19	tcgttcacactcaactccca	agcgagatcccatgactgac	Intron target
CIT02g-20	cgtgccttccattgttgtat	tgttcacacaagcagcaagtt	Intron target
CIT02g-21	tttgggcctgcgacgatc	acggtgttattcctagcatgc	Intron target
CIT02g-22	ctctacgagctgtcttcgct	tcccttggtagtacttggaca	Intron target
CISNP02g-1	aaggaaatcaagaacaggaggataaaa	aataatcaactcatacgacttgtggtc	SNP
	ggaagaaggtagtgtatatgaagattgg	tatgttctaccttcttccttttgctaat	
CISNP02g-2	aggcagaccttcttacagtggctatt	aagatttactctcctgactttgcact	SNP
	tgaaattgacgttttatttttgtactatt	atagtctgatggagatacccagtgatt	
CISSR02g-1	tgtcatttactcgtgtgcttca	ccttacgctttcctcataaacc	SSR
CISSR02g-2	gactacaactaccttcccgtgg	aggatgaaaacctcgacacact	SSR
CISSR02g-3	ctaaaccataagcaatcccctg	gtctacaactaccttcccgtgg	SSR
CISSR02g-4	ttcgtaggttttgtgcatgttc	agttagggtaggaagaggtggg	SSR
CISSR02g-5	acttccagcaaatgttgtagcc	gtcgagagttgagggtcgtc	SSR
CISSR02g-6	taagcaacatctcatccccttt	gaatacgcctccactcatacct	SSR

nulli-tetrasomic lines confirmed that one of the specific IT markers, *CIT02g-14*$_{\text{-900bp}}$, could be used to differentiate four sub-genomes (Fig. 2).

Thirty-seven 2G$_{\text{_465}}$-specific markers (30 developed in this research and seven reported by Qin et al. 2011) were assigned to the Ta_2BL reference sequence of Chinese

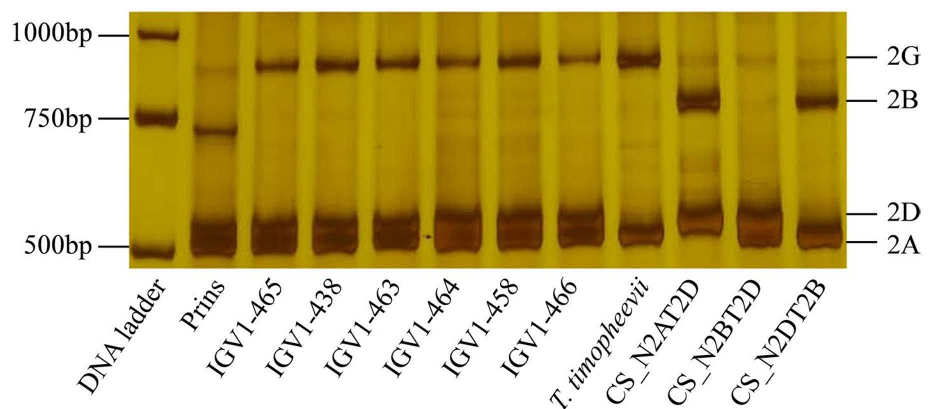


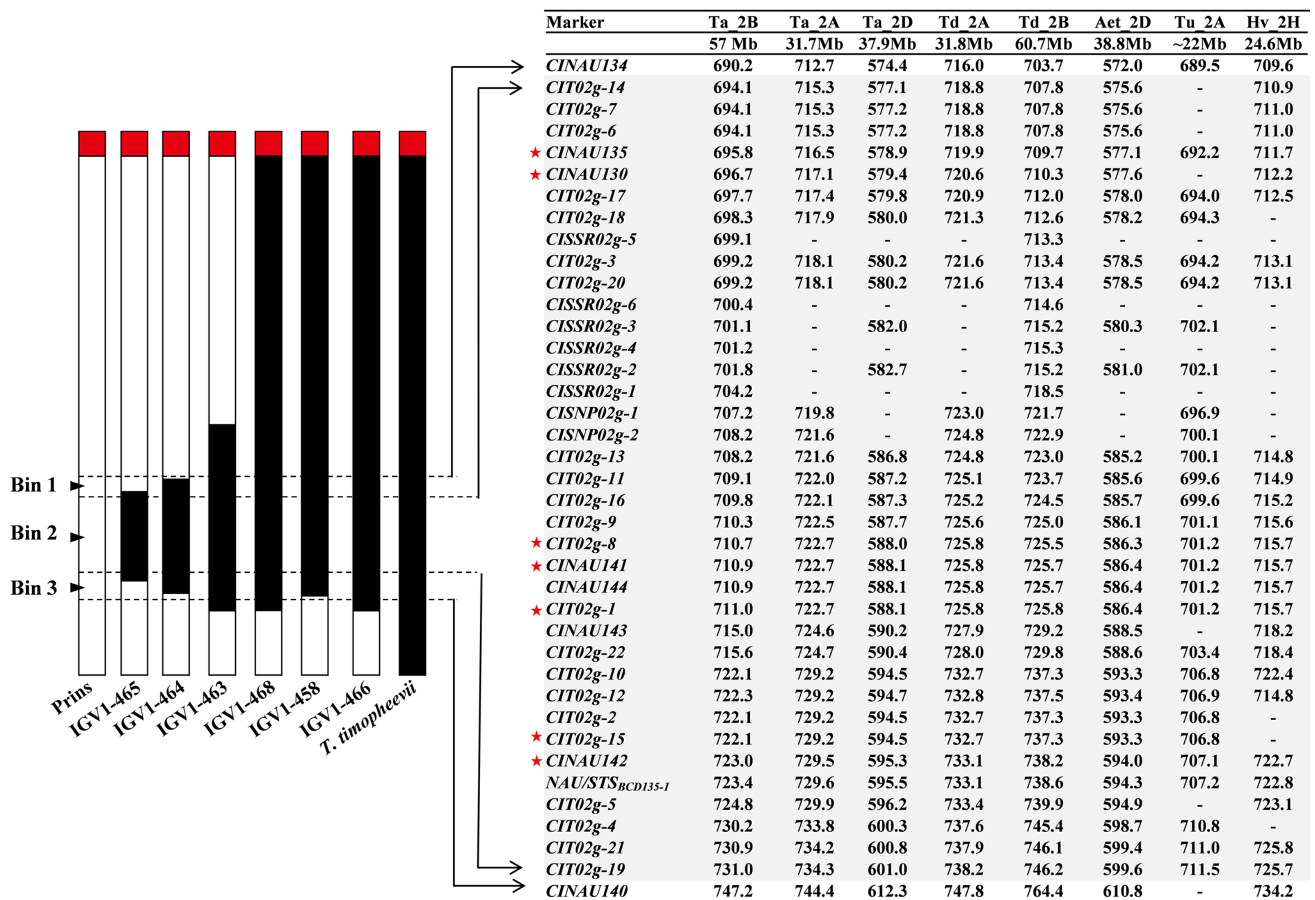
Fig. 2 Validation of the specificity of the IT marker *CIT02g-14* for the introgressed 2G fragment. Prins: the receptor of *Pm6*; IGV1-465 to IGV1-466: the introgression lines with different 2G chromosome segment sizes containing *Pm6*. *T. timopheevii*: the donor of *Pm6*. N2AT2D, N2BT2D and N2DT2B are three nulli-tetrasomic lines of Chinese Spring. The arrow indicates the specific bands for each subgenome

Spring. It was found that *CIT02g-14*$_{\text{-900bp}}$ and *CIT02g-19*$_{\text{-750bp}}$ were the most proximal and distal to the centromere, covering a 36.9 Mb physical distance (Fig. 3). The physical distance was similar among the sub-genomes of the same origin and their progenitor but varied in different genomes/sub-genomes, with the maximum distance being observed in Td_2BL (38 Mb) of wheat and the minimum in Hv_2HL (15 Mb) of barley (Fig. 3). The genome region bordering *Pm6* was determined by the two nearest neighbor markers outside 2G$_{\text{-465}}$ segment, *CINAU134* for *CIT02g-14*$_{\text{-900bp}}$ and *CINAU140* for *CIT02g-19*$_{\text{-750bp}}$. Among the six introgression lines with the exception of IGV1-464 and IGV1-465, *CINAU134* amplified the specific band for *T. timopheevii* and *CINAU140* amplified the specific band for *T. timopheevii* in only three lines, i.e., IGV1-463, IGV1-468 and IGV1-466. Therefore, we presumed that *Pm6* was most likely located in Bin2, not in Bin1 and Bin 3 (Fig. 3), and *CIT02g-14*$_{\text{-900bp}}$ and *CIT02g-19*$_{\text{-750bp}}$ were used for the screening of recombinants from the segregation populations for the *Pm6* locus.

Fine mapping of *Pm6* by generating allogeneic chromosome recombination induced by the *Ph1* pairing control system

A total of 34 BC_1F_1 individuals that were heterozygous for *Pm6* and homozygous for *phlb* were derived from the IGV1-465/CS *ph1b*//CS *ph1b* crosses. They were self-pollinated to generate BC_1F_2 or BC_1F_2 equivalents in the subsequent generations for recombinant screening.

Recombinant screening was performed in two rounds. Two flanking markers, *CIT02g-14*$_{\text{-900bp}}$ and *CIT02g-19*$_{\text{-750bp}}$, were used for the first round of screening. A total of 164 recombinants were identified from 820 $BC_1F_{2:3}$ individuals derived from 214 RHLs in BC_1F_2. The recombinants could be classified into ten haplotypes by genotyping using another 35 specific markers (28 developed in this study and seven from previous study) (Fig. 4 II). The recombinants were evaluated for *Pm* resistance by inoculating the single *Bgt* isolate E26 at the fifth leaf stage. The results showed



Marker	Ta_2B	Ta_2A	Ta_2D	Td_2A	Td_2B	Aet_2D	Tu_2A	Hv_2H
	57 Mb	31.7Mb	37.9Mb	31.8Mb	60.7Mb	38.8Mb	~22Mb	24.6Mb
CINAU134	690.2	712.7	574.4	716.0	703.7	572.0	689.5	709.6
CIT02g-14	694.1	715.3	577.1	718.8	707.8	575.6	-	710.9
CIT02g-7	694.1	715.3	577.2	718.8	707.8	575.6	-	711.0
CIT02g-6	694.1	715.3	577.2	718.8	707.8	575.6	-	711.0
★ *CINAU135*	695.8	716.5	578.9	719.9	709.7	577.1	692.2	711.7
★ *CINAU130*	696.7	717.1	579.4	720.6	710.3	577.6	-	712.2
CIT02g-17	697.7	717.4	579.8	720.9	712.0	578.0	694.0	712.5
CIT02g-18	698.3	717.9	580.0	721.3	712.6	578.2	694.3	-
CISSR02g-5	699.1	-	-	-	713.3	-	-	-
CIT02g-3	699.2	718.1	580.2	721.6	713.4	578.5	694.2	713.1
CIT02g-20	699.2	718.1	580.2	721.6	713.4	578.5	694.2	713.1
CISSR02g-6	700.4	-	-	-	714.6	-	-	-
CISSR02g-3	701.1	-	582.0	-	715.2	580.3	702.1	-
CISSR02g-4	701.2	-	-	-	715.3	-	-	-
CISSR02g-2	701.8	-	582.7	-	715.2	581.0	702.1	-
CISSR02g-1	704.2	-	-	-	718.5	-	-	-
CISNP02g-1	707.2	719.8	-	723.0	721.7	-	696.9	-
CISNP02g-2	708.2	721.6	-	724.8	722.9	-	700.1	-
CIT02g-13	708.2	721.6	586.8	724.8	723.0	585.2	700.1	714.8
CIT02g-11	709.1	722.0	587.2	725.1	723.7	585.6	699.6	714.9
CIT02g-16	709.8	722.1	587.3	725.2	724.5	585.7	699.6	715.2
CIT02g-9	710.3	722.5	587.7	725.6	725.0	586.1	701.1	715.6
★ *CIT02g-8*	710.7	722.7	588.0	725.8	725.5	586.3	701.2	715.7
★ *CINAU141*	710.9	722.7	588.1	725.8	725.7	586.4	701.2	715.7
CINAU144	710.9	722.7	588.1	725.8	725.7	586.4	701.2	715.7
★ *CIT02g-1*	711.0	722.7	588.1	725.8	725.8	586.4	701.2	715.7
CINAU143	715.0	724.6	590.2	727.9	729.2	588.5	-	718.2
CIT02g-22	715.6	724.7	590.4	728.0	729.8	588.6	703.4	718.4
CIT02g-10	722.1	729.2	594.5	732.7	737.3	593.3	706.8	722.4
CIT02g-12	722.3	729.2	594.7	732.8	737.5	593.4	706.9	714.8
CIT02g-2	722.1	729.2	594.5	732.7	737.3	593.3	706.8	-
★ *CIT02g-15*	722.1	729.2	594.5	732.7	737.3	593.3	706.8	-
★ *CINAU142*	723.0	729.5	595.3	733.1	738.2	594.0	707.1	722.7
NAU/STS$_{BCD135-1}$	723.4	729.6	595.5	733.1	738.6	594.3	707.2	722.8
CIT02g-5	724.8	729.9	596.2	733.4	739.9	594.9	-	723.1
CIT02g-4	730.2	733.8	600.3	737.6	745.4	598.7	710.8	-
CIT02g-21	730.9	734.2	600.8	737.9	746.1	599.4	711.0	725.8
CIT02g-19	731.0	734.3	601.0	738.2	746.2	599.6	711.5	725.7
CINAU140	747.2	744.4	612.3	747.8	764.4	610.8	-	734.2

Fig. 3 Microcolinearity analysis of the *Pm6* region using the developed 2G-specific markers. Left: The schema graph of chromosome 2BL of Prins, *T. timopheevii* and six *T. aestivum*–*T. timopheevii* introgression lines; white boxes indicate chromatin from the 2B Prins; black boxes indicate 2G introgression fragments from *T. timopheevii*; the red box indicates the centromere. Right: Physical location of markers used for the fine mapping of *Pm6*; the markers indicated with asterisks were reported by Ji et al. (2006) and Qin et al. (2011); "–" represents an absence of positional information in the reference genome

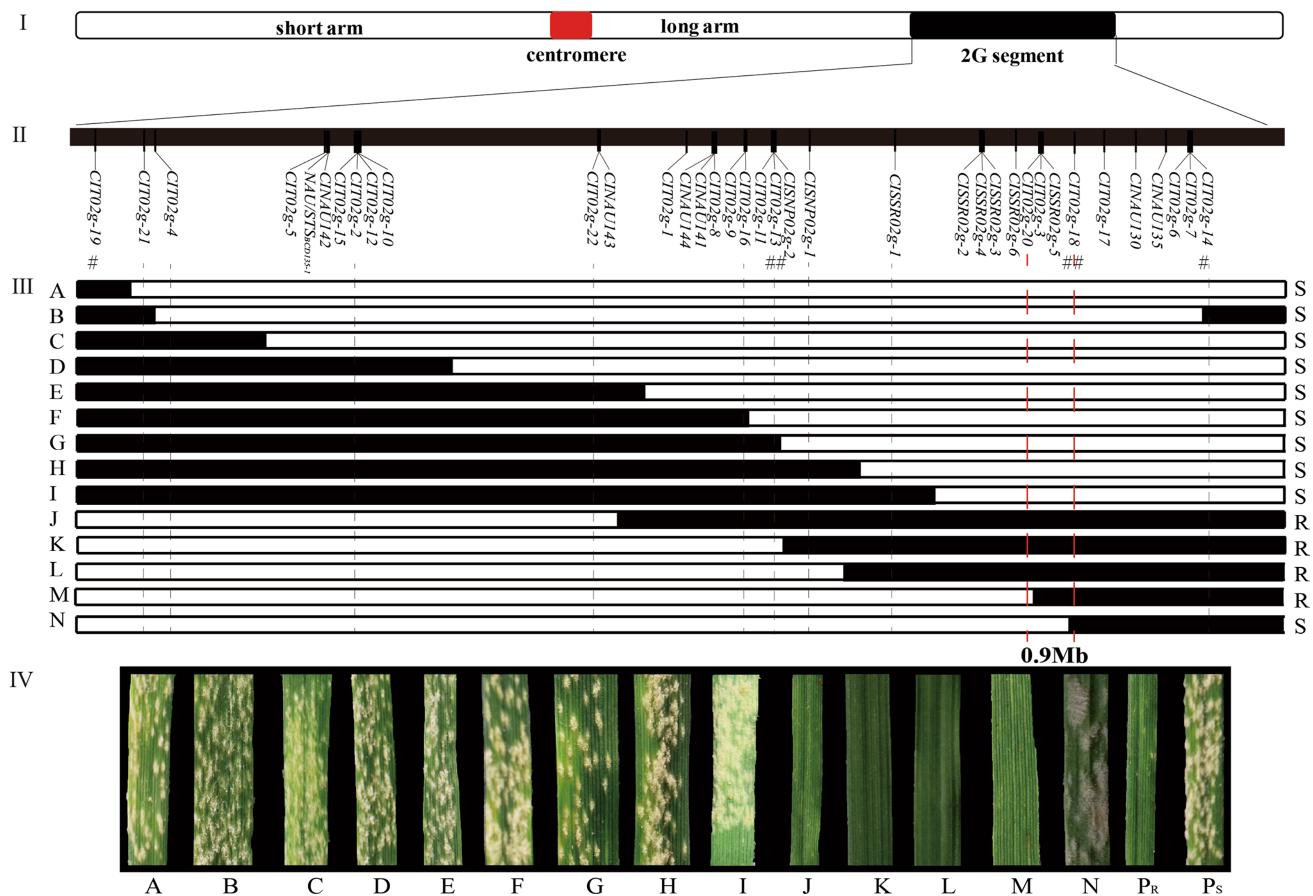


Fig. 4 Fine mapping of *Pm6* using the identified recombinants. **I** The 2B/2G introgression chromosome in IGV1-465. The red box indicates the centromere of chromosome 2B; the black box indicates the introgressed 2G segment from *T. timopheevii*. **II** The distribution of chromosome 2G-specific markers in the 2G introgression segment. Markers above # and ## are those markers that were used for the first and second rounds of recombinant screening, respectively. **III** Recombinant haplotypes classified through marker analysis. White boxes indicate chromatin from wheat 2B; black boxes indicate chromatin from *T. timopheevii* 2G; A to N represent 14 recombinant haplotypes classified through marker analysis; S and R represent susceptible and resistant recombinants, respectively; black dotted lines indicate the positions of the markers. The region between the two dotted red lines represents the physical region of *Pm6*. **IV** *Pm* resistance of different haplotypes. A to N: 14 recombinant haplotypes; P_R: IGV1-465 (resistant parent); P_S: CS *ph1b* (susceptible parent) (color figure online)

that only haplotypes J and K were resistant and the remaining eight haplotypes were susceptible (Fig. 4 III, IV, A–G, N). Hence, *Pm6* was narrowed to a physical region flanked by two markers, *CIT02g-13*$_{-500bp}$ and *CIT02g-18*$_{-300bp}$. The corresponding physical distances were 4 Mb in Ta_2AL, 10 Mb in Ta_2BL and 7 Mb in Ta_2DL. Due to the limited number of recombinants, ten markers within the genome region flanked by *CIT02g-13*$_{-500bp}$ and *CIT02g-18*$_{-300bp}$ co-segregated with *Pm* resistance (Fig. 4 II). This region covers a physical distance of 9 Mb.

The second round of recombinant screening was performed using the flanking markers, *CIT02g-13*$_{-500bp}$ and *CIT02g-18*$_{-300bp}$. Eighteen recombinants were identified from 642 $BC_1F_{2:4}$ individuals derived from 134 RHLs in $BC_1F_{2:3}$. The recombinants could be classified into four haplotypes by genotyping using the other ten specific markers. These recombinants were evaluated for their resistance to isolate E26 at the fifth leaf stage. Two haplotypes were resistant (Fig. 4 III, IV, L and M), and the remaining two were susceptible (Fig. 4 III, IV, H and I). *Pm6* was further mapped to the genome region flanked by *CIT02g-20*$_{-490bp}$ and *CIT02g-18*$_{-300bp}$, and the physical distance was narrowed to 0.9 Mb on chromosome 2B (Fig. 4 III).

The homoeologous recombination frequency and recombination frequency per million base pairs (RPM) were compared. The homoeologous recombination frequency and RPM were 20.000% and 0.541%, respectively, for the first round of screening using the $BC_1F_{2:3}$ population and 2.803% and 0.280% for the second round of screening using the $BC_1F_{2:4}$ population (Table 2). In the presence of *Ph1b*, recombination between 2B and 2G was extremely low (Qin et al. 2011). Our results indicated that the introduction of

Table 2 Comparison of the recombination frequency of 2B/2G chromatin at *Pm6* region in CS *ph1b* background with that of wild-type *Ph1b*

Recombination	Population	No. of lines	Recombinants	Physical distances of *Pm6* region (Mb)	Recombination frequency (%)	Recombination frequency per Mb (%)
IGV1-465/Prins (*Ph1bPh1b*)	F_2	1816	0	28	0	0
IGV1-466/Prins (*Ph1bPh1b*)	F_2	891	6	139	0.67	0.005
IGV1-465/CS (*ph1bph1b*)//CS (*ph1b-ph1b*)	$BC_1F_{2:3}$	820	164	37	20.00	0.540
IGV1-465/CS (*ph1bph1b*)//CS (*ph1b-ph1b*)	$BC_1F_{2:4}$	642	18	10	2.80	0.280

ph1b significantly increased the 2B/2G recombination frequency in the *Pm6* region. The narrowing of this region laid a solid foundation for *Pm6* candidate gene prediction and the final cloning of *Pm6*.

Identification of *Pm6* in a natural population and validation of its contribution to *Pm* resistance

The *Pm6* flanking markers *CISSR02g-100*$_{\text{-350bp}}$ and *CIT02g-18*$_{\text{-300bp}}$ were used to genotype a natural population consisting of 387 varieties. The specific products for both markers could be amplified from six of the varieties (Mianmai 40, Mianmai 48, Xingyi 4, Zhongyou 206, Jimai 44 and Jimai 53) (Fig. S3), and we presumed that *Pm6* was present in these varieties. Through genotyping using specific markers for six other known *Pm* resistance genes (*Pm2*, *Pm3b*, *Pm4*, *Pm8*, *Pm21* and *Pm38*), we found that 92 of 387 varieties harbored none of the above seven *Pm* resistance genes (data not shown). When the single *Bgt* isolate E26 was inoculated at the adult stage, the above six *Pm6*-containing varieties were all found to be highly resistant, while 70 out of 92 non-*Pm* varieties were *Pm* susceptible. When the *Bgt* mixture was inoculated at the adult stage, Mianmai 40, Mianmai 48 and Xingyi 4 were found to be highly resistant, Zhongyou 206 and Jimai 44 were moderately resistant, and Jimai 53 was susceptible. Eighty-eight out of the 92 varieties were moderately or highly susceptible to *Pm*. Considering the average *Pm* resistance level against either E26 or the *Bgt* mixture, *Pm6*-containing varieties were more resistant to *Pm* than 92 non-*Pm* varieties (Fig. S4). This implied that *Pm6* was still effective against the tested *Bgt* mixture or E26 isolate and that the use of the *Pm6* flanking markers in molecular-assisted selection was effective for *Pm* resistance improvement.

Discussion

The transfer of favorable genes from wild relatives is an effective way to broaden the genetic diversity of cultivated wheat for the improvement of wheat cultivars. The cloning of target genes from alien species through a map-based cloning approach is extremely difficult for two main reasons: the relatively low diversity of the target traits among the collections of specific wild species (Cao et al. 2011) and recombination suppression between alien and corresponding wheat chromosomes which makes it difficult to narrow the target region (Jayatilake et al. 2013; Wang et al. 2014; Xie et al. 2012). The suppression of recombination between chromosomes from cultivated wheat and wild wheat species has largely limited the efforts to clone alien genes by using a map-based strategy. The powdery mildew resistance genes *Pm12* and *Pm27* introgressed into wheat chromosome 6B of *Ae*. *speltoides* and *T*. *timopheevii*, respectively, show no recombination between the alien segments (6S or 6G) and wheat chromosome 6B (Järve et al. 2000; Jia et al. 1996). In the wheat–*H*. *villosa* translocation line T6VS/6AL, the recombination of chromosome arm 6VS with its wheat counterpart is completely suppressed (Xie et al. 2012). An introgression from an unidentified wild relative species carrying the multiple disease resistance locus *Lr20*/*Sr15*/*Pm1* also showed no recombination between the alien and wheat chromatin (Neu et al. 2002).

The deletion of the *Ph1* locus allows homoeologous pairing to occur relatively more easily, implying that the *ph1b* mutant could be exploited to overcome recombination suppression between chromosomes from common wheat and its relatives. The *ph1b* mutant has been applied to improve the wheat–alien recombination frequency. The effects of the *ph1b* mutant vary for different alien chromosomes (arms or fragments). The recombination frequencies in the homozygous *ph1b* background for wheat–rye translocation chromosomes involving 2RS and 2RL are 0.62% and 16.3%, respectively (Lukaszewski et al. 2004); the recombination frequencies of 4VS and 6VL in wheat–*H*. *villosa* translocation with wheat counterparts are 2.19% (Zhao et al. 2013) and 6.81% (data unpublished), respectively; the recombination frequencies of $4H^{ch}$, $5H^{ch}$ and $7H^{ch}$ in wheat–barley substitution lines with wheat chromosomes range from 3.3 to 7.5% (Rey et al. 2015).

In previous work, Qin et al. (2011) tried to screen recombinants in the *Pm6* region from two segregating populations

from a cross between IGV1-465/Prins and IGV1-466/Prins. IGV1-466 contains a larger introgressed 2G segment ($2G_{_466}$) than that in IGV1-465. Initially, two flanking markers *CINAU117* and *CINAU139* were used, and both markers were present within $2G_{_466}$ rather than $2G_{_465}$. Among the 1816 F_2 individuals from the IGV1-465/Prins cross, 36 were deemed recombinants. However, no recombination site occurred within $2G_{_465}$ (Fig. S1), indicating that there was no crossover between 2G and 2B chromatin in this population. For the IGV1-466/Prins population, six of 891 F_2 individuals were identified as recombinants and the recombination sites were located within $2G_{_466}$, indicating an extremely low recombination frequency (0.005% RPM) between allogeneic 2G and 2B (Table 2). In this study, we proved that the introduction of *ph1b* could greatly increase the allogeneic recombination frequency, with recombination frequencies of 0.54% and 0.28% RPM being observed in the first and second rounds of recombinant screening, respectively. Through this approach, *Pm6* was fine mapped within less than 1 Mb of the genome region. This again demonstrated the effectiveness of the *Ph* control pairing system in both reducing linkage drag and the fine mapping of target genes from an alien species in a wheat background.

A high-density map lays a foundation for the map-based cloning of target genes (Xue et al. 2008). The DNA markers used for the detection of alien chromatin introgressed into wheat germplasm show the distinct characteristic of being cross species specific. The ability to detect polymorphic loci across species is most likely attributed to the primers being positioned within regions of the genome that exhibit consistent sequence conservation. Common wheat presents a very high repetitive DNA content, accounting for 80–90% of the genome, and these repetitive DNA sequences are typically species specific (Mayer et al. 2014). Therefore, repetitive element-derived molecular markers, such as SSR, are likely not applicable to the detection of alien chromatin. A low interspecific transferability of cereal SSRs has been reported in rye species (Li et al. 2018). In this study, a total of 100 SSR primers were designed according to the sequence of wheat chromosome 2B in accordance with the homologous *Pm6* region of 2G. Only six SSR markers could be used for the analysis of introgression lines, implying a low efficiency of genomic SSRs across plant species. We checked the primers for these six applicable SSR markers and found that they could be uniquely aligned within the intergenic region of the survey sequence of flow-sorted 2G (data not shown), which explains their transferability. Although the transferability of genomic SSRs across plant species is unexpected, we still found collinearity in the non-gene-coding region, implying a close relationship between the G genome of *T. timopheevii* and the wheat B genome (Kilian et al. 2007).

EST-SSR and IT markers are two priority options for efficient polymorphic marker development (Ishikawa et al. 2007; Yu et al. 2004; Zhang et al. 2017). A high degree of synteny exists between wheat and its wild relatives within the same homoeologous groups, and ESTs or functional genes constitute high-synteny region. The gene- or EST-based markers in conserved regions are transferable when used for the detection of alien chromatin from related species (Wang et al. 2017; Xiao et al. 2017). In addition, when these markers are genetically associated with a trait of interest, it will be helpful to predict the candidate genes by referring to the target genome region where the marker sequences are allocated (Thiel et al. 2003). We found that 22 IT markers could amplify specific amplicons in the *Pm6* region, suggesting that they have become essential tools for the advanced fine mapping and map-based cloning of *Pm6*.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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