Theor Appl Genet (2006) 114:165–175
DOI 10.1007/s00122-006-0420-1

ORIGINAL PAPER

Development of functional markers specific for seven *Pm3* resistance alleles and their validation in the bread wheat gene pool

L. Tommasini · N. Yahiaoui · P. Srichumpa · B. Keller

Received: 12 May 2006 / Accepted: 30 September 2006 / Published online: 25 October 2006

Abstract In the ideal case, molecular markers used for marker-assisted selection are allele-specific even if the alleles differ only by a few nucleotide polymorphisms within the coding sequence of target genes. Such 'perfect' markers are completely correlated with the trait of interest. In hexaploid wheat (*Triticum aestivum* L.) the *Pm3* locus encodes seven alleles (*Pm3a–Pm3g*) conferring resistance to different races of *Blumeria graminis* f.sp. *tritici*, the agent of powdery mildew, a major disease of bread wheat. All *Pm3* alleles are known at the molecular level. Here, we generated specific markers for the *Pm3* alleles based on nucleotide polymorphisms of coding and adjacent non-coding regions. The specificity of these markers was validated in a collection of 93 modern or historically important cultivars and breeding lines of wheat and spelt (*Triticum spelta* L.). These markers confirmed the presence of the predicted *Pm3* alleles in 31 varieties and lines known to carry *Pm3* resistance alleles. In a few varieties, *Pm3* alleles different from alleles previously described based on pathogenicity tests or tightly linked markers were observed. In all these cases, the identity of the marker-detected *Pm3* alleles was confirmed by DNA sequence analysis. *Pm3* markers confirmed the absence of known *Pm3* resistance alleles in 54 European wheat and spelt varieties in which *Pm3* alleles had not been previously identified. These results indicate that the developed markers are highly diagnostic for specific *Pm3* resistance alleles in a wide range of varieties and breeding lines, and will be useful (1) for identifying *Pm3* alleles in the wheat gene pool, (2) for efficient marker-assisted selection of these genes, and (3) for combining multiple *Pm3* alleles within a single cultivar through transgenic approaches.

Communicated by P. Langridge.

Electronic supplementary material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00122-006-0420-1 and is accessible for authorized users.

L. Tommasini · N. Yahiaoui · P. Srichumpa · B. Keller (✉)
Plant Molecular Biology Department,
Institute of Plant Biology, University of Zürich,
Zollikerstr. 107, 8008 Zürich, Switzerland
e-mail: bkeller@botinst.unizh.ch

Present Address:
L. Tommasini
Department of Botany and Plant Sciences,
University of California, Riverside,
2150 Batchelor Hall, Riverside,
CA 92521-0124, USA

Present Address:
P. Srichumpa
Ubon Ratchathani Rice Research Center,
P.O. Box 65, Muang District,
34000 Ubon Ratchathani, Thailand

Introduction

Powdery mildew, caused by the biotrophic fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*), is a prevalent wheat (*Triticum aestivum* L., $2n = 42$, AABBDD) disease occurring world-wide in temperate climates. Cultivation of varieties with natural resistance is the most effective way to control the powdery mildew disease, while the fungicides currently used are not affordable in many wheat production areas. In addition, fungicides can cause environmental problems and their extensive use may speed up the selection for resistant pathogen races (Bennett 1984; Zeller and Hsam 1998).

Wheat powdery mildew (*Pm*) resistance genes are race-specific, follow the gene-for-gene model (Flor 1971) and the resistance reaction is associated with rapid host cell death. Thirty-two race-specific powdery mildew resistance genes (*Pm1–Pm32*) have been identified so far in the winter and spring hexaploid wheat gene pool, including land races and wild relatives of wheat (Huang and Röder 2004). *Pm3* was one of the first described loci among the *Pm* genes (Briggle and Sears 1966). *Pm3* is a single, dominant locus on the short arm of wheat chromosome 1A, and carries a higher number of alleles than other *Pm* genes. In fact, ten different resistance specificities (*Pm3a–Pm3j*) originating from the five continents have been identified in the wheat germplasm by classical genetic analysis (Zeller and Hsam 1998). *Pm3* alleles have been widely and successfully employed in breeding programs. Some of these alleles have remained effective in conferring resistance (http://www.racchangins.ch/doc/fr/chercheurs/amelior/datamoni/resul_monitor_f.html 2005; HGCA Annual Report 2005; Hsam and Zeller 2002; Bougot et al. 2002; Szunics et al. 2001; Svec and Miklovicova 1998).

The characteristics of most of the documented *Pm* resistance genes have mainly been determined by traditional genetic analyses including allelism tests, using a set of differential powdery mildew isolates that have been characterised for avirulence/virulence and lines with differential resistance (Zeller et al. 1993; Zeller and Hsam 1998). Classical genetic analyses do not always provide reliable information on resistance genes, due to (1) low genetic recombination between tightly linked genes, (2) limited number of pathogen isolates available to distinguish between different alleles and between multiple *Pm* genes with partially overlapping effects, and (3) environmental influences. In addition, these analyses require considerable resources for the management and maintenance of numerous isolate stocks and cumbersome infection procedures. On the contrary, genetic resistance analyses based on molecular markers are not affected by environmental variation, can be examined at any stage of plants' life cycle, provide higher genetic resolution, and are not affected by gene interaction, thus increasing the efficiency of gene identification and selection (Lande and Thompson 1990; Langridge et al. 2001; Feuillet and Keller 2004).

Different marker technologies have been used to tag resistance genes in wheat. The first type of molecular markers was represented mainly by restriction fragment length polymorphisms (RFLPs). These were subsequently replaced by PCR-based markers, particularly simple sequence repeats (SSRs) which are more abundant, require lower amount of DNA and are amenable to high throughput methods (Feuillet and Keller 2004). RFLP and SSR markers tightly linked to *Pm3* were previously identified (Hartl et al. 1993; Ma et al. 1994; Huang et al. 2004; Bougot et al. 2002), but were not specific enough to distinguish between some *Pm3* alleles (Bougot et al. 2002). The diagnostic power of these markers is limited by genetic recombination between marker and target locus. On the contrary, functional markers (Andersen and Lübberstedt 2003) derived from polymorphic sites within gene coding sequences causally affecting phenotypic trait variation, are more efficient than linked markers for gene identification and selection.

In different crop species, markers were successfully designed within coding sequences of resistance genes to detect allelic variation, e.g. for the *L* locus, conferring rust resistance in flax (Hausner et al. 1999) and for the *pvr1* gene for potyvirus resistance in *Capsicum* (Yeam et al. 2005).

Until recently, developing functional markers for *Pm* genes was not possible, because no *Pm* gene was isolated. However, the recent cloning of *Pm3b* by map-based cloning (Yahiaoui et al. 2004) followed by molecular and functional characterization of all *Pm3* resistance alleles (Srichumpa et al. 2005; Yahiaoui et al. 2006) opened up the possibility to design functional markers based on the coding region of each *Pm3* allele. *Pm3* genes encode a coiled-coil, nucleotide binding site, leucine-rich repeat (CC-NBS-LRR) type of disease resistance protein (Yahiaoui et al. 2004). *Pm3* is a member of a large gene family cluster that shows 84–94% sequence identity among its members and is spread over a region of 1 Mb on chromosomes 1A, 1B and 1D (Yahiaoui et al. 2004). Haplotype and sequence analysis of the *Pm3* alleles showed that *Pm3h*, *Pm3i* and *Pm3j* were identical to *Pm3d, Pm3c* and *Pm3b*, respectively, and that *Pm3* has seven different allelic resistance specificities (*Pm3a–Pm3g*). *Pm3a–Pm3g* alleles share 98.5% nucleotide sequence identity and 97% amino acid sequence identity (Srichumpa 2005; Yahiaoui et al. 2006). Haplotype analysis at the *Pm3* locus of two wheat lines susceptible to powdery mildew, the landrace Chinese Spring and the European cultivar Kanzler, indicated the presence of a *Pm3* allele in these lines showing 97–99% sequence identity to the *Pm3* resistance alleles (Yahiaoui et al. 2006). This susceptible allele, named *Pm3CS*, has probably been at the origin of the known *Pm3* resistance alleles (Yahiaoui et al. 2006).

Markers specific for each *Pm3* resistance allele would have important practical applications in wheat breeding. However, the high sequence similarity among *Pm3* alleles and among members of the *Pm3* gene family, the

large size of the wheat genome and its hexaploidy represent considerable challenges to the development of gene-specific markers. In this study, we developed seven *Pm3* allele-specific markers. The specificity of these markers was validated in a large collection of wheat and spelt varieties from throughout the world.

Materials and methods

Plant material for marker development

For developing allele-specific markers, 12 differential lines were used (Table 1). For the four *Pm3a*, *Pm3b*, *Pm3c* and *Pm3f* alleles, donor wheat lines (Asosan, Chul, Sonora and Michigan Amber, respectively) and four Near Isogenic Lines (NILs) developed from these lines in the genetic background of the susceptible wheat cultivar Chancellor (CC, Briggle 1969) were used. For *Pm3c*, Triticale/8*Chancellor was also used. The German Spring wheat 'Kolibri' (*Pm3d*), the Australian wheat line W150 (*Pm3e*, Zeller et al. 1993; Zeller and Hsam 1998) and the French wheat cultivar 'Aristide' (*Pm3g*, Sourdille et al. 1999) were donors of the other alleles. In addition, three wheat powdery mildew susceptible lines (Kanzler, Chancellor and Chinese Spring) were used as negative controls for primer development. Finally, an aneuploid nullitetrasomic line of Chinese Spring lacking chromosome 1A, but containing four copies of chromosome 1B (N1A/T1B) was also used (Sears 1966).

Wheat varieties used for the validation of the *Pm3* allele-specific markers

Validation of *Pm3* allele-specific markers was carried out using a wide range of agriculturally important wheat (*Triticum aestivum* L.) and spelt (*Triticum spelta* L.) varieties and their main progenitors. Thirty-three varieties known to contain *Pm3* alleles were identified based on database searches (European wheat database, http://www.genbank.vurv.cz/wheat/pedigree/gene1_2.asp, and germplasm resources information network, GRIN, http://www.ars-grin.gov/npgs/acc/acc_queries.html). For these accessions, seeds were obtained from the gene banks or directly from breeders.

The *Pm3* varieties examined in this study represent a large part of the global wheat material carrying *Pm3* alleles. Among these varieties, *Pm3a* and *Pm3c* are found in many lines from different countries. In contrast, very few lines are predicted to carry the *Pm3b* (Chul, Melon and GUS122), *Pm3e* (line W150 from Australia) and *Pm3f* alleles (Michigan Amber, USA). *Pm3d* is the most frequent allele in European varieties and is mainly present in lines from Germany. *Pm3g* is found with high frequency only in varieties from France.

A set of eight highly powdery mildew resistant breeding lines (Bougot et al. 2002) was provided by Dr. Trottet and Dr. Koenig. Their specific resistance genes were unknown because of the lack of isolates with corresponding virulence genes (Bougot et al. 2002). Finally, 52 varieties without *Pm3* alleles, representing part of the current and historically important European breeding germplasm from diverse genetic origin were screened (Siedler et al. 1994). These included winter and spring wheat, as well as spelt varieties.

Primer design and PCR conditions

Wheat genomic DNA was isolated according to the method used by Stein et al. (2001). The *Pm3* gene was amplified and sequenced as described in Srichumpa

Table 1 List of *Pm3* differential lines with known *Pm3* resistance alleles and of *Pm3* Near Isogenic Lines (NILs, Briggle et al. 1966) used for allele-specific primer development

Line name	Allele for powdery mildew resistance at the *Pm3* locus	Country of origin	Accession number of *Pm3* allele
Asosan	*Pm3a*	Japan	AY939880; DQ071555
Asosan/8*Chancellor[a]	*Pm3a*		–
Chul	*Pm3b*	Russia	AY325736
Chul/8*Chancellor[a]	*Pm3b*		–
Sonora	*Pm3c*	Mexico	DQ251487; DQ517917
Sonora/8*Chancellor[a]	*Pm3c*		–
Triticale/8*Chancellor[a]	*Pm3c*	USA	DQ251487
Kolibri	*Pm3d*	Germany	AY939881; AY605285
W150	*Pm3e*	Australia	DQ251488; DQ517518
Michigan Amber	*Pm3f*	USA	DQ071554; DQ0711556
Michigan Amber/8*Chancellor[a]	*Pm3f*		–
Aristide	*Pm3g*	France	DQ251489; DQ517919

[a] NILs eight times backcrossed into Chancellor (*CC*)

et al. (2005). RACE PCR was performed as described by Srichumpa et al. (2005). The 3′UTR of the remaining *Pm3* alleles were previously sequenced by Srichumpa et al. (2005). Accession numbers of *Pm3* allelic sequences are given in Table 1. Sequence assembly was performed using the Staden Package (http://www.staden.sourceforge.net/). Nucleotide sequences of *Pm3a–g* alleles were aligned using ClustalX (Thompson et al. 1997). Amino acid alignments were visualised and analysed using the software GeneDoc (http://www.psc.edu/biomed/genedoc/).

PCR thermal cycler conditions were optimised to obtain the lowest possible number of unspecific bands and the highest possible amplification signal for each allele-specific band on a set of differential lines (Table 1). The PCR reagents mixture (25 µl total volume) contained 65 ng wheat genomic DNA template, 0.2 µM forward primer, 0.2 µM reverse primer, 0.1 mM of each dNTP (Peqlab), 1.5 U Taq DNA polymerase (Sigma) and 1× PCR buffer (10 mM Tris–HCl pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatine). PCR amplifications were performed using a PTC-200 thermal cycler (MJResearch). The PCR cycling conditions were as follows: an initial denaturation step at 94°C for 3 min followed by 30 cycles of 94°C for 45 s, an annealing step at variable annealing temperatures depending on the primer pairs for 35 s, an elongation step of 1 min per kb at 72°C; and a final extension step at 72°C for 10 min. PCR-amplified *Pm3* allele bands were detected by standard gel electrophoresis on 1–1.2% agarose gels. In this paper allele names are indicated using italic font style, while primer names are indicated with the corresponding allele name followed by -/F for forward primers or -/R for reverse primers, using regular font style.

A primer pair UP3B (5′TGGTTGCACAGACA ATCC3′) and UP1A (5′GAAACCCGGCATAAGG AG3′) located in the *Pm3* promoter region, 4,360 bp upstream from the *Pm3* ATG start codon (Yahiaoui et al. 2006) was used to examine all the lines of this study to determine the presence or absence of a *Pm3* type of gene.

Results

Development of diagnostic markers for *Pm3* alleles

To develop seven *Pm3* allele-specific markers, each one being diagnostic for a specific resistance allele, we aligned and compared nucleotide coding sequences and the adjacent untranslated regions (UTRs) of the *Pm3* resistance alleles and of the *Pm3CS* susceptible allele. This allowed the identification of nucleotide polymorphisms that uniquely characterise each allele. The sequence alignments that were used for the design of primers specific for each *Pm3* resistance allele are presented in Suppl. Fig. 1.

Overall, a high level of similarity was observed among *Pm3* resistance allele sequences, corresponding to 98.5% nucleotide sequence identity and 97% amino acid sequence identity in the coding sequence and complete identity in an adjacent 4 kb of 5′ non-coding region (Yahiaoui et al. 2006). The information on sequence polymorphism was used to develop specific markers that allowed the amplification of PCR products of the optimal size for analysis on agarose gels. To obtain the highest allele specificity, primers were designed on the regions with highest nucleotide variation across *Pm3* alleles, within the desired size range of amplification products.

The *Pm3a, Pm3b, Pm3c*, and *Pm3f* alleles show blocks of sequence polymorphisms which allowed the design of primers containing several specific nucleotides. The *Pm3a* and *Pm3f* alleles were highly similar to each other and showed polymorphic sequence blocks which are only found in the LRR region of these two alleles. This feature was used to design one primer specific to both alleles (Pm3a/R and Pm3f/R, Fig. 1). To distinguish them, a second primer was developed on the unique LRR region which is different between the two genes (Fig. 1). The *Pm3b* marker was designed by combining a primer in the 5′ non-coding region

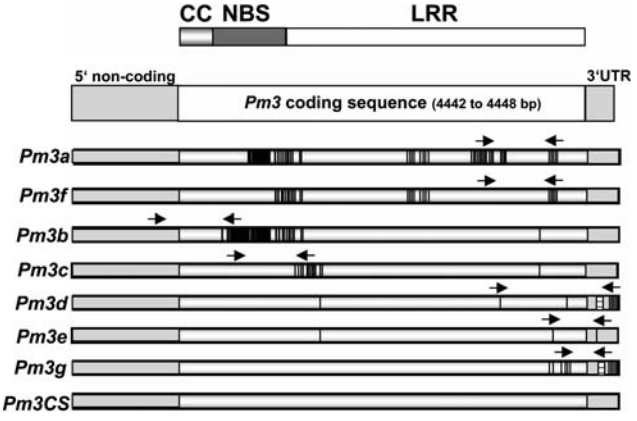

Fig. 1 Position of specific primers for *Pm3* resistance alleles. The genomic sequences of each *Pm3* allele (*Pm3a–Pm3g* and *Pm3CS*) including flanking 5′ non-coding and 3′ untranslated region (*UTR*) are shown. *Black bars* represent nucleotide polymorphisms in *Pm3* allelic sequences. The *striped box* in the 3′UTR of *Pm3d* and *Pm3g* represents a 10 bp insertion in these sequences compared to other *Pm3* alleles. A schematic representation of the encoded protein sequence is presented at the *top*. The location of Pm3 allele-specific primers is indicated by *arrows*. Size proportions of different parts of *Pm3* are not maintained for clarity of the figure

(300 bp upstream of the start codon) and a second primer in the NBS-coding region specific for this allele (Fig. 1). Similarly, the *Pm3c* specific marker was designed using a polymorphic sequence only present in this allele at the beginning of the LRR coding region.

The high sequence conservation between some alleles posed challenges to obtain markers specific for each of them. The *Pm3e*, *Pm3d* and *Pm3g* alleles show very few specific nucleotides in their coding sequences and they are also very close in sequence to the susceptible allele *Pm3CS* (Yahiaoui et al. 2006). To identify additional polymorphisms, the 3′UTRs of the *Pm3c*, *Pm3e* and *Pm3g* alleles were amplified using RACE PCR. The complete set of 3′UTR sequences was aligned and compared (Suppl. Fig. 1). Polymorphic regions included point and multiple mutations, and one small (10 bp) insertion in the 3′UTR unique to *Pm3d* and *Pm3g* compared to other *Pm3* alleles. For all three *Pm3* alleles (*Pm3d, Pm3e* and *Pm3g*) specific markers were developed by combining primers based on single nucleotide polymorphisms in the last third of the LRR-encoding region and primers based on polymorphic regions in the 3′UTR. As little as two nucleotide polymorphisms within the primers specific to *Pm3e* (Pm3e/F and Pm3e/R sequences), allowed to distinguish *Pm3e* from other *Pm3* alleles. The 10 bp InDel in the 3′UTR region of *Pm3* alleles was useful for designing the marker specific to the *Pm3g* allele. Thus, *Pm3* allele-specific markers were generated for each of the seven resistance alleles (*Pm3a–Pm3g*, Table 2, Fig. 2a–g).

The PCR profile for each allele-specific primer pair was optimised (Table 2) using differential *Pm3* donor and near-isogenic lines (Table 1) and negative control lines (Chinese Spring carrying *Pm3CS*, Kanzler carrying *Pm3CS*, and Chancellor which does not carry any *Pm3* allele). As a positive control for PCR amplification, a marker specific to the *Pm3*-haplotype was used in all lines tested with the *Pm3* allele-specific primers (UP3B/UP1A, Srichumpa et al. 2005; Yahiaoui et al. 2006). This *Pm3*-haplotype specific marker is located in the 5′ non-coding region of the *Pm3* gene. A maximum of two PCR fragments of different size are generated from each line by the *Pm3*-haplotype specific marker (Fig. 2h). The 0.9 kb fragment maps to chromosome 1A (A band) and indicates the presence of the *Pm3* haplotype, and the second fragment corresponds to a *Pm3* homoeologous gene on wheat chromosome 1B. The characteristic fragments amplified from differential lines by *Pm3* allele-specific markers are shown in Fig. 2. The size of allele-specific amplification products ranged from 524 bp for *Pm3e* to 1,382 bp for *Pm3b* (Table 2) and were easily resolved on 1.0–1.2% agarose gels. *Pm3* allele-specific bands showed complete reproducibility.

Validation of *Pm3* marker specificity in the wheat gene pool

Validation of the *Pm3* allele-specific markers developed above was performed using a set of 93 accessions of winter wheat and spelt, with lines and varieties derived from private and public sector breeding programs. This material includes a large part of the global wheat material carrying *Pm3* alleles (Tables 3, 4, Suppl. Table 1) together with lines not known to carry *Pm3* resistance alleles. In all the accessions screened with the *Pm3* allele-specific markers, not more than one allelic band per variety was amplified, confirming that *Pm3a–Pm3g* form a true allelic series. Genotype scores based on *Pm3* allele-specific markers were compared with data from previous studies based on phenotypic assessment and tightly linked SSR markers. In 28 out of 33

Table 2 Sequences of *Pm3* allele-specific primer sets used in this study

Marker function	Primer name	Primer sequence	Annealing temperature (°C)	PCR product size (bp)
Specific for *Pm3a*	Pm3a/F	gga gtc tct tcg cat aga	53	624
	Pm3a/R	cag ctt cta aga tca agg at		
Specific for *Pm3b*	Pm3b/F	ggc aca gac aaa gct ctg	58	1382
	Pm3b/R	tcg agt agc tcg gga atc		
Specific for *Pm3c*	Pm3c/F	cta gtg gag gta gtt gac	55	846
	Pm3c/R	agt cgt tca aga gaa cgg c		
Specific for *Pm3d*	Pm3d/F	tga cta ttc gtg ggt gca	58	1109
	Pm3d/R	gac tgc ggc aca gtt cag c		
Specific for *Pm3e*	Pm3e/F	gga atc cct ttg gct tgt	55	524
	Pm3e/R	cta gca gag cag tgc aag		
Specific for *Pm3f*	Pm3f/F	gga gtc tct ttg ctt aag	54	624
	Pm3f/R	cag ctt cta aga tca agg at		
Specific for *Pm3g*	Pm3g/F	gaa tcc ctt tat ctt gac	52	540
	Pm3g/R	att ccc cta gca gag cag aa		

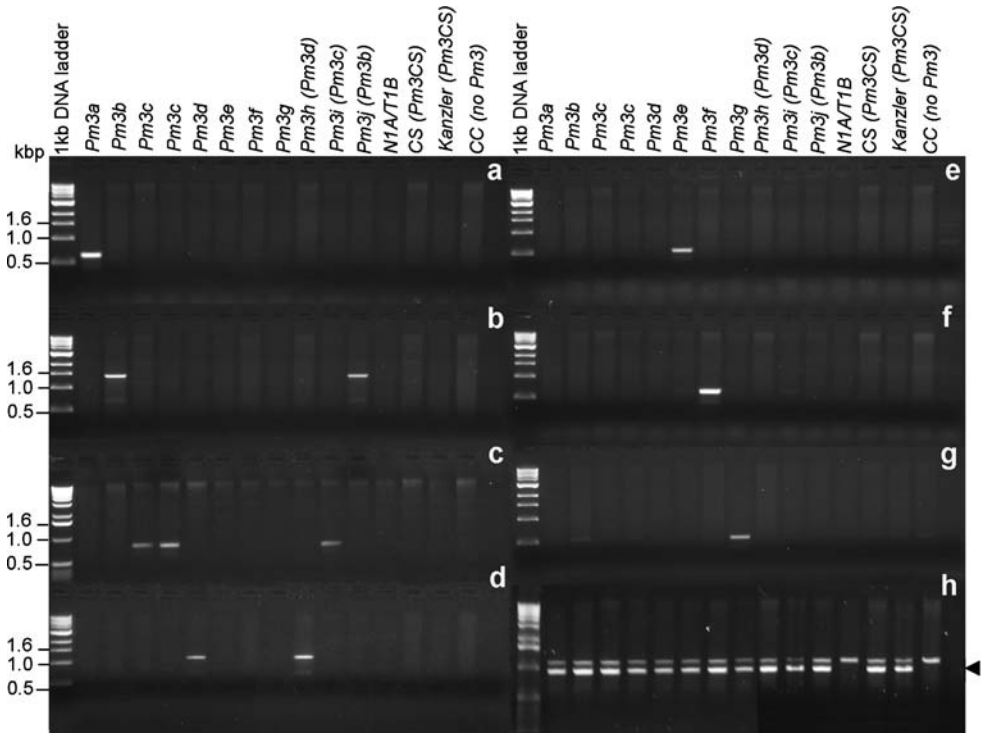

Fig. 2 *Pm3* allele-specific PCR markers amplify specific fragments in the *Pm3* differential lines. *Pm3* allele-specific marker bands amplified fragments ranging from 524 bp (for *Pm3e*) to 1,382 bp (for *Pm3b*) in differential lines. **a–g** Results of PCR analysis with markers for *Pm3a–Pm3g*. **h** Analysis of the *Pm3* haplotype using primer pair UP3B/UP1A. The *arrowhead* indicates the 0.9 kb fragment indicative of a *Pm3* haplotype on wheat chromosome 1A. The *upper* (1.1 kb) fragment indicates a *Pm3*-homoeologous gene amplified from chromosome 1B. The following wheat lines with different *Pm3* alleles were used: Asosan/8*CC (*Pm3a*), Chul/8*CC (*Pm3b*), Sonora/8*CC (*Pm3c*), Triticale/8*CC (*Pm3c*), Kolibri (*Pm3d*), W150 (*Pm3e*), Michigan Amber/8*CC (*Pm3f*), Aristide (*Pm3g*), Abessi (*Pm3h*), N324 (*Pm3i*), GUS122 (*Pm3j*). N1A/T1B is a nullisomic/tetrasomic line of Chinese Spring where chromosome 1A is replaced by an additional set of 1B chromosomes. Absence of a fragment in this line indicates that it is located on the 1A chromosome. The susceptible *Pm3CS* allele is present in the lines *CS* (Chinese Spring) and Kanzler. The line Chancellor (*CC*) does not have a *Pm3* gene. Sizes of the amplified PCR fragments are indicated in Table 2

varieties with known *Pm3* alleles and in seven out of eight highly resistant breeding lines, the *Pm3* allele-specific marker analysis agreed with earlier studies (Tables 3, 4). In some cases, the *Pm3*-marker analysis provided more precise information in terms of *Pm3* allelic content. For example, in four highly resistant breeding lines, for which a marker tightly linked to *Pm3*, SSR PSP2999 (Bougot et al. 2002) indicated 'no *Pm3c* and *Pm3d*', the *Pm3* allele-specific markers indeed amplified in each variety one specific *Pm3* allelic band different from *Pm3c* and *Pm3d* (Table 4). Similarly, in two other resistant lines, for which PSP2999 indicated 'probably *Pm3d*', a specific *Pm3d* allelic band was amplified by the *Pm3d* marker, whereas the other *Pm3* markers did not amplify any allele-specific band (Table 4). In one line (Oïd-90HD4-234), in which PSP2999 indicated 'no *Pm3c* and *Pm3d*, the *Pm3* allele-specific markers did not amplify any band from a *Pm3* resistance allele. In agreement with this result, sequence analysis revealed the presence of the susceptible *Pm3CS* allele (Table 4). We conclude that results from *Pm3* allele-specific markers are in good agreement with results from genetic and phytopathology studies.

In a few varieties, *Pm3* alleles which were not detected in earlier studies were amplified by the developed markers and were subsequently confirmed by sequence analysis. Infection tests suggested the presence of the *Pm3a* resistance in cultivar Florida302 (Leath and Heun 1990). In this line, the *Pm3* allele-specific markers did not amplify any characteristic *Pm3* allelic band (Table 3). In agreement with these results, sequence analysis revealed the presence of the susceptible allele *Pm3CS* (Table 3). Cultivar NCBGTA5 was predicted to have *Pm3a* based on pathogenicity tests (Shi et al. 1998; Murphy et al. 1999), whereas the *Pm3e* marker and sequence analysis of the *Pm3* allele in this cultivar confirmed the presence of a *Pm3e* allele (Table 3). This suggests that classical phytopathology tests were not sufficient for a precise characterization of the *Pm3* allele in this line. In one powdery mildew resistant breeding line, 90RHD4-219, for which microsatellite PSP2999 indicated 'no *Pm3c* and no *Pm3d*',

Table 3 Genotypes of *Pm3* wheat cultivars used for validating *Pm3* allele-specific markers

Variety Name	Expected *Pm3* allele	*Pm3* allele band amplified	Countr of origin	*Pm3* haplotype-specific marker (UP3B/UP1A)
Coker 797	*Pm3a*	*Pm3a*	USA	AB
Florida302	*Pm3a*[a]	*Chinese Spring*	USA	A
Hadden	*Pm3a*	*Pm3a*	USA	AB
Norin 29	*Pm3a*	*Pm3a*	Japan	AB
Norin 3	*Pm3a*	*Pm3a*	Japan	A
Saluda	*Pm3a*	*Pm3a*	USA	AB
NC96BGTA5	*Pm3a*[b]	*Pm3e*	USA	AB
Soprano	*Pm3a*	*Pm3a*	France	AB
OK-75-R-3645	allelic or closely linked to *Pm3*[c]	–	USA	B
Glory	*Pm3a*	*Pm3a*	USA	AB
GUS122	*Pm3b (Pm3j)* [d]	*Pm3b*	Russia	AB
Melon	*Pm3b*[e]	–	Germany	B
Cawnpore	*Pm3c*	*Pm3c*	USA	AB
Indian	*Pm3c*	*Pm3c*	USA	AB
Sturgeon	*Pm3c*	*Pm3c*	USA	AB
N324	*Pm3c (Pm3i)*[d]	*Pm3c*	Nepal	AB
Wolkoren	*Pm3c*	*Pm3c*	South Africa	AB
Borenos	*Pm3c*	*Pm3c*	Germany	AB
Abessi	*Pm3d (Pm3h)* [d]	*Pm3d*	Germany	AB
Lavett	*Pm3d*	*Pm3d*	Sweden	AB
Munk	*Pm3d*	*Pm3d*	Germany	AB
Quattro	*Pm3d*	*Pm3d*	Germany	AB
Ralle	*Pm3d*	*Pm3d*	Germany	AB
Mieka	*Pm3d*	*Pm3d*	Germany	AB
Axona	*Pm3d*	*Pm3d*	Netherlands	AB
Devon	*Pm3d*	*Pm3d*	UK	AB
Gullen	*Pm3e*[f]	–	Australia	B
ABO (= ABO772)	*Pm3g*	*Pm3g*	France	AB
Champetre	*Pm3g*[g]	–	France	B
Courtot	*Pm3g*	*Pm3g*	France	AB
Lutin	*Pm3g*	*Pm3g*	France	AB
Rubens	*Pm3g*	*Pm3g*	France	AB
Soissons	*Pm3g*	*Pm3g*	France	AB

A is a PCR band indicative of the *Pm3*-haplotype, amplified from chromosome 1A; *B* is a PCR band indicative of a *Pm3*-homoeologous gene amplified from chromosome 1B; - no amplification of *Pm3* allele band. References for the expected alleles can be found at the European wheat database (http://www.genbank.vurv.cz/wheat/pedigree/gene1_2.asp) and at the germplasm resources information network (GRIN) website, at: http://www.ars-grin.gov/npgs/acc/acc_queries.html)

[a] Murphy et al. (1999); [b] Shi et al. (1998); [c] Chung and Griffey (1995); [d] Yahiaoui et al. (2006); Huang et al. (2004); [e] Beschreibende Sortenliste Getreide (1997); [f] Zeller et al. (1993); [g] McIntosh at al. (2003)

Table 4 Molecular identification of *Pm3* alleles in highly powdery mildew resistant breeding lines (Bougot et al. 2002) using *Pm3* allele-specific markers and a *Pm3*-haplotype specific marker

Variety name	Expected *Pm3* allele	*Pm3* allele found	Country of origin	*Pm3* haplotype-specific marker (UP3B/UP1A)
Oïd 91-35	*noPm3c* and *Pm3d*	*Pm3e*	USA	AB
Oïd 92-35	*no Pm3c* and *Pm3d*	*Pm3a*	USA	AB
90RHD4-215	*no Pm3c* and *Pm3d*	*Pm3g*	France	AB
90RHD4-219	*no Pm3c* and *Pm3d*	*Pm3d*	France	A
90RHD4-225	*no Pm3c* and *Pm3d*	*Pm3g*	France	AB
90RHD4-234	*no Pm3c* and *Pm3d*	*Pm3CS*	France	AB
90RHD4-266	*Pm3d?*	*Pm3d*	France	AB
90RHD4-273	*Pm3d?*	*Pm3d*	France	A

A is a PCR band indicative of the *Pm3*-haplotype, amplified from chromosome 1A; *B* is a PCR band indicative of a *Pm3* homoeologous gene amplified from chromosome 1B

the *Pm3d* marker amplified a characteristic *Pm3d* fragment (Table 4). Finally, in the case of OK-75-R-3645, Melon, Gullen and Champetre, where the *Pm3a* (OK-75-R-3645, http://www.genbank.vurv.cz), *Pm3b* (Melon, http://www.genbank.vurv.cz), *Pm3e* (Gullen, http://www.genbank.vurv.cz) and *Pm3g* (Champetre, Bougot et al. 2002) alleles were respectively detected based on classical genetic analyses, *Pm3* allele-specific markers did not amplify any characteristic *Pm3* allelic band (Table 3). This could be due to the absence of the *Pm3* gene in these lines. In the varieties found to carry *Pm3* alleles (33 varieties and eight highly resistant breeding lines, Tables 3, 4), the *Pm3* haplotype-specific marker (UP3B/UP1A) always amplified the characteristic *Pm3*-haplotype band. In the case of OK-75-R-3645, Melon, Gullen and Champetre, the UP3B/UP1A marker did not amplify the 0.9 kb fragment from the *Pm3* promoter identifying the *Pm3* haplotype, in agreement with the absence of amplification of *Pm3* allele-specific markers (Table 3). This confirms that no *Pm3* resistance allele is present in these lines. We conclude that *Pm3* allele-specific markers were highly specific within the complete set of lines screened.

Among the 52 varieties not known for carrying *Pm3* alleles and analysed here (Suppl. Table 1), (1) Ten varieties showed neither the characteristic *Pm3*-haplotype band, nor the *Pm3*-homoeologous band on chromosome 1B (Suppl. Table 1), (2) 19 were missing the characteristic *Pm3*-haplotype band and showed only the *Pm3* homoeologous band on chromosome 1B, and (3) 22 showed the characteristic *Pm3*-haplotype band. Our markers for the *Pm3* resistance alleles did not detect any of the known *Pm3* resistance alleles (*Pm3a–Pm3g*) in these lines. Several of these lines were shown to carry either the susceptible *Pm3CS* allele or new *Pm3* sequences (Yahiaoui et al. 2006). We conclude that the indications from *Pm3* allele-specific markers were in agreement with the *Pm3* haplotype-specific markers and with sequence analysis of the *Pm3* genes in different wheat lines.

Discussion

We have developed seven PCR-based markers which successfully distinguished the allelic series of powdery mildew resistance genes at the locus *Pm3* in wheat (*Pm3a–Pm3g*). These markers were designed on polymorphisms within *Pm3* coding and adjacent non-coding regions, including single and multiple nucleotide polymorphisms and a small InDel.

The existence of a *Pm3* gene family on the three group 1 wheat chromosomes together with the high level of nucleotide sequence conservation among *Pm3* alleles made the development of allele-specific markers challenging. To develop allele-specific primers, various primer combinations were tested. Primers were designed mainly in the terminal parts of the *Pm3* coding region and in the 3′UTR region, due to the higher sequence polymorphism of these regions. Similarly, the 3′terminus of the *L* locus was more informative regarding the development of markers specific for the *L* alleles conferring rust resistance specificity in flax (Hausner et al. 1999). The adjustment of primer annealing temperatures was critical to primer specificity and allowed to eliminate unspecific bands and to obtain strong signals for specific allelic fragments. This was also observed in the development of allele-specific markers for the *FAD2* gene controlling oleic acid in spring turnip rape (Tanhuanpää et al. 1998).

The application of the *Pm3* allele-specific markers will possibly require a high throughput, and it might be desirable to screen each sample with multiple *Pm3* markers. Therefore, the development of multiplex PCR for *Pm3* alleles would be advantageous. Markers with similar annealing temperatures but major size differences of the amplified fragments could possibly be combined in one reaction. Preliminary experiments in our lab have shown that markers for *Pm3a* and *Pm3c*, as well as *Pm3b* and *Pm3d*, can be multiplexed (N. Yahiaoui, unpublished data).

Validation of the *Pm3* allele-specific markers developed above was performed using a large set of wheat cultivars and breeding lines, including most of the varieties known for carrying *Pm3* alleles. Genotype scores based on *Pm3* allele-specific markers were compared with the *Pm3* determination based on phenotypic assessment and tightly linked markers from previous studies, and a good agreement was found. In the few cases of discrepancy between *Pm3* allele-specific markers and phenotypic assessment, the specificity of *Pm3* allele markers was always confirmed by *Pm3* sequence analysis or, in the varieties where the *Pm3* locus was absent, by the *Pm3* haplotype-specific marker. For example, Florida302, showed the presence of the susceptible allele *Pm3CS* instead of the previously predicted *Pm3a* allele (Leath and Heun 1990). Therefore, in Florida302 *Pm* resistance must derive from a gene different from *Pm3* or from a combination of different *Pm* genes. Accordingly, Leath and Heun (1990) suggested that Florida302, besides *Pm3a*, should carry further *Pm* resistance, as shown by comparison with reactions to cultivars virulent to *Pm3a*. In the case of OK-75-R-3645, Melon, Gullen and Champetre, different *Pm3* alleles were detected based on classical genetic analyses in previous studies, whereas *Pm3*

allele-specific markers and the *Pm3*-haplotype specific marker did not amplify any characteristic *Pm3* fragment. Therefore, in these varieties, *Pm* resistance must also derive from a gene different from *Pm3* or from a combination of different *Pm* genes. The difficulty of an accurate evaluation of *Pm* genes based on phenotypic assays lies in the complexity of the genetic control of powdery mildew resistance in wheat, increased by the complexity of avirulence and virulence factors of powdery mildew isolates. *Pm* genes can interact and mask each other, making the identification and selection for *Pm* genes daunting or impossible based only on phenotypic evaluation. Therefore, to unravel the genetics of *Pm* resistance in wheat, a high number of powdery mildew isolates is required (Zeller and Hsam 1998), but in some cases a sufficient number of isolates is not available. E.g., the fact that infection tests with *Pm3* race-specific isolates in Florida302 and NCBGTA5 were carried out with a limited number of isolates specific only for *Pm3a, Pm3b* and *Pm3c* (Shi et al. 1998; Leath and Heun 1990) could explain discrepancies between molecular and phenotypic assessment in these varieties. Alternatively, discrepancies between molecular data from our study and phenotypic data from previous studies could be caused by genetic diversity within varieties or breeding material classified with the same name.

We could compare in this work the information provided by a closely linked molecular marker and the allele specific *Pm3* markers. The microsatellite PSP2999 previously described as a marker for the *Pm3* locus did provide reliable information on the *Pm3* allelic content of wheat lines. However, this information was only partial and in many cases only suggested presence or absence of a group of alleles (e.g. no *Pm3c* and *Pm3d* in Oïd 91-35 and others). The specific markers for the *Pm3* alleles allowed the precise characterization of the genotype at the *Pm3* locus of these lines. In a few cases of disagreement between *Pm3* allele-specific markers and tightly linked molecular markers (for instance, in line 90RHD4-219), the specificity of *Pm3* allele-specific markers was always confirmed by *Pm3* sequence analysis. Discrepancies between *Pm3* allele-specific markers and tightly linked markers, such as PSP2999, are probably due to recombination between *Pm3* and these marker loci or to mutations at SSR loci, which generally evolve faster than their target gene located nearby. While tightly linked molecular markers are not able to predict the target trait with complete accuracy, markers developed on polymorphims within gene coding regions, as the *Pm3* allele-specific markers developed here, are completely accurate in detecting the trait of interest. In fact, if alleles are very closely related such as in the case of *Pm3* resistance genes, tightly linked markers will never be completely diagnostic and knowledge of the target sequence is essential. Previous studies demonstrated the advantages of allele specific markers in several crop species. For instance, markers were successfully designed within coding sequences of different alleles of the *L* locus, a rust resistance gene in flax (Hausner et al. 1999) and for the *pvr1* gene for potyvirus resistance in *Capsicum* (Yeam et al. 2005). In hexaploid wheat, markers were designed within the coding region of the *puroindoline b* gene controlling grain texture (Huang and Röder 2005), within *Rht1* and *Rht2* controlling dwarfism (Ellis et al. 2002) and within *Gli-1* coding for a γ-gliadin (Zhang et al. 2003). Similarly, in this study we developed allele-specific markers within coding regions of the *Pm3* alleles, representing the first example of functional allelic markers for a disease resistance gene in wheat. By validating these markers in a large germplasm collection, we demonstrated that they were highly specific to *Pm3* alleles amongst a wide selection of wheat and spelt cultivars. Therefore, the developed markers will improve accuracy in identifying loci for powdery mildew resistance in the wheat gene pool and in selecting resistant cultivars within genetic improvement programs. In a particular example, the characterization of the new powdery mildew resistance in the lines Abessi (*Pm3h*), N324 (*Pm3i*), and GUS122 (*Pm3j*) could have been facilitated by the use of the *Pm3* markers. New powdery mildew resistance was identified in these lines and assigned to the *Pm3* locus suggesting the presence of new *Pm3* alleles (Huang et al. 2004). Sequence analysis showed that the *Pm3d, Pm3c* and *Pm3b* alleles were in fact present respectively in the Abessi, N324 and GUS122, suggesting that the new resistance in these lines is conferred by an additional locus. The presence of *Pm3d, Pm3c* and *Pm3b* alleles probably interfered with the characterization of the additional resistance present in these lines.

Considering that *Pm3* resistance alleles have been deployed globally in wheat breeding for about 70 years (Hsam and Zeller 2002), *Pm3* allele-specific molecular markers are likely to be widely relevant in public and private sector wheat-breeding programs. In this study, large part of the global wheat material carrying *Pm3* alleles was screened with allele-specific markers. In this material, one group of *Pm3* alleles (*Pm3a*, *Pm3c, Pm3e* and *Pm3f*) is mainly present in varieties deriving from regions outside Europe and rarely used in European breeding programs, suggesting that they could provide effective resistance in European countries in combination with other *Pm* genes (http://www.racchangins.ch/doc/fr/chercheurs/amelior/datamoni/resul_monitor_f.html, 2005; Szunics et al. 2001; Svec and Miklovicova 1998).

A second group of alleles (*Pm3b, Pm3d* and *Pm3g*) is predominantly found in European-derived varieties. The wide cultivation of *Pm3d* and *Pm3g* varieties throughout Europe suggests that higher virulence rates for these alleles than for the first group of alleles might be present in Europe, although limited evidence from recent virulence surveys is available (http://www.racchangins.ch/doc/fr/chercheurs/amelior/datamoni/resul_monitor_f.html, 2005). Until now, breeding strategies for improving powdery mildew resistance have mainly relied on the use of single major resistance genes, due to the technical difficulties of combining multiple resistance genes within single cultivars based on classical breeding approaches and of identifying multiple disease resistance genes based on classical genetic approaches. However, for obtaining a wider resistance spectrum to several powdery mildew races and durable resistance, it will be necessary to combine multiple *Pm* genes within single cultivars (Punja 2001). The molecular markers developed here open up the opportunity to (1) efficiently identify *Pm3* alleles in the wheat gene pool, (2) select for *Pm3* alleles through classical breeding methods, (3) control the deployment of *Pm3* genes in strategies for crop disease control, such as in cultivations of mixtures of varieties carrying different resistance specificities, (4) combine different *Pm3* alleles within single lines through transgenic technology, and (5) study transgenic expression and stability of transgenic lines across generations.

Acknowledgments This work was supported by grant no. 3100AD-105620 of the Swiss National Science Foundation and by the University of Zurich. We thank Dr. D. Fossati, FAL Reckenholz, the National Small Grains Research Facility (Aberdeen, Idaho), Dr. F.J. Zeller, Dr. R. Parks, Dr. J. Enjalbert, Prof. W. E. Weber, Dr. A. Spanakakis, Dr. M. Trottet and Dr. J. Koenig who kindly supplied us with seeds of the varieties screened.

References

Andersen JR, Lübberstedt T (2003) Functional markers in plants. Trends Plant Sci 8:1360–1385

Bennett FGA (1984) Resistance to powdery mildew in wheat: a review of its use in agriculture and breeding programmes. Plant Pathol 33:279–300

Beschreibende Sortenliste Getreide (1997) Mais, Oelfruechte, Leguminosen, Hackfruechte. Herausgegeben vom Bundessortenamt. Hannover

Bougot Y, Lemoine J, Pavoine MT, Barloy D, Doussinault G (2002) Identification of a microsatellite marker associated with *Pm3* resistance alleles to powdery mildew in wheat. Plant Breed 121:325–329

Briggle LW (1969) Near-Isogenic lines of wheat with genes for resistance to *Erysiphe graminis* f. sp. *tritici*. Crop Sci 9:70–72

Briggle LW, Sears ER (1966) Linkage of resistance to *Erysiphe graminis* f. sp. *tritici* (*Pm3*) and hairy glume (*Hg*) on chromosome 1A of wheat. Crop Sci 6:559

Chung YS, Griffey CA (1995) Powdery mildew resistance in winter wheat: II. Identity of resistance genes. Crop Sci 35:383–388

Ellis MH, Spielmeyer W, Gale KR, Rebetzke GJ, Richards RA (2002) "Perfect" markers for the *Rht-B1b* and *Rht-D1b* dwarfing genes in wheat. Theor Appl Genet 105:1038–1042

Feuillet C, Keller B (2004) Molecular markers for disease resistance: the example wheat. In: H. Lörz and G. Wenzel (eds) Biotechnol Agric For, Vol. 55, Springer Berlin Heidelberg New York, pp 353–364

Flor HH (1971) Current status of gene-for-gene concept. Annu Rev Phytopathol 9:275–296

Hartl L, Weiss H, Zeller FJ, Jahoor A (1993) The use of RFLP markers for the identification of alleles of the *Pm* locus conferring powdery mildew resistance in wheat (*Triticum aestivum* L.). Theor Appl Genet 86:959–963

Hausner G, Rashid KY, Kenaschuk EO, Procunier JD (1999) The development of codominant PCR/RFLP based markers for the flax rust-resistance alleles at the *L* locus. Genome 42:1–8

Hsam SLK, Zeller FJ (2002) Breeding for powdery mildew resistance in common wheat (*Triticum aestivum* L.). In: Bléanger RR, Bushnell WR, Dik AJ, Carver TLW (eds) The powdery mildews a comprehensive treatise. APS Press, St Paul, pp 219–238

Huang X-Q, Röder MS (2004) Molecular mapping of powdery mildew resistance genes in wheat: a review. Euphytica 137:203–223

Huang XQ, Röder MS (2005) Development of SNP assays for genotyping the *puroindoline b* gene for grain hardness in wheat using pyrosequencing. J Agric Food Chem 53:2070–2075

Huang X-Q, Hsam SLK, Mohler V, Röder MS, Zeller FJ (2004) Genetic mapping of three alleles at the *Pm3* locus conferring powdery mildew resistance in common wheat (*Triticum aestivum* L.). Genome 47:1130–1136

Lande R, Thompson R (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743–756

Langridge P, Lagudah ES, Holton TA, Appels R, Sharp PJ et al. (2001) Trends in genetic and genome analyses in wheat: a review. Aust J Agric Res 52:1043–1077

Leath S, Heun M (1990) Identification of wheat powdery mildew resistance genes in cultivars of soft red winter wheat. Plant Dis 74:747–752

Ma ZQ, Sorrels ME, Tanksley SD (1994) RFLP markers linked to powdery mildew resistance genes *Pm1, Pm2, Pm3*, and *Pm4* in wheat. Genome 37:871–875

McIntosh RA, Devos KM, Dubcovsky J, Morris CF, Rogers WJ (2003) Catalogue of gene symbols for wheat. http://www.wheat.pw.usda.gov/ggpages/wgc/2003upd.html

Murphy JP, Leath S, Huynh D, Navarro RA, Shi A (1999) Registration of NC96BGTA4, NC96BGTA5, and NC96BGTA6 wheat germplasm. Crop Sci 39:883

Punja ZK (2001) Genetic engineering of plants to enhance resistance to fungal pathogens—a review of progress and future prospects. Can J Plant Pathol 23:216–235

Sears ER (1966) Nullisomic-tetrasomic combinations in hexaploid wheat. In: Riley R, Lewis KR (eds) Chromosome manipulations and plant genetics. Oliver and Boyd, Edinburgh, pp 29–45

Shi AN, Leath S, Murphy JP (1998) A major gene for powdery mildew resistance transferred to common wheat from wild Einkorn wheat. Phytopathology 88:144–147

Siedler H, Messmer MM, Schachermayr GM, Winzeler H, Winzeler M et al. (1994) Genetic diversity in European wheat and spelt breeding material based on RFLP data. Theor Appl Genet 88:994–1003

Sourdille P, Robe P, Tixier MH, Doussinault G, Pavoine MT et al. (1999) Location of *Pm3g*, a powdery mildew resistance allele in wheat, by using a monosomic analysis and by identifying associated molecular markers. Euphytica 110:193–198

Srichumpa P, Brunner S, Keller B, Yahiaoui N (2005) Allelic series of four powdery mildew resistance genes at the *Pm3* locus in hexaploid bread wheat. Plant Physiol 139:885–895

Stein N, Herren G, Keller B (2001) A new DNA extraction method for high-throughput marker analysis in a large-genome species such as *Triticum aestivum*. Plant Breed 120:354–356

Svec M, Miklovicova M (1998) Structure of populations of wheat powdery mildew (*Erysiphe graminis* DC f.sp. *tritici* Marchal) in Central Europe in 1993–1996: I. Dynamics of virulence. Eur J Plant Pathol 104:537–544

Szunics L, Szunics L, Vida G, Bedo Z, Svec M (2001) Dynamics of changes in the races and virulence of wheat powdery mildew in Hungary between 1971 and 1999. Euphytica 119:143–147

Tanhuanpää P, Vilkki J, Vihinen M (1998) Mapping and cloning of *FAD2* gene to develop allele-specific PCR for oleic acid in spring turnip rape (*Brassica rapa* ssp. *oleifera*). Mol Breed 4:543–550

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. Nucl Acids Res 25:4876–4882

Yahiaoui N, Srichumpa P, Dudler R, Keller B (2004) Genome analysis at different ploidy levels allows cloning of the powdery mildew resistant gene *Pm3b* from hexaploid wheat. Plant J 37:528–538

Yahiaoui N, Brunner S, Keller B (2006) Rapid generation of new powdery mildew resistance genes after wheat domestication. Plant J 47:85–98

Yeam I, Kang BC, Lindeman W, Frantz JD, Faber N et al. (2005) Allele-specific CAPS markers based on point mutations in resistance alleles at the *pvr1* locus encoding eIF4E in *Capsicum*. Theor Appl Genet 112:178–186

Zeller FJ, Hsam SLK (1998) Progress in breeding for resistance to powdery mildew in common wheat (*Triticum aestivum* L.). In: Slinkard AE (ed) Proceedings of the 9th international wheat genetics symposium, vol 1. University Extension Press, Saskatoon, pp 178–180

Zeller FJ, Lutz J, Stephan U (1993) Chromosome location of genes for resistance to powdery mildew in common wheat (*Triticum aestivum* L.). 1. *Milk* and other alleles at the *Pm3* locus. Euphytica 68:223–229

Zhang W, Gianibelli MC, Ma W, Rampling L, Gale KR (2003) Identification of SNPs and development of allele-specific PCR markers for gamma-gliadin alleles in *Triticum aestivum*. Theor Appl Genet 107:130–138