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A "one-marker-for-two-genes" approach for efficient molecular discrimination of *Pm12* and *Pm21* conferring resistance to powdery mildew in wheat

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Abstract Powdery mildew, caused by *Blumeria graminis* f. sp. *tritici*, is one of the most important wheat diseases worldwide. Pyramiding different resistance genes into single cultivar has been proposed as one remedy to provide durable resistance. Powdery mildew resistance genes *Pm12* (T6BS-6SS.6SL), transferred from *Aegilops speltoides* to wheat cv. Wembley, and *Pm21* (T6VS.6AL), introduced from *Dasypyrum villosum* to wheat cv. Yangmai5, conferred broad-spectrum resistance to *B. graminis* f. sp. *tritici*. Both *Pm12* and *Pm21* genes are located on the short arms of homologous group six involved translocated chromosomes 6SS.6BL and 6VS.6AL, respectively. Simple sequence repeat motifs of wheat

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simple sequence repeat (SSR) and expressed sequence tag (EST) sequences on the short arm of homologous group six chromosomes were analyzed to develop molecular markers for discriminating chromosome arms 6AS, 6BS, 6DS, 6VS, and 6SS. One EST–SSR marker, *Xcau127*, was polymorphic, and therefore can be used to distinguish the two resistance genes and the respective susceptible alleles. This marker allowed us to develop an efficient "one-marker-for-two-genes" procedure for identifying powdery mildew resistance genes *Pm12* and *Pm21* for marker-assisted selection and gene pyramiding in wheat breeding programs.

Keywords *Pm12* · *Pm21* · Powdery mildew · SSR · Marker-assisted selection

Introduction

Wheat is one of the most important cereal crops worldwide, providing around 35% of food for human consumption. Powdery mildew, caused by *Blumeria graminis* f. sp. *tritici*, is a major disease of wheat, particularly in areas with cool or maritime climates. Deployment of diversified powdery mildew resistance genes proved an economical and environmentally sound method to control the disease. Pyramiding resistance genes into a single genotype was proposed as an effective way to achieve durable resistance. However, conventional breeding to combine resistance genes is a tedious process, which can be made more efficient by the use of molecular markers tightly linked to the resistance genes (Tanksley et al. 1989). To date, 39 powdery mildew resistance gene loci have been assigned to specific chromosomes and formally designated. Among them, 24 resistance genes/alleles have been tagged by molecular markers (Huang and Röder 2004; McIntosh et al. 2008).

Pm12, present in a wheat—*Aegilops speltoides* translocation (T6BS-6SS.6SL) (Miller et al. 1988) and *Pm21* in a series of wheat—*Dasypyrum villosum* translocation (T6VS.6AL) lines (Chen et al. 1995) confer resistance to all isolates of *B. graminis* f. sp. *tritici* tested in China (Duan et al. 1998), Europe (Huang and Röder 2004), and the USA (Niewoehner and Leath 1998). The translocation line carrying *Pm12* was tagged with a restriction fragment length polymorphism (RFLP) marker (Jia et al. 1996) and then with SSR and EST–SSR markers (Song et al. 2007), whereas *Pm21* has been tagged with RFLP (Li et al. 1995, 2005), random amplification of polymorphic DNA (RAPD) (Qi et al. 1996), sequence-characterized amplified region (SCAR) (Liu et al. 1999), and resistance gene analog (RGA) (Cao et al. 2006) markers. All these markers are translocation chromosome (arm) specific and therefore identify only *Pm12* or *Pm21*.

SSRs (or microsatellites), simple sequence length polymorphisms containing tandem repeats of basic motifs <6 bp, are abundant and highly polymorphic in eukaryotic genomes. High-density wheat SSR maps have been published (Röder et al. 1998; Somers et al. 2004; Song et al. 2005). Comparative genetic maps reveal close relationships between homoeologous chromosomes within hexaploid wheat and with other *Triticeae* species. Study of homoeologous group six RFLP markers indicated homoeologous relationships between *D. villosum* 6V and wheat chromosomes 6A, 6B, and 6D (Qi et al. 1999). *A. speltoides* chromosome 6S is very closely related to wheat chromosome 6B even though the species is longer considered to be the B genome donor of polyploid wheat (Kimber and Athwal 1972; Dvorak and Zhang 1990; Huang et al. 2002). The transferability of wheat genomic and EST SSR data to related cereals and grasses has been confirmed by various workers (Gupta et al. 2003; Kuleung et al. 2004; Zhang et al. 2005). Compared with genomic SSRs, EST–SSRs show a very high level of transferability across closely related species because they originate from transcribed regions that are conserved between genomes (Yu et al. 2004). In this paper we describe the results of a comparative sequence analysis of SSR repeat motifs between chromosomes 6AS, 6BS, 6DS, 6SS, and 6VS leading to development of a "one-marker-for-two-genes" assay, thus providing an efficient means to identify and combine *Pm12* and *Pm21* located in different alien segments.

Materials and methods

Plant materials

The *Pm12* and *Pm21* powdery mildew resistance gene donors Line #31 (6BS-6SS.6SL) and R149

(6VS.6AL) were kindly provided by Dr. X. Y. Duan, Chinese Academy of Agricultural Sciences, Beijing, and Prof. D. J. Liu, Nanjing Agricultural University. Common wheat cv. Jing 411 was used as the susceptible recurrent parent in backcrosses with Line #31 and R149 to develop near-isogenic lines (NILs), Line#31/8*Jing 411 with *Pm12* and R149/8*Jing 411 carrying *Pm21*. The BC_8F_2 segregating populations consisting of 190 and 72 individuals and their BC_8F_3 progenies were used for marker analysis of *Pm12* and *Pm21*, respectively. A F_2 population consisting of 85 individuals derived from intercross Line#31/8*Jing 411//R149/8*Jing 411 was selected for marker validation and gene pyramiding. *Pm21* cosegregated $SCAR_{1400}$ (Liu et al. 1999) and *Pm12*-linked SSR markers (Song et al. 2007) were used to identify the presence of 6VS and 6SS chromosome arms in each F_2 individuals. Polymorphic marker was also tested in diversified lines/cultivars containing different powdery mildew genes for allelic diversity (Table 2).

Chinese Spring (CS) ditelosomic lines (Dt6AS, Dt6AL, Dt6BS, Dt6BL, Dt6DS, and Dt6DL) of homoeologous group six (kindly provided by Drs. W. J. Raupp and B. S. Gill, Wheat Genetics Resource Centre, Kansas State University, USA) were used for chromosomal arm assignments of polymorphic markers.

Powdery mildew disease evaluation

Powdery mildew seedling reactions were determined as described by Liu et al. (2002). *B. graminis* f. sp. *tritici* race E09 was used for inoculations. Reactions were scored on a 0, 0;, and 1–4 infection type (IT) scale, with 0 representing no visible symptoms, 0; representing necrotic flecks, and 1, 2, 3, 4 for highly resistant, resistant, susceptible, and highly susceptible reactions, respectively. Resistant reaction of 20 seedlings of each F_3 progenies were tested to classify the F_2 individuals into three types, namely homozygous resistant (RR), heterozygous resistant (Rr), and homozygous susceptible (rr). Due to the complete resistance of *Pm12* and *Pm21*, the reactions of tested seedlings were almost all 0; (resistant) or 4 (susceptible) on this scale.

Genomic DNA isolation and PCR analysis

Genomic DNA was extracted from uninfected leaves by the cetyl trimethylammonium bromide (CTAB) method (Saghai-Maroof et al. 1984). Wheat SSR and EST markers physically mapped on homologous group six (http://wheat.pw.usda.gov, Randhawa et al. 2004) were screened to find polymorphisms between the NILs (Line#31/8*Jing 411 and R149/8*Jing 411) and recurrent parent Jing 411 DNA samples. Each polymerase chain reaction (PCR) was performed in a total volume of 10 μl containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dNTP, 25 ng of each primer, 50–100 ng genomic DNA, and 0.75 U Taq DNA polymerase. Amplifications were performed at 94°C for 3 min, followed by 45 cycles at 94°C for 1 min, 50–60°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. PCR products were separated on 8% nondenaturing polyacrylamide gels. Gels were silver-stained and photographed.

Chromosomal arm assignments of the polymorphic marker loci

Polymorphic SSR loci between the NILs and Jing 411 were assigned to chromosome arms using Chinese Spring homoeologous group six nullisomic–tetrasomics and ditelosomics.

Results

Polymorphic marker between *Pm12*, *Pm21*, and Jing 411

Among 180 genomic and EST–SSR primer pairs screened, EST–SSR marker *Xcau127* detected stable polymorphic DNA fragments differentiating the three genotypes, *Pm12* (Line#31/8*Jing 411), *Pm21* (R149/8*Jing 411), and Jing 411. The CAU127 primer pair amplified 6AS-, 6DS-, and 6SS-specific DNA bands in Line#31 and Line#31/8*Jing 411, but 6BS-, 6DS-, and 6VS-specific DNA bands in R149 and R149/8*Jing 411 (Fig. 1). After testing in two BC_8F_2 segregating populations, *Xcau127* was found to be cosegregated with the powdery mildew resistance genes, indicating the presence of *Pm12* and *Pm21* genes in the resistant plants, respectively.

Xcau127 was developed from wheat EST sequence CA640857 containing a trinucleotide simple sequence repeat motif $(CAG)_8$. Sequence comparison of CAU127 amplicons on *Pm12*, *Pm21*, and Jing 411 revealed simple sequence repeat motif $(CAG)_n$ variations

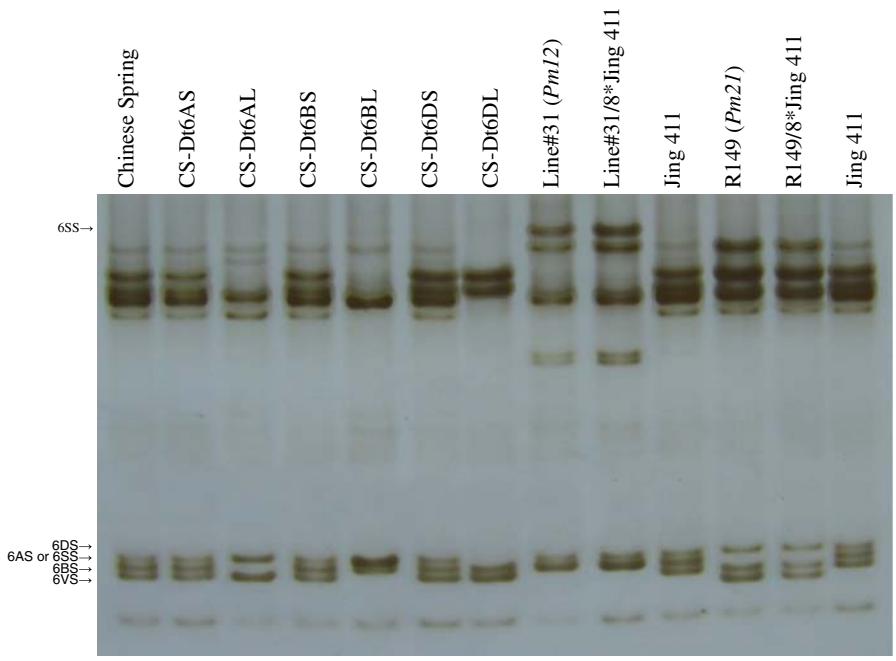

Fig. 1 Amplification pattern of EST–SSR primer CAU127 on Chinese Spring ditelosomics of homoeologous group six, the *Pm12*, *Pm21 donor lines*, and the NILs

between chromosome arms 6AS, 6BS, 6DS, 6SS, and 6VS (Fig. 2). CAU127 amplified 147-, 153-, 159-, 159-, and 165-bp DNA fragments in 6VS, 6BS, 6AS, 6SS, and 6DS, respectively. Both fragments amplified in 6AS and 6SS arms sized 159 bp which could not be distinguished by gel analysis. However, another polymorphic DNA

Fig. 2 imple sequence repeat (SSR) motif differences between CAU127 amplicons from 6DS, 6SS, 6AS, 6BS, and 6VS

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6DS   AAAGCAGCAGCAG - - - CTTGATCAGCAGCAGCAGCAGCAGCAGTCC
6SS   AAAGCAGCAGCAG - - - CTTGATCAGCAGCAGCAGCAG - - - - - - - TCC
6AS   AAAGCAGCAGCAGCAGCTTGATCAGCAGCAGCAGCAG - - - - - - - - - - TCC
6BS   AAAGCAGCAG - - - - - - - CTTGATCAGCAGCAGCAGCAG - - - - - - - - - - TCC
6VS   AAAGCAGCAG - - - - - - - CTTGATCAGCAGCAG - - - - - - - - - - - - - - - - - TCC
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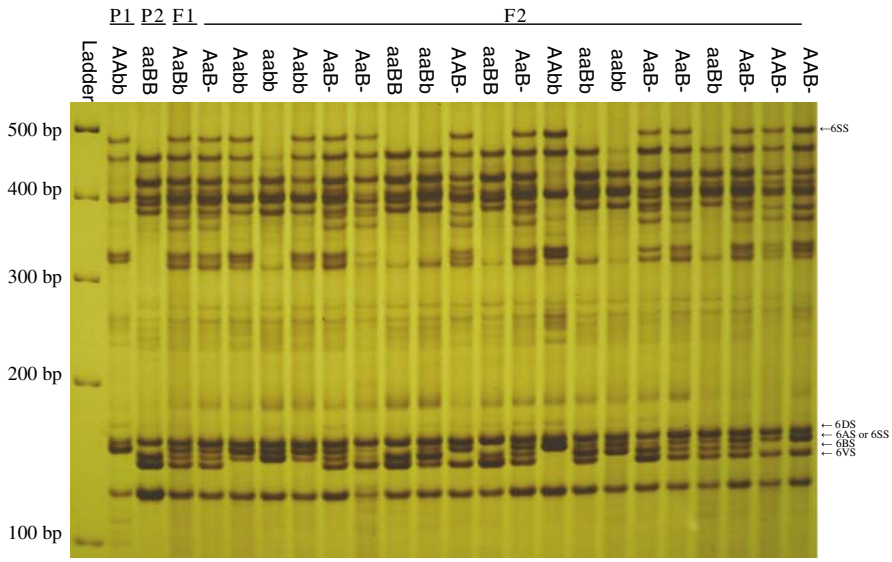

Fig. 3 Amplification patterns of EST–SSR CAU127 on Line#31/8*Jing 411 (P1, AAbb, *Pm12*), R149/8*Jing 411 (P2, aaBB, *Pm21*), Line#31/8*Jing 411//R149/8*Jing 411 (F_1, AaBb, *Pm12* + *Pm21*) and their F_2 different genotypes. *AA*, *Aa*, *bb*, *BB*, *Bb*, *bb* represent the homozygous resistant, heterozygous resistant, and homozygous susceptible genotypes of *Pm12* and *Pm21* alleles, respectively

band between 6SS and other chromosome arms could be detected on nondenaturing polyacrylamide gel (Fig. 1).

"One-marker-for-two-genes" approach for molecular discrimination of *Pm12* and *Pm21* genes

To test if SSR primer pair CAU127 could be used as molecular marker for simultaneously selection of *Pm12* and *Pm21*, the primer was used to amplify the F_2 individuals from Line#31/8*Jing 411//R149/8*Jing 411. *Xcau127* was able to identify both resistant alleles of *Pm12* (AAbb) and *Pm21* (aaBB) as well as the susceptible allele of Jing 411 (aabb) in the F_2 population. Among the nine possible genotypes derived from intercross *Pm12* (AAbb)/*Pm21* (aaBB), seven genotypes (AAB_, AaB_, AAbb, Aabb, aaBB, aaBb, and aabb) could be distinguished by amplification patterns of marker *Xcau127* in a single gel (Fig. 3; Table 1). The observed individuals number of genotypes A_B_, A_bb, aaB_, and aabb were 50, 14, 17, and 4, which is in agreement with two different genes segregating ratio of 9:3:3:1 ($x^2 = 0.59$). Presence of 6VS (*Pm21*) and 6SS (*Pm12*) chromosome arms in each F_2 plants was conformed by *Pm21* cosegregated $SCAR_{1400}$ and *Pm12*-linked SSR markers, respectively. Powdery mildew testing was also conducted on the F_3 progenies to identify the homozygous and heterozygous genotypes of each F_2 individual. Both phenotypic test and molecular markers results are in agreement with the *Xcau127* screening pattern.

Marker *Xcau127* was further validated in diversified wheat lines containing different powdery mildew resistance genes (Table 2). The *A. speltoides* 6SS- and

Table 1 The genotypes and phenotypes of 85 F_2 individuals derived from Line#31/8*Jing 411//R149/8*Jing 411

Genotype	Plant number	Infection type
AAB_	15	0;
AaB_	35	0;
AAbb	1	0;
Aabb	13	0;
aaBB	8	0;
aaBb	9	0;
aabb	4	4
Total	85	

Table 2 Allelic variation of *Xcau127* on diversified wheat lines containing different powdery mildew resistance genes

Lines/cultivars	*Pm* gene	IT	*Xcau127*
Chancellor	–	4	aabb
Axminster/8*Cc	1a	4	aabb
Ulka/8*Cc	2	0;	aabb
Asosan/8*Cc	3a	4	aabb
Chul/8*Cc	3b	4	aabb
Sonora/8*Cc	3c	4	aabb
Kolibri	3d	4	aabb
W150	3e	4	aabb
Mich. Amber/8*Cc	3f	4	aabb
Khapli/8*Cc	4a	0;	aabb
Armada	4b	0;	aabb
Hope/8*Cc	5a	4	aabb
Aquila	5b	0;	aabb
Coker 983	5a + 6	0;	aabb
Timgalen	6	2	aabb
Coker 747	6	2	aabb
CI 14189	7	1	aabb
Kavkaz	8	4	aabb
Wembley	12	0;	AAbb
R4A	13	0;	aabb
Brigand	16	0;	aabb
Amigo	17	1–	2
aabb			
XX186	19	4	aabb
KS93WGRC28	20	0;	aabb
Yangmai5—Sub 6V	21	0;	aaBB
NC96BGTA5	25	0;	aabb
87-1/C20//2*8866	30	0;	aabb
Maris Dove	2 + Mld	0;	aabb
Mission	4b + 5b	0;	aabb
Normandie	1 + 2 + 9	0;	aabb
Maris Huntsman	2 + 6	0;	aabb
Baimian 3	4 + 8	0;	aabb
Xiaobaidong	XBD	0;	aabb
Line#31/6*J190	12	0;	AAbb
Line#31/8*Jing 411	12	0;	AAbb
R149/7*Wen6	21	0;	aaBB
R149/8*Jing 411	21	0;	aaBB
R149/8*8866//*Pm30*/8*8866	21 + 30	0;	aaBB
Line#31/6*190//R137/4*J190	12 + 21	0;	A_B_
Line#31/4*207//R137/5*207	12 + 21	0;	A_B_
Line#31/8*Jing 411//R149/8*Jing 411	12 + 21	0;	A_B_

D. villosum 6VS-specific DNA fragments were found only on lines carrying genes *Pm12*, *Pm21* or both. No 6SS- or 6VS-specific DNA fragments were detected in any wheat cultivars or lines without *Pm12* or *Pm21* in their pedigrees in our breeding program, indicating a very low allelic diversity for *Xcau127* loci in wheat genome.

Discussion

While RFLP markers linked to *Pm12* (Jia et al. 1996) and *Pm21* (Li et al. 1995) have been reported, their use for marker-assisted selection is impractical in breeding program. PCR-based markers are more efficient as well as being safe and relatively inexpensive. RAPD, SCAR, and RGA markers linked to *Pm21* (Qi et al. 1996; Liu et al. 1999; Cao et al. 2006) have been developed and used extensively in marker-assisted selection of *Pm21* in wheat breeding programs. SSR markers linked to *Pm12* were also identified recently and used to select a series of introgression lines (Song et al. 2007). While being acceptable for selection of *Pm12* or *Pm21* alone, these markers are source specific and must be used separately for selection each gene. The marker *Xcau127* identified here has the advantage of enabling selection of both genes in a single reaction. *Pm12* and *Pm21* are highly effective powdery mildew resistance genes against all isolates of *B. graminis* f. sp. *tritici* tested in China (Duan et al. 1998), Europe (Huang and Röder 2004), and the USA (Niewoehner and Leath 1998). This "one-marker-for-two-genes" approach provides a highly efficient system for identifying and combining *Pm12* and *Pm21*. The low allelic variations and highly specific *Pm12* and *Pm21* polymorphic marker *Xcau127* provide potential applicability of this system for a wide variety of genetic backgrounds in breeding programs.

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