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ORIGINAL PAPER

Collinearity-based marker mining for the fine mapping of *Pm6*, a powdery mildew resistance gene in wheat

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Abstract The genome sequences of rice (*Oryza sativa* L.) and *Brachypodium distachyon* and the comprehensive Triticeae EST (Expressed Sequence Tag) resources provide invaluable information for comparative genomics analysis. The powdery mildew resistance gene, *Pm6*, which was introgressed into common wheat from *Triticum timopheevii*, was previously mapped to the wheat chromosome bin of 2BL [fraction length (FL) 0.50–1.00] with limited DNA markers. In this study, we saturated the *Pm6* locus in wheat using the collinearity-based markers by extensively exploiting these genomic resources. All wheat ESTs located in the bin 2BL FL 0.50–1.00 and their corresponding orthologous genes on rice chromosome 4 were firstly used to develop STS (Sequence Tagged Site) markers. Those identified markers that flanked the *Pm6* locus were then used to identify the collinear regions in the genomes of rice and *Brachypodium*. Triticeae ESTs with orthologous genes in these collinear regions were further used to develop new conserved markers for the fine mapping of *Pm6*. Using two F_2 populations derived from crosses of IGVI-465 × Prins and IGVI-466 × Prins, we mapped a total of 29 markers to the *Pm6* locus. Among them, 14 markers were co-segregated with *Pm6* in the IGVI-466/Prins population. Comparative genome analysis showed that the collinear region of the 29 linked markers covers a ~5.6-Mb region in chromosome 5L of *Brachypodium* and a ~6.0-Mb region in chromosome 4L of rice. The marker order is conserved between rice and *Brachypodium*, but re-arrangements are present in wheat. Comparative mapping in the two populations showed that two conserved markers (CINAU123 and CINAU127) flanked the *Pm6* locus, and an LRR-receptor-like protein kinase cluster was identified in the collinear regions of *Brachypodium* and rice. This putative resistance gene cluster provides a potential target site for further fine mapping and cloning of *Pm6*. Moreover, the newly developed conserved markers closely linked to *Pm6* can be used for the marker-assisted selection (MAS) of *Pm6* in wheat breeding programs.

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Introduction

Powdery mildew, which is caused by *Blumeria graminis* f.sp. *tritici* (*Bgt*), is one of the most devastating diseases of wheat worldwide. This biotrophic fungus reduces grain yields by as much as 48% in susceptible cultivars during years of severe epidemics (Everts and Leath 1992). Compared with the application of fungicides, using resistant cultivars is a more effective and environmentally safe approach to control the disease. To date, 60 powdery mildew resistance genes/alleles have been reported in wheat, and most of them have been mapped to specific chromosomes or chromosome regions (Zhang et al. 2010). These genes have been extensively used in breeding, but only the *Pm3* gene has been cloned. The cloning of more

Pm genes will be helpful both for improving powdery mildew resistance by a transgenic approach and for understanding the mechanism of resistance at the molecular level. However, the cloning of genes in wheat is very difficult because of the following features of the wheat genome: its polyploid nature, its large size (17 Gb), and its possession of highly repetitive sequences (>80%).

The tetraploid *Triticum timopheevii* (2n = 4x = 28, genome AAGG) is a valuable resource for powdery mildew resistance. Four resistance genes have been transferred from *T. timopheevii* into hexaploid common wheat (*T. aestivum* L., 2n = 6x = 42, AABBDD), including *Pm6* (Jorgensen and Jensen 1973), *Pm27* (Peusha et al. 2000), *Pm37* (Perugini et al. 2008), and *MlAG12* (Maxwell et al. 2009). *Pm6* has been widely and successfully used in wheat breeding for powdery mildew resistance; it is best expressed from the third leaf stage and thereafter, and it is moderately effective, but recognizable, at the seedling stage (Bennett 1984). Virulence matching the *Pm6* gene has occurred in many regions; nevertheless, *Pm6* is still effective in the field in some areas, especially when it is used in combination with other *Pm* genes such as *Pm2* (Švec and Miklovičová 1998; Cai et al. 2005; Costamilan 2005; Věchet 2006; Shi et al. 2009; Purnhauser et al. 2010). The *Pm6* gene was previously mapped to the long arm of chromosome 2B (2BL) and linked with an RFLP (Restriction Fragment Length Polymorphism) marker, BCD135 (Tao et al. 2000). Ji et al. (2008) converted this RFLP marker into two STS (Sequence Tagged Site) markers, NAU/$STS_{BCD135-1}$ and NAU/$STS_{BCD135-2}$, which are closely linked to *Pm6* with a genetic distance of 0.8 cM. However, the number of molecular markers that have been mapped to the *Pm6* region is still limited; the saturation of the genetic map of this region will be extremely important both for molecular marker-assisted selection (MAS) and the further map-based cloning of this gene.

According to Tao et al. (2000) and Ji et al. (2007, 2008), *Pm6* was assigned into the bin 2BL [fraction length (FL) 0.50–1.00], a region with high gene density (Conley et al. 2004). A total of 274 wheat ESTs have been mapped to this region (Qi et al. 2004); these mapped ESTs are particularly useful for developing PCR-based markers for the fine mapping of *Pm6*. Moreover, there is good collinearity among species in the grass family, and the collinearity of wheat chromosomes and the chromosome regions of model species, such as rice and *Brachypodium*, have been well characterized (Feuillet et al. 2003; La Rota and Sorrells 2004; Bossolini et al. 2007; Krattinger et al. 2009; The International Brachypodium Initiative 2010). The available genomic sequences of rice and *Brachypodium* provide parallel maps, and these are useful for generating markers in Triticeae-targeted regions, and in some cases, for identifying candidate genes. By comparing sequences from putatively syntenic loci in rice and other grass species, it was demonstrated that the sequence conservation between these species is mainly restricted to coding regions (Avramova et al. 1996; Tikhonov et al. 1999; Dubcovsky et al. 2001). From this point of view, the comprehensive Triticeae EST datasets, which represent portions of the coding regions, are rich resources for genomic mapping. Therefore, the genomic sequences of model species and the Triticeae EST sequences serve as invaluable resources for the marker saturation of a syntenic target region and may accelerate the attempts at synteny-based positional cloning of agronomically important genes from large-genome cereal species. Quraishi et al. (2009) identified 695 conserved orthologous set (COS) sequences to design primers based on the synteny between rice genes and wheat ESTs that have been mapped physically. These COS markers provide useful resources for the fine mapping of genes and QTLs in common wheat. This comparative genomics approach has been used for most of the positional cloning projects in wheat, including *Yr36* (Fu et al. 2009), *Lr34* (Krattinger et al. 2009), and *Lr10* (Feuillet et al. 2003).

In order to facilitate the deployment of the *Pm6* gene in wheat breeding and further map-based cloning of this gene, in the present research, we will: [1] develop a high-density genetic linkage map of the *Pm6* locus through comparative genomics analysis using the genome sequences of rice and *Brachypodium* and the available Triticeae ESTs, and [2] characterize the collinearity of the *Pm6* region in wheat genome and those in rice and *Brachypodium* genome.

Materials and methods

Plant materials

The Swedish spring common wheat variety Prins, which is susceptible to powdery mildew, one *T. timopheevii* accession with powdery mildew resistance, and nine *Pm6*-carrying *T. aestivum–T. timopheevii* introgression lines (IGVI-448, IGVI-458, IGVI-463, IGVI-464, IGVI-465 [FAO 163b/7*Prins], IGVI-466 [Kenya Lemphi 50-13596/7*Prins], IGVI-468, IGVI-474, and Coker747) were kindly provided by Dr. J. MacKey, Swedish Agricultural University, Uppsala, Sweden. Two F_2 populations, including 1,816 individuals from the cross of IGVI-465 × Prins and 891 individuals from the cross of IGVI-466 × Prins, were generated and used for the genetic mapping of the *Pm6* gene. The local wheat variety Sumai 3, which is highly susceptible to powdery mildew, was used for the live production and preservation of the *Bgt* pathogen. Another wheat cultivar, Chancellor, was used as the susceptible control.

Eight wheat varieties with known powdery mildew resistance genes (Coker983 [*Pm5* + *6*], Baitu 3 [*Pm4* + *8*],

Chul/8*Cc [*Pm3b*], Khapli/8*Cc [*Pm4a*], Ulka/8*Cc [*Pm2*], Hope/8*Cc [*Pm5*], Kavkaz [*Pm8*], Nannong 9918 [*Pm21*]) were used to evaluate the *Pm6*-linked markers for their potential use in MAS.

Evaluation of powdery mildew resistance

All of the tested individuals and the susceptible controls were planted under the controlled greenhouse conditions. To determine an optimal growth stage for evaluating the *Pm6* resistance phenotype, the chronological growth stage of Prins and IGVI-465 were planted at intervals of 10 days so that *Bgt* could be inoculated at the same designated growth stage for both genotypes. Seedlings were inoculated with *Bgt* by brushing conidia from neighbor sporulating susceptible seedlings of Sumai 3 onto the tested seedlings; the inoculations were repeated after 48 h. The *Bgt* population used in this study was a native population of *Bgt* collected from Nanjing, Jiangsu province, China. At 15 days post-inoculation when the susceptible variety, Prins, became severely infected, photos were taken and disease reactions were scored. For Prins and IGVI-465 at different growth stages and the other *Pm6*-carrying lines, twenty plants were scored for each line at each growth stage. The plants were rated as resistant (R) or susceptible (S), wherein the resistant phenotypes showed either no visible symptoms or necrotic flecks, and the susceptible phenotypes had no necrosis and showed high to full sporulation. For the intermediate type, which was difficult to rate as resistant or susceptible, 30 individuals of each of the $F_{2:3}$ families were further evaluated to deduce the genotypes of their corresponding F_2 individuals.

The development of molecular markers

EST-STS markers

Pm6 was previously mapped to the chromosome bin 2BL FL 0.50–1.00, which has good synteny with rice chromosome 4 (Tao et al. 2000; Sorrells et al. 2003; Ji et al. 2008). Thus, wheat ESTs that mapped to bin 2BL FL 0.50–1.00 were aligned to the rice genome sequences using BLASTN. ESTs with orthologous genes in the syntenic region of rice chromosome 4 were used for developing STS markers with Primer 3 Software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) (Rozen and Skaletsky 2000).

Conserved markers and COS markers

Comparison of the sequences from putatively syntenic loci of rice and other grass species has demonstrated that conservation is maintained in the exons but not in the introns (Avramova et al. 1998; Tikhonov et al. 2000; Dubcovsky et al. 2001). Therefore, primers designed to amplify the intronic sequences can greatly improve the identification of polymorphisms in species like wheat (Feuillet and Salse 2009). Such software (i.e., the Conserved Primers 2.0 Software) has been developed and released by You et al. (2009); thus, it is possible to design primers that are located in the conserved coding regions but that amplify the intron sequences. Such primers are also called conserved primers, intron-flanking primers (Wei et al. 2005; You et al. 2008, 2009), or exon priming intron crossing (EPIC) primers (Palumbi and Baker 1994; Hassen et al. 2002).

All of the ESTs from the Triticeae (including wheat, barley, rye and *Aegilops tauschii*) that were found to be homologous to *Brachypodium* and rice genes within the syntenic regions were used to design conserved primers using the Conserved Primers 2.0 Software. The default primer-designing parameters used in the pipeline were as follows: primer length of 18–25 bp with the optimum 20 bp, Tm of 55–65°C with optimum 60°C, GC content of 20–80%, and an 800-bp optimum product size with a range from 400 to 1,500 bp. The expected value of $1E^{-10}$ was used as the default in the BLASTN search, and the intron length was restricted to no more than 1,500 bp to ensure high efficiency of amplification by PCR. Usually, only one primer pair is chosen for each EST; two or more primer pair candidates may be obtained if multiple unique collinear exon blocks are found within an EST. In this study, a total of 338 conserved markers were designed based on the identified Triticeae ESTs. In addition, 39 COS markers assigned to the syntenic region of the rice genome were also selected from Quraishi et al. (2009) and used to screen markers linked to *Pm6*. The information regarding those primers that are polymorphic between the parents of the mapping populations is provided in Table 1.

Polymerase chain reaction (PCR)

Genomic DNA was extracted from young leaves as previously described (Qi et al. 1997), and PCR was performed following the procedure of Ji et al. (2008). The PCR products were separated on 8% non-denaturing polyacrylamide gels (Acr:Bis = 19:1 or 39:1) at room temperature with 1 × TBE buffer and visualized by silver staining (Bassam et al. 1991).

Genetic mapping

The linkage map was constructed with the JoinMap 4.0 software (Van Ooijen 2006) using Kosambi's mapping function (Kosambi 1944) and a LOD score of 3.0.

Table 1 Molecular markers mapped to the *Pm6* region

Marker	Marker type	Forward primer	Reverse primer	Annealing temperature (°C)	Expected size (bp)
CINAU117	STS	GACCCAAGAGGCGTTGATTA	CATGTGTGCCAAATTCAAGC	55	250
CINAU118	CP	GCTGTGACTGCTGGATTCAA	ACCGGGACTGTGTAGACTGG	53	871
CINAU119	CP	CTTCGTTGCTCGAAAGGTTC	CGGGTGAAACATCTTCTGGT	55	348
CINAU120	CP	GCCATGGCTAAGGAAGAAGA	ACCTTGGCGAGCTTCTTGAC	53	197
CINAU121	CP	CCTAGACTGGCCAAGACGAT	ATGGTTTGATTCACCAGCAA	53	350
CINAU122	COS	CACCTACCTCGTCAACGG	GCCGAGAGCTCGATCAGG	53	758
CINAU123	CP	TTGTACGCCATCGACACATT	CCGAACAGAGTTTTGCCTTC	52	470
CINAU124	COS	GAGTGCTCCACTGTAAAGCC	CACCTTTGTAGACAGTCCCG	55	546
CINAU125	COS	CCTCTTCCTGACCATCTTCC	TGACAGTCACTCCAATCACG	53	292
CINAU126	COS	TCATTTGGTTGCATAGTTGC	AATTTAGCAGTATTCTTAGCTTCCC	55	358
CINAU127	CP	ATGCCCACAAGTGCCTAAAC	ATGGGCCGTACAAGAAAGTG	55	1,900
CINAU128	CP	TCGAACATGGCTGTGATGAT	GGCTCAGCTTTACCAAGAGC	55	315
CINAU129	CP	ATCTTGCAGCTTTTGCGTTT	GCTCCCTGACACTCTTGAGG	55	649
CINAU130	CP	GGCGAGAAAATGTTGTCCAT	AGAAGAGCTGGAGCACCTTG	57	645
CINAU131	CP	CAACTGCTGGCTCTTCTTCC	GGAACAGCAGCGTCTTCTTC	55	336
CINAU132	STS	GTGGCTACACCCAAACGG	CAGATCAACGGGAGACATCAC	51	246
CINAU133	STS	AAGAACCATATCTGGGCTGTC	TACAACAAGATGCCGCAGGCTAACA	52	515
CINAU134	COS	ATCAACAAGATCTTCGACGG	CTTTGTCTGAACATTGCTGC	55	849
CINAU135	STS	TTGGTGACGCAGTAATGGAA	TGTGACAGAGCTAGGGCAAG	55	250
CINAU136	STS	CTGACTGCGCCTTATGTTGA	CCGTGGCTTGATGGAGTCATA	51	412
CINAU137	COS	GGACAATGAGAAAGCAAAGG	CTTTGCAAGAGCATCAGAGG	55	309
CINAU138	CP	TTCCCGAAGGACTACCATTG	TCCAGTCACCTCTGGAGCTT	53	761
CINAU139	STS	CAAAGGAGCCTTTCGATGAG	GGATTCGGGTAGCTTGCATA	57	500
CINAU140	COS	CACGGTGGAAGTCACTAACC	CAGTTTCCAAGGCATAGGG	55	498
CINAU141	COS	CACACATGGCAAGTTACAGG	ATCAGACTTGCTTGCTCACC	55	426
CINAU142	STS	CGACTACGTGACGCTCAAGA	ACTTGTCGTCGAGGAGGATG	65	300
CINAU143	COS	GTTGGTGGTTGAAAAGATGG	AGTATGCACCTTCGATTTGC	53	601
CINAU144	STS	GCTCCTCAGCAAATGCCTAC	GATGAAGTGGTGAGCAAGCA	57	1,000

marker type: *STS* STS-derived marker, *CP* conserved marker designed by Conserved Primers 2.0, *COS* COS marker

Comparative genomics analysis and gene identification

All of the corresponding EST sequences of polymorphic markers were annotated and ordered based on the comparison with the rice pseudomolecule, release 6.1 (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml), the 8 X *Brachypodium distachyon* genome assemblies v1.0 (http://www.brachybase.org/blast/), the rice gene database (the Rice Genome Annotation Project-MSU Rice Genome Annotation [Osa1] Release 6.1, http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/), and the *Brachypodium* gene database (the *Brachypodium distachyon* Genome Annotation-Release 1.0, http://www.modelcrop.org/cgi-bin/gbrowse/brachyv1/). BLASTN was performed to search against the rice and *Brachypodium* databases with cutoff parameters of E-value $\leq 1E^{-10}$, identity $\geq 80\%$ and a minimum of a 100-bp match length. When several significant hits were found, only the best hit was adopted. TBLASTX and BLASTX searches were further carried out against the NCBI nr protein database to identify the putative proteins. A significant match for a protein was detected based on a minimum of 80% amino acid identity for at least 50 residues of the protein sequence and an E-value of less than $1E^{-7}$.

The genome sequence similarity between rice and *Brachypodium* within the orthologous region of *Pm6* was calculated using the Artemis Comparison Tool (http://www.sanger.ac.uk/Software/ACT) (Carver et al. 2005) with a default cutoff score of 250 for the BLASTN algorithm.

Results

Evaluation of *Pm6* resistance at different growth stages

To determine an optimal stage for the evaluation of the *Pm6*-resistant phenotype, different growth stages of the introgression line (IGVI-465) and the susceptible parent (Prins) were inoculated at the same time with the native *Bgt* population collected from Nanjing, Jiangsu province, China, and evaluated at 15 days post-inoculation (Fig. 1). Prins was found to be susceptible at all the tested stages. However, at the first leaf stage, IGVI-465 was susceptible, but showed gradually increased resistance from the second leaf stage and reached complete resistance at the fourth leaf stage. The same phenomenon was observed in *T. timopheevii* and all of the nine *Pm6*-carrying lines (Fig. 2), suggesting that *Pm6* resistance was fully expressed from the fourth leaf stage and thereafter. Thus, in this study, all of the F_2 plants and their $F_{2:3}$ progenies were inoculated and evaluated at the fourth leaf stage.

Segregation of *Pm6* in the two mapping populations

All the individuals from the two F_2 populations and their parents were evaluated for their powdery mildew resistance. Among a total of 1,816 F_2 plants of the IGVI-465/Prins population, 1,376 were resistant and 440 were susceptible, which fits a 3:1 Mendelian segregation ratio ($P = 0.44$) (Table 2). The segregation of the co-dominant marker allele CINAU133 linked to *Pm6* also fits with the expected Mendelian segregation ratio of 1:2:1 in this population ($P = 0.82$) (Table 3). However, for the IGVI-466/Prins F_2 population, 713 plants were resistant and 178 were susceptible, and the segregation ratio was significantly distorted from the 3:1 ratio ($P < 0.01$) (Table 2). In agreement with the phenotypic data, segregation of the two dominant marker alleles, CINAU139 and NAU/$STS_{BCD135\text{-}2}$, and the co-dominant marker allele, CINAU117, linked to *Pm6* were also significantly distorted from the expected Mendelian ratio in the IGVI-466/Prins population ($P < 0.01$) (Table 3). It has been reported that IGVI-465 contains the smallest 2G chromosome segment without a centromere from 2G, whereas IGVI-466 has the largest 2G chromosome segment including the centromere (Ji et al. 2007). Therefore, we suggest that the distorted segregation might be due to the preferential transmission of the 2BS-2GS·2GL·2BL chromosome in IGVI-466 from the F_1 to the F_2 generation.

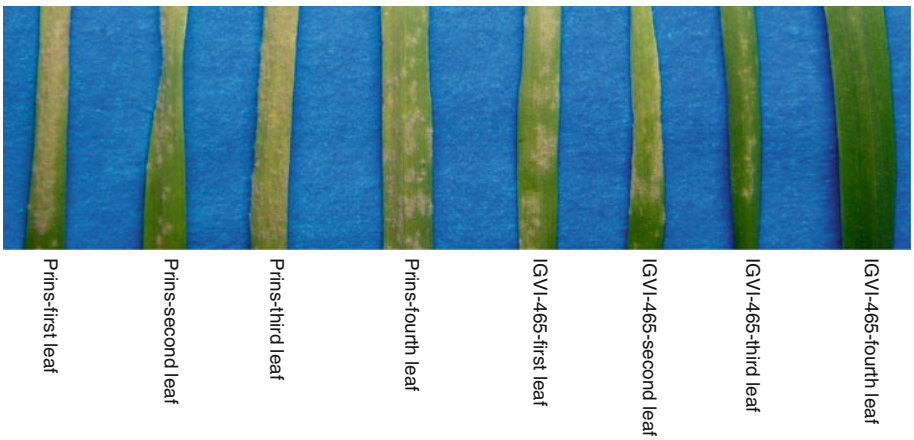

Fig. 1 Powdery mildew resistance of Prins and IGVI-465 at four different growth stages. The two lines at different growth stages were inoculated with *Bgt* at the same time, and the photo was taken 15 days post-inoculation. For each line at each stage, 20 plants were scored

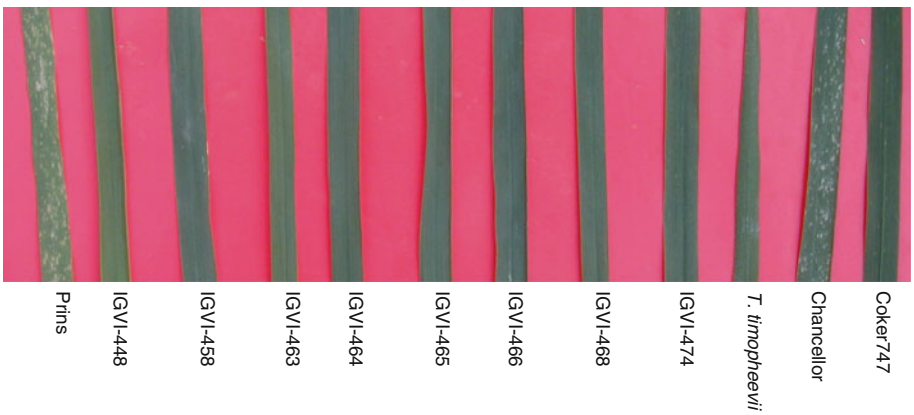

Fig. 2 Powdery mildew resistance of ten *Pm6*-carrying lines at their fourth leaf stage. Prins and Chancellor were used as susceptible controls

Table 2 Segregation ratios of *Pm6* in the two F_2 populations

Population	Number of the F_2 plants	Observed ratio of R:S	Expected ratio (R:S)	χ^2	*P* value
IGVI-465/Prins	1816	1376:440	3:1	0.596	0.44
IGVI-466/Prins	891	713:178	3:1	11.987	<0.01

R resistant *S* susceptible

Table 3 Segregation of markers flanking the *Pm6* locus in the two F_2 populations

Population	Marker	Total	Observed ratio[a]			Expected Ratio (R:Seg:S or R:S)	χ^2	*P* value
			R	Seg	S			
IGVI-465/Prins	CINAU133	1816	464	907	445	1:2:1	0.3979	0.82
IGVI-466/Prins	CINAU139	891	280	430	178	1:2:1	24.2435	<0.01
IGVI-466/Prins	CINAU117	891	713	–	178	3:1	11.9869	<0.01
IGVI-466/Prins	$NAU/STS_{BCD135-2}$	891	712	–	179	3:1	11.4572	<0.01

[a] The segregation ratio for dominant markers in F_2 populations indicates *Pm6_* plants: *pm6pm6* plants, and the segregation ratio for co-dominant markers includes non-segregating resistant families (*Pm6Pm6*), segregating families (*Pm6pm6*) and non-segregating susceptible families (*pm6 pm6*). *R* resistant, *Seg* segregating, and *S* susceptible

Identification of STS markers flanking the *Pm6* locus and the screening of recombinants

A total of 164 STS markers were designed based on the sequences of the wheat ESTs assigned to the deletion bins 2BL4 FL 0.50–0.89 and 2BL6 FL 0.89–1.00. Among them, eight STS markers (CINAU117, CINAU132, CINAU133, CINAU135, CINAU136, CINAU139, CINAU142 and CINAU144) were closely linked to *Pm6* (Table 1). Two of them, CINAU117 and CINAU139, flanked the *Pm6* locus in the IGVI-466/Prins population (Fig. 3b). In the IGVI-465/Prins population, however, the marker CINAU117 showed no polymorphism, and the other seven markers were mapped to one distal side of the *Pm6* (Fig. 3a).

The two flanking markers, CINAU117 and CINAU139, were used to amplify the F_2 populations and identify recombinants near the *Pm6* locus. A total of 36 recombinants between the interval of *Pm6* and marker CINAU139 were identified among the 1,861 F_2 plants of the IGVI-465/Prins population, and six recombinants were identified in the *Pm6* region flanked by CINAU117 and CINAU139 in the IGVI-466/Prins population. Altogether, 42 recombinants were identified and used for the fine mapping of the *Pm6* gene.

Collinearity-based marker mining for fine mapping of *Pm6*

Although 274 mapped ESTs have been physically assigned to the chromosome bin 2BL FL 0.50–1.00 (Qi et al. 2004), only eight markers are linked to the *Pm6* locus. The low marker density in this region is insufficient for the fine mapping and map-based cloning of the *Pm6* gene. Fortunately, abundant Triticeae EST resources (including wheat, barley, rye and *Ae. tauschii*) and the model genome sequences of rice and *Brachypodium* can be utilized to increase the marker density. To identify the collinear regions of *Pm6* in the model genomes of rice and *Brachypodium*, the corresponding eight EST sequences of the STS markers linked to *Pm6* were used as queries to perform a BLAST search against the genome sequences of rice and *Brachypodium*, and their orthologs were found in both species. The collinear region covered ~5.6 Mb of chromosome 5L in *Brachypodium* and ~6.0 Mb of chromosome 4L in rice (Fig. 3c, d). Genes located in the two collinear genome regions were selected to develop new markers for saturating the *Pm6* locus. These gene sequences were then used to search against Triticeae ESTs to identify the corresponding orthologs. Based on the identified Triticeae ESTs, a total of 338 conserved primers were designed using the ConservedPrimers2.0 Software (You et al. 2009). Another 39 COS markers assigned to the collinear region of rice genome (Quraishi et al. 2009) were also selected for the fine mapping of the *Pm6* gene. These 377 markers were then used to detect polymorphisms in the two resistant introgression lines and the susceptible variety Prins. A total of 11 conserved markers and 9 COS markers were found to be polymorphic (Table 1).

Fine mapping of *Pm6* using the newly developed markers

All of the 42 recombinants were genotyped with the 20 newly developed polymorphic markers. All of the 20

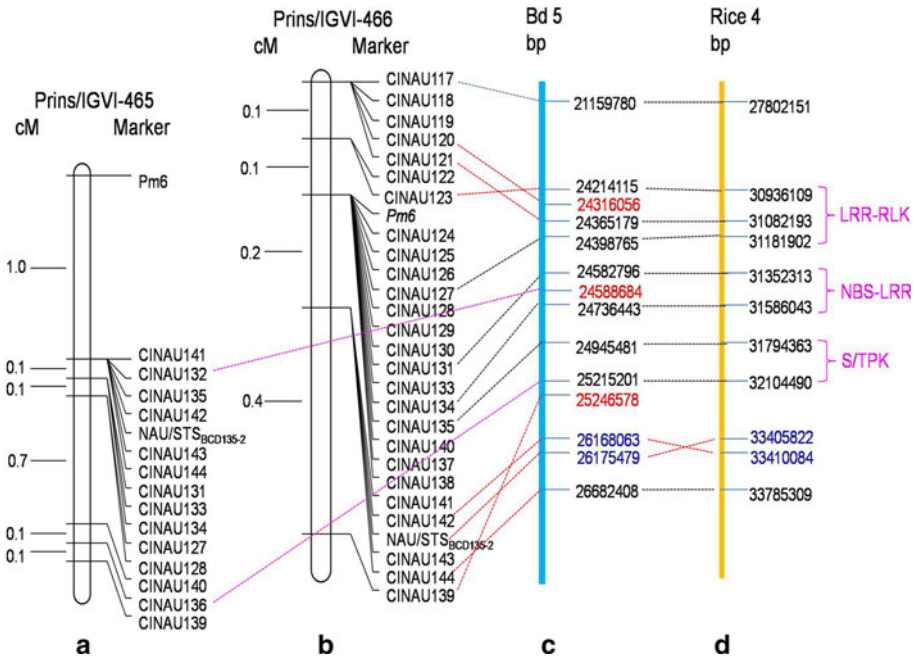

Fig. 3 Fine mapping and comparative mapping of the *Pm6* gene. Shown are the linkage maps of the *Pm6* gene in **a** the F_2 populations of IGVI-465/Prins (including 1816 individuals) and **b** the F_2 populations of IGVI-466/Prins (including 891 individuals); and **c** the collinearity of the *Pm6* region of wheat with the corresponding genomic region of *Brachypodium* chromosome Bd5 and **d** rice chromosome 4

markers were found to be closely linked to *Pm6* using the IGVI-466/Prins population, and 14 of them co-segregated with *Pm6* (Fig. 3b). However, only seven of the 20 markers could be mapped using the IGVI-465/Prins population, and all of them were mapped to one distal side of *Pm6*, with the smallest genetic distance of 1.0 cM (Fig. 3a). Comparative mapping in the two populations revealed that marker CINAU127 was 1.1 cM from the distal end of *Pm6* in the IGVI-465/Prins population but co-segregated with *Pm6* in the IGVI-466/Prins population, whereas marker CINAU123 delimited the proximal end and was 0.1 cM from *Pm6* in the IGVI-466/Prins population. Combined with the comparative analysis in rice and *Brachypodium*, the two conserved markers are the most closely linked markers that flank the *Pm6* locus (Fig. 3).

Several markers co-segregated in both populations (Fig. 3a, b). For example, CINAU135 and CINAU142 co-segregated in the IGVI-465/Prins population, but their orthologs are ~1.0 and ~1.6 Mb apart from each other in the collinear regions of *Brachypodium* and rice, respectively. Another two markers, CINAU117 and CINAU121, co-segregated in the IGVI-466/Prins population, and their orthologs are ~3.2 and ~3.3 Mb apart from each other in the collinear regions of *Brachypodium* and rice, respectively.

Collinearity analysis of the *Pm6* region of wheat with that in rice and *Brachypodium*

Totally, we mapped 29 markers (including the marker NAU/$STS_{BCD135-2}$ developed by Ji et al. (2008)) to the *Pm6* locus (Fig. 3a, b). All these corresponding EST sequences of *Pm6*-linked markers were used as queries to perform a BLAST search against the genome sequences of rice and *Brachypodium*; 89.6 and 79.3% of these ESTs had their orthologs in chromosome 5L of *Brachypodium* and chromosome 4L of rice. The order of these markers has remained highly conserved between rice and *Brachypodium*. However, there are re-arrangements between wheat and both rice and *Brachypodium*. For example, in the IGVI-466/Prins population, re-arrangements were observed between markers CINAU120 and CINAU123 and between CINAU142 and CINAU139 (Fig. 3).

Among the 14 markers co-segregating with *Pm6* in the IGVI-466/Prins population, 12 markers had their orthologs in the collinear regions of *Brachypodium* and rice. The collinear region covers ~2.2 Mb of genome sequence in *Brachypodium* and ~2.4 Mb of genome sequence in rice. In the ~2.4 Mb collinear region of rice, there are three putative resistance gene analog (RGA) clusters (Fig. 3d), including an LRR-receptor-like protein kinase cluster (LRR-RLK, containing five members), an NBS-LRR cluster (containing six members) and a serine/threonine protein kinase cluster (S/TPK, five members) (Fig. 3d). The LRR-RLK cluster is flanked by markers CINAU123 and CINAU127, the NBS-LRR cluster is flanked by markers CINAU131 and CINAU134, and the S/TPK cluster is flanked by markers CINAU135 and CINAU136 (Fig. 3). In the collinear region of *Brachypodium,* the rice LRR-RLK and S/TPK clusters are also present, but their members are reduced to two and four, respectively, while the NBS-LRR family is completely absent. Comparative mapping in the two mapping populations showed that four markers, CINAU131, CINAU134, CINAU135 and

CINAU136, covering the NBS-LRR and S/TPK clusters, were mapped to one distal side of *Pm6* (Fig. 3a, d), whereas markers CINAU123 and CINAU127, covering the LRR-RLK cluster, could span the *Pm6* locus (Fig. 3b, d). It was deduced that the identified LRR-RLK cluster in the orthologous regions of rice and *Brachypodium* might be the candidate orthologous genes for *Pm6*.

The orthologous regions of rice (a region of ~250 kb from 30,934,293 to 31,184,382 bp of chromosome 4) and *Brachypodium* (a region of ~190 kb from 24,210,919 to 24,401,317 bp of chromosome Bd5) flanked by two conserved markers, CINAU123 and CINAU127, were further analyzed using the Artemis Comparison Tool (Carver et al. 2005). The two regions were found to be well conserved both for gene number (28 for *Brachypodium* and 29 for rice) and gene order (Fig. 4). Five members of the LRR-receptor-like protein kinase gene cluster are present in the rice orthologous region, whereas only two members are present in *Brachypodium* (Fig. 4).

PCR-based markers for the marker-assisted selection of *Pm6*

The two most closely linked markers flanking the *Pm6* locus (Fig. 3), CINAU123 and CINAU127, were used for amplification in different varieties and lines possessing known powdery mildew resistance genes to verify their potential use in MAS of *Pm6* in wheat breeding. The results showed that only *T. timopheevii* and *Pm6*-carrying genotypes produced the expected band of ~470 bp for CINAU123 and the expected band of ~1,900 bp for CINAU127, whereas these bands were not observed in any of the genotypes without the *Pm6* gene (Fig. 5). Therefore, both markers could be helpful in the selection of the *Pm6* gene in breeding programs for powdery mildew resistance.

Discussion

Pm6 was previously mapped to the wheat chromosome bin of 2BL FL 0.50–1.00 with limited DNA markers (Tao et al. 2000; Ji et al. 2007, 2008). In the present study, we successfully developed and mapped 28 new markers to this region, and 14 of them were co-segregated with *Pm6* in the IGVI-466/Prins population. Comparative analysis with the genomes of rice and *Brachypodium* enabled us to identify two conserved markers (CINAU123 and CINAU127), which flank the *Pm6* locus, and the orthologous regions in the two model genomes. Our study is a successful example of using a comparative genomics approach to fine map genes in the non-sequenced wheat genome. The establishment of a high-resolution synteny picture between sequenced genomes such as rice, sorghum, maize, and *Brachypodium* and non-sequenced cereal genomes such as the Triticeae opens up exciting perspectives for using the model genomes to support the positional cloning of genes from the other genomes by a so-called cross-genome map-based cloning approach (Salse et al. 2009a, b). The positional cloning of vernalization genes (*Vrn1*, *Vrn2* and *Vrn3*) (Yan et al. 2003, 2004, 2006) and a high grain protein content gene (*GPC-1B*) (Distelfeld et al. 2004; Uauy et al. 2006) in wheat have benefited greatly from comparative genome analysis with the rice genome sequence. However, the isolation of the wheat disease resistance genes *Lr21* (Huang et al. 2003), *Lr10* (Feuillet et al. 2003) and *Pm3b* (Yahiaoui et al. 2004) have shown that wheat and rice have very limited collinearity in these regions due to massive genomic re-arrangements (Keller et al. 2005). The recent sequencing of the *Brachypodium* genome provides a new comparative genomics approach for the fine mapping and cloning of wheat genes. *Brachypodium* is more closely related to temperate cereals such as wheat than to tropical rice, both in whole-genome

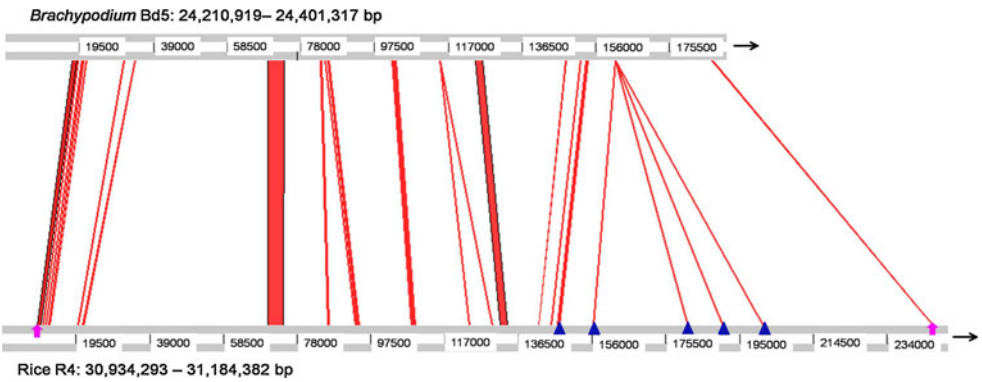

Fig. 4 Comparison of orthologous regions in rice and *Brachypodium* flanked by two conserved markers, CINAU123 and CINAU127, using the ACT tool (Carver et al. 2005). The lines on end connect the orthologous genes present in the same orientation; the *horizontal arrows* point toward the telomeres. The rice genes corresponding to markers, CINAU123 and CINAU127, are indicated by *vertical arrows*. Five members of the LRR-receptor-like protein kinase gene cluster in rice are indicated on their orthologous regions by *triangles*

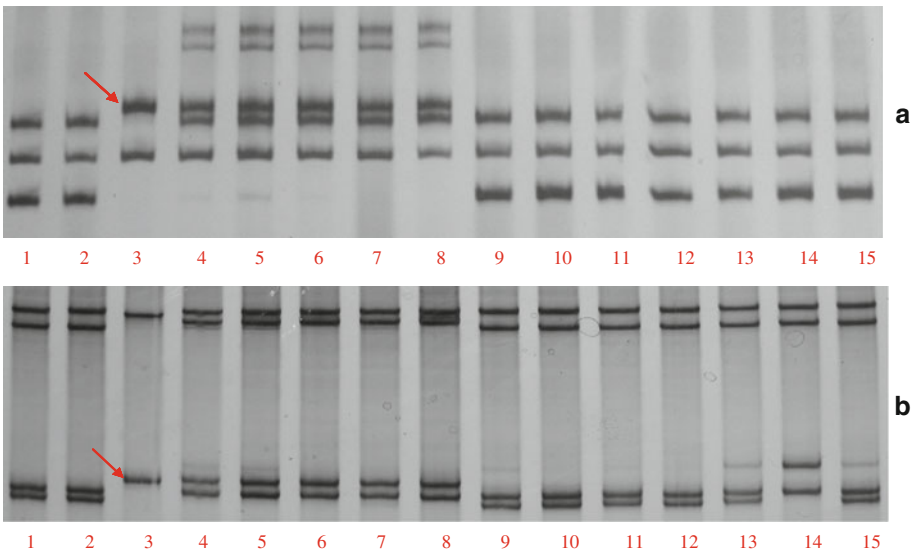

Fig. 5 Amplification of two conserved markers, **a** CINAU123 and **b** CINAU127, in 15 genotypes. The *arrows* show the specific bands amplified in the *Pm6*-carrying lines. *Lanes* 1–15: 1. Prins; 2. Chancellor; 3. *T. timopheevii* (*Pm6*); 4. IGVI-465 (*Pm6*); 5. IGVI-466 (*Pm6*); 6. IGVI-468 (*Pm6*); 7. IGVI-474 (*Pm6*); 8. Coker983 (*Pm5* + *6*); 9. Baitu 3 (*Pm4* + *8*); 10. Chul/8*Cc (*Pm3b*); 11. Khapli/8*Cc (*Pm4a*); 12. Ulka/8*Cc (*Pm2*); 13. Hope/8*Cc (*Pm5*); 14. Kavkaz (*Pm8*); and 15. Nannong 9918 (*Pm21*)

comparisons (The International Brachypodium Initiative 2010) and detailed analyses of particular gene regions (Bossolini et al. 2007). Both the rice and *Brachypodium* genomes were used to develop markers with which to saturate the wheat *Ph1* region and to reveal its gene content. *Ph1* orthologs were identified in the syntenic regions of *Brachypodium* and rice genomes (Griffiths et al. 2006). Thus, many genes and QTLs involved in developmental processes show good conservation between cereal genomes and are good candidates for direct 'cross-genome map-based cloning'. In our study, 89.6% and 79.3% of the corresponding EST sequences of the *Pm6*-linked markers had orthologs on *Brachypodium* chromosome 5L and rice chromosome 4L, respectively, suggesting that *Brachypodium* may be a better structural model. Although local inversions and re-arrangements were observed for the *Pm6* region in wheat (Fig. 3), including the rice genome in a three-way comparison with *Brachypodium* and wheat showed that flanking genes are conserved enough to provide a source of conserved markers (or COS markers) that can be used to saturate the target gene region in wheat.

Gene deletions and insertions were observed while comparing the orthologous regions of rice and *Brachypodium*. In the region between markers CINAU123 and CINAU127, nine genes found in rice had no orthologs in the corresponding region of the *Brachypodium* genome, and six predicted genes from the orthologous region of *Brachypodium* had no orthologs in the corresponding region of the rice genome (data not shown). The NBS-LRR gene cluster is completely deleted from the *Brachypodium* genome. Comparative mapping in the two populations and syntenic analyses between the three genomes helped us to identify a known LRR-RLK resistance gene analog cluster at the *Pm6* locus. Further efforts will be made to reveal the relationship between these putative LRR-RLK genes and *Pm6*.

Most of the race-specific genes are expressed throughout the entire vegetative life cycle of wheat; however, a few genes such as *Pm5* and *Pm6* are only fully expressed from the third to fifth leaf stage. In this study, we also observed that *Pm6* carriers were fully susceptible at the first leaf stage, but at the fourth leaf stage and later, they were stably resistant to the present *Bgt* population from Nanjing, Jiangsu province, China, indicating that these *Pm6*-carrying lines show a type of adult plant resistance (APR). Despite high frequency of virulence against the *Pm6* gene at the seedling stage, varieties carrying *Pm6* still possess an intermediate or high effective against the *Bgt* population in the field in some areas (Švec and Miklovičová 1998; Věchet 2006; Ji et al. 2008; Shi et al. 2009; Purnhauser et al. 2010). This difference between high virulence at the seedling stage and the low degree of infection in the field may be a consequence of higher resistance of adult plants depending on nonspecific resistance gene expression (Švec and Miklovičová 1998). QTLs for APR to powdery mildew have been mapped to the region near the *Pm6* gene from a Japanese wheat variety (Fukuho-komugi), a soft red winter wheat variety (Massey), and a French winter bread wheat line (RE9001) (Liu et al. 2001; Bougot et al. 2006; Liang et al. 2006). We couldn't exclude the possibility that the 2G chromosome segment of *T. timopheevii* present in the *Pm6*-carrying lines may also include a QTL for APR to powdery mildew, however, the genetic relationship of the race-specific resistance gene *Pm6* and the possible QTLs conferring APR to powdery mildew is worth to study in the future.

In this study, *Pm6* was inherited as a single dominant gene in the IGVI-465/Prins population. This result is in agreement with those of Ji et al. (2008), who used the

IGVI-465/Prins population and Tao et al. (2000), who used the IGVI-463/Prins population. The segregation for both the resistance phenotype and the co-dominant marker fit the expected 3:1 and 1:2:1 ratios, respectively, in the IGVI-465/Prins population; however, the segregation displayed a distorted pattern in the IGVI-466/Prins population, which indicated the preferential transmission of the *Pm6*-containing segment over the non-*Pm6* segment ($P < 0.01$) (Tables 2, 3). According to Tao et al. (2000) and Ji et al. (2007), the introgression lines IGVI-463 and IGVI-465 only contain a small segment of the long arm of chromosome 2G, whereas IGVI-466 contains segments from both arms as well as the centromere of chromosome 2G. The distorted segregation in the IGVI-466/Prins population might be due to the preferential transmission of the 2BS-2GS·2GL·2BL chromosome. Brown-Guedira et al. (1996) found that 2G (2B) substitution lines had normal morphology and fertility, but chromosome 2G was preferentially transmitted in the cytogenetic stocks. Similarly, previous reports have indicated that a *Sr36*-containing 2G chromosome segment exhibits preferential transmission, whereas the *Sr40*-containing 2G chromosome segment has shown inconsistent results in two different populations (Allard and Shands 1954; Brown-Guedira et al. 2003; Wu et al. 2009). Group-2 chromosomes with gametocidal genes may show preferential transmission and cause breaks in wheat chromosomes, and they have been isolated from several species (Tsujimoto 1995; Endo and Gill 1996). The chromosome 2G of *T. timopheevii* may also have a gametocidal gene. However, the exact mechanism causing the preferential transmission of the *T. timopheevii* chromosome segment remains unclear.

In spite of the distorted segregation of the population derived from the cross of IGVI-466 and Prins, this population was used successfully to increase the polymorphism near the *Pm6* locus and to identify markers flanking *Pm6* at the proximal side. Comparison and integration of the genetic maps obtained from the two populations enabled us to identify flanking markers to narrow the *Pm6* interval.

In this study, recombination was found to occur between the *Pm6* gene and all of its linked markers in the IGVI-465/Prins population, whereas some of these markers co-segregated with *Pm6* in the IGVI-466/Prins population, which indicates that there was significant suppression of recombination in the *Pm6* region. As there is a large 2G segment present in the introgression line IGVI-466, the relatively low homology between chromosomes 2B and 2G are likely the main cause of the substantial recombination suppression in the IGVI-466/Prins population. A similar compression of map distance has also been observed for the *Sr40*- and *Sr36*-containing 2G chromosome segments (Tsilo et al. 2008; Wu et al. 2009). It has been reported that different marker order was found for chromosomes 2B and 2G (Salina et al. 2006). We also observed a difference in the marker order for the two populations (Fig. 3a, b). This implies that there might be re-arrangements between chromosomes 2B and 2G.

Both the suppression of recombination and the complicated structure near the *Pm6* region make it difficult to find molecular markers that are physically close to or co-segregate with *Pm6*. We introduced different accessions of *T. timopheevii*, but we failed to identify any available accessions that were susceptible to powdery mildew. This hindered our attempt to construct a new population using *T. timopheevii* accessions for further fine mapping and map-based cloning of the *Pm6* gene. However, we have treated the resistant *T. timopheevii* with the mutagen EMS, and the identification of induced susceptible mutants is in progress.

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