



Molecular cloning, functional verification, and evolution of *TmPm3*, the powdery mildew resistance gene of *Triticum monococcum* L.

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ABSTRACT. Powdery mildew (Pm) is one of the most harmful diseases in wheat. Three *Pm*-resistance genes, *Pm3*, *Pm21*, and *Pm8*, have been cloned but most *Pm3/Pm8* alleles have lost their resistance to Pm in hexaploid wheat. In this study, a new *Pm3* homolog gene (*TmPm3*) was isolated from *Triticum monococcum* L. using a homology-based cloning strategy, being the first report of a functional *Pm3* homolog gene from a diploid wheat species. The transient expression of *TmPm3* in leaf epidermal cells showed that over-expressed *TmPm3* could significantly inhibit the penetration of *Blumeria graminis* f. sp. *tritici*

conidia spores and the formation of haustoria. Sequence analysis of *Pm3* alleles shed new light on the evolution of *Pm3* genes, providing a better understanding of the molecular basis of disease resistance. This study also suggested that homology-based cloning of resistance genes is a feasible method for the isolation of functional resistance genes from wheat germplasm.

Key words: Plant resistance breeding; *Pm3*; Powdery mildew; Resistance gene; *Triticum monococcum*; Wheat

INTRODUCTION

Wheat is widely cultivated around the world and represents more than one-third of the staple food of humans. Common wheat (*Triticum aestivum* L.) is an allohexaploid species that emerged about 10,000 years ago from the spontaneous hybridization of the tetraploid wheat *T. turgidum* L. with the diploid goatgrass *Aegilops tauschii* Coss. (Dvorák et al., 1993). A small number of elite cultivars were widely planted according to modern wheat production practices, resulting in frequent diseases and pest infestations (McDonald and Linde, 2002). Breeding-resistant crop species relies on the continual identification of resistance (R) genes and on the integration of these novel sources of resistance into breeding programs. The genetic diversity found in the germplasm of closely related wild and domesticated crop species has proven to be a hugely valuable source of genetic material for improving wheat resistance to biotic stresses (Mago et al., 2005; Singh et al., 2012). Wheat closest relatives also contain many powdery mildew (Pm)-resistance genes/alleles, and more than 30 of these, including *Pm13*, *Pm19*, and *Pm35* from *A. tauschii* (Miranda et al., 2007), and *Pm30* from *Triticum dicoccoides* Schrank ex Schübl. (Liu et al., 2002), were introgressed from wild species.

Powdery mildew, caused by *Blumeria graminis* f. sp. *tritici*, is a highly destructive disease of common wheat. Nearly 70 *Pm*-resistance genes/alleles have been identified in wheat's germplasm, and many of them have been successfully used in breeding programs. However, only three of these genes have been cloned: *Pm3*, *Pm8*, and *Pm21* (Yahiaoui et al., 2004; Cao et al., 2011; Hurni et al., 2013). *Pm3* is located on the short arm of wheat's chromosome 1A and encodes a coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR)-resistance protein. *Pm8* was mapped to the short arm of rye's chromosome 1R, and cloned using a homology-based approach (Hurni et al., 2013).

Cultivated einkorn wheat (*Triticum monococcum* L., A^m), the first domesticated wheat species, has been cultivated for centuries in the Middle East, Central Asia, Europe, and North Africa. Today, einkorn wheat is a valuable genetic resource for wheat improvement programs and is a useful model species for comparative wheat genomic studies. *T. monococcum* has excellent resistance to stem rust isolates *Sr21*, *Sr22*, and *Sr35* (Paull et al., 1994; Saintenac et al., 2013; Zaharieva and Monneveux, 2014), leaf rust isolates *Lr50* and *Lr63* (Jahoor et al., 2005), and Pm isolates *Pm1b* and *Pm25* (Huang and Der, 2004; Zaharieva and Monneveux, 2014). Studies in comparative genomics indicated that the pseudogene *TmRGL-1* (a *Pm3* homologous gene, AY146588) has undergone one major deletion, three frame shifts, and four in-frame stop codon mutations during its rapid evolution (Wicker et al., 2003, 2007a). Consequently, *TmRGL-1* does not confer resistance to powdery mildew.

In the present study, a new functional *Pm3* allele, *TmPm3* from *T. monococcum*, was

obtained using a homology-based cloning strategy. The function of *TmPm3* in resistance to powdery mildew was confirmed via the transient expression of this gene in the epidermal cells of wheat leaves.

MATERIAL AND METHODS

Plant materials

The Plant Germplasm Institute of Kyoto University (Japan) provided the 33 einkorn wheat lines used in this study, which included seven *T. urartu* Thumanjan ex Gandilyan, three *T. boeoticum* Boiss., and 23 *T. monococcum* accessions ([Table S1](#)). Prof. Xiayu Duan of the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences provided the powdery mildew isolate E09.

DNA extraction and PCR amplification

Total DNA was extracted using the modified CTAB method previously described by Porebski et al. (1997). The specific primers for the *Pm3* alleles used in the PCR were originally designed and reported by Tommasini et al. (2006). The full-length and partial sequences of *TmPm3* were amplified using PrimeSTAR HS DNA Polymerase.

Expression vector construction and single-cell transient expression assay

The full-length sequence of *TmPm3* was amplified using the primers pMDC32-B16F (Table 1, *Bam*HI site underlined) and pMDC32-B16R (Table 1, *Pac*I site underlined). PCR products were then cloned into the pEASYTM-Blunt Zero Cloning Vector (TransGen Biotech, Beijing, China). After sequencing and confirming the presence of *TmPm3*, this gene was excised from the cloning vector and ligated into the pMDC32 plasmid under the control of the CaMV-35S promoter. The newly constructed plasmid was named pMDC32-*TmPm3*.

To validate the role of *TmPm3* in conferring resistance to powdery mildew, a transient single-cell assay was performed according the method described in previous studies (Panstruga, 2004). This approach has been shown to be effective in studies assessing the function of powdery mildew-resistance genes in wheat (Yahiaoui et al., 2004; Hurni et al., 2013). The plasmid DNA of pMDC32-*TmPm3* and the pAHC25 vector carrying the beta-glucuronidase gene (*GUS*) gene, also under the control of the CaMV-35s promoter, were co-introduced into leaves' epidermal cells of the susceptible wheat cultivar Xueza0 using the biolistic bombardment method. Empty pMDC32 and pAHC25 plasmids were used as the control. After inoculating the leaves of three biological replicates with the mildew isolate E09, the haustorium index (HI) was calculated based on the observation of *GUS*-expressing cells under a light microscope.

Sequence analysis

Pm3-CS, *Pm3a-g*, *Pm8*, *AetPm3*, *HvPm3*, and *Pm3-1B* sequences were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/) and DNA sequence assembly was conducted using the Vector NTI Suite 8.0 (<http://www.invitrogen.com>). Multiple-sequence alignment

was performed in ClustalW (Thompson et al., 1994); nucleotide diversity was calculated with DNASP version 5.0 (Librado and Rozas, 2009) and phylogenetic relationships were analyzed in MEGA 6 (Tamura et al., 2013).

RESULTS

Pm3 allele detection in *T. monococcum*

A large number of *Pm3* alleles have been identified in the short arm of wheat chromosome 1A, where the *Pm3* locus was defined. The *Pm3* locus has also been detected in bread wheat chromosomes 1B and 1D and in rice chromosome 1, implying collinearity of wheat and rice genomes. Based on these results, we hypothesized the existence of a *Pm3* orthologous gene in *T. monococcum*. The UP3B/UP1A primer pair was used to detect *Pm3* in 33 accessions of einkorn wheat (Yahiaoui et al., 2004); the present study revealed that 12 of the 33 accessions had a *Pm3* locus. To identify the *Pm3* alleles in these 12 einkorn wheat lines, specific markers for *Pm3a-*, *b-*, *c-*, *d-*, *e-*, *f-*, and *g-*resistance alleles were used in a PCR-based analysis (Tommasini et al., 2006). The primers for the *Pm3f* allele generated a clear band in accession 3AA28 and this product was cloned for sequencing. The sequence of this fragment was highly homologous to that of the *Pm3f* allele (90.1% identity in the mapped range) suggesting the presence of a *Pm3f* homologous gene in *T. monococcum*.

Homology-based cloning of the *Pm3* candidate gene from *T. monococcum*

To obtain the full sequence of the *Pm3* candidate gene in *T. monococcum* accession 3AA28, a series of primers were used to clone it. The primers were designed according to the information obtained from sequencing the *Pm3f* candidate gene. Five independent fragments, obtained from PCR using primers Phrase2, L318F, L71RV, L61RV, and 3AA28 specific primers (Figure 1A and Table 1), were sequenced, identified, and assembled into a long contig using the Vector NTI software package.

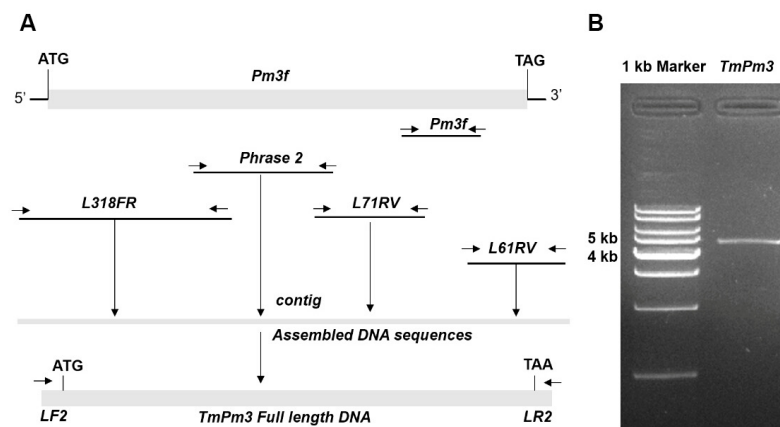


Figure 1. Homology-based cloning of *TmPm3* from *Triticum monococcum*. **A.** PCR amplification of several genomic fragments of *Pm3f* and subsequent assembly of the full-length DNA sequence of *TmPm3*. **B.** Confirmation of the PCR amplification of *TmPm3* full-DNA sequence using the primers LF2 and LR2.

Table 1. PCR primers used for cloning of the *TmPm3* gene expression analysis, and construction of the expression vector.

Description	Primer name	Primer sequence
Specific for <i>Pm3f</i>	Pm3f/F	GGAGTCTCTTTGCTTAAG
	Pm3f/R	CAGCTTCTAAGATCAAGGA
<i>Phrase2</i> fragment	p5944-1619bpF2	GCTTCCAGATACTGCTCG
	p7562-1619bpR2	CAAATAGTCCCACGGCT
<i>L318</i> fragment	2pm3b2118F1	GGCAAGCTCGTGAAGGTA
	pPhrase2R1	ATGCCGAGCAGTATCTGG
<i>L71RV</i> fragment	pPhrase3F2	ATTACTGGCAACAGTGGG
	pPhrase3R2	CAACAATCAGGGATCAGG
<i>L61RV</i> fragment	pPhrase3F2	ATTACTGGCAACAGTGGG
	pPhrase3R2	CAACAATCAGGGATCAGG
Full-length genomic DNA	pLF2	AAAGTTTCAGCCAAGTTCAA
	pLR2	AGGCGAGTGTCTTCTCAGTT
Full-length cDNA	cDNAF	TGCCCTTAAAGTTTCAGCC
	cDNAR	ATGCCGTTTCTACGTGGTT
Construct for expression vector	pMDC32-B16F	CGCGGATCCTGCCCTTAAAGTTTCAGCC
	pMDC32-B16R	CCTTAAATTAATGCCGTTTCTACGTGGTT

Based on the successful assembly of the above fragments, a full-length gene was deduced and a new primer pair (LF2, LR2) was designed (Table 1). A PCR using these primers amplified a specific fragment about 5 kb in length (Figure 1B), and a subsequent reverse transcription-PCR allowed the generation of its full-length cDNA sequence. Sequence alignment with known *Pm3* alleles confirmed that this gene was a novel *Pm3* homolog, having 79% similarity with *Pm3CS* and 68% similarity with *TmRGL-1* (Figure S1), which we named as *TmPm3*. Sequence comparison between the full-length cDNA and the genomic DNA of the *TmPm3* gene indicated that *TmPm3* was 4452 bp long and contained one 165-bp intron near the stop codon in the 3'-region (NCBI GenBank accession No. KR873101). *TmPm3* encoded a 1428-amino acids protein.

Nucleotide diversity in *TmPm3* and other *Pm3* alleles

Pm3CS is considered the ancestor of the *Pm3*-resistance alleles in the current model of *Pm3* gene evolution (Yahiaoui et al., 2006). As this is the case, the nucleotide diversity of the *Pm3* alleles was compared to that of *Pm3b*, *Pm8*, *AetPm3*, *HvPm3*, and *Pm3-1B* coding sequences, and the nucleotide diversity of *TmPm3* with that of *Pm3CS*. DNA sequence comparison showed that, similarly to the other *Pm3* homolog genes, the newly identified *TmPm3* gene encoded a resistance protein containing CC, NBS, ARC1 (the abbreviation of “and regulators of cell death”), ARC2, SPA (sequence between ARC2 and LRR domain), and LRR domains (Figure 2).

The CC domain of the *TmPm3*-encoded protein was 164 amino acids long, meaning this region of *TmPm3* had seven more amino acids than the *Pm3* homolog proteins, owing to the presence of a 21-bp insertion in the N terminal. Twenty-eight LRR motifs were present in the *TmPm3* protein, similar to the number found in most of the other *Pm3* homolog proteins, with the exception of *Pm8* and *AetPm3*. Among these motifs, LRR7 was only found in *Pm3CS*, *Pm3b*, and *AetPm3*, while LRR13 was only found in *TmPm3*, *Hvp3*, and *Pm3-1B*. Neither LRR7 nor LRR13 were found in *Pm8*. Owing to the deletion of a large fragment in the C-terminal region, LRR28 and LRR29 were not present in *HvPm3* (Figure S2). DNA

sequence comparison between all alleles revealed polymorphic sites in all the domains of *TmPm3*, *Pm8*, *AetPm3*, *HvPm3*, and *Pm3-1B*, while in *Pm3b* and *Pm3CS* polymorphic sites only existed in NBS and SPA regions.

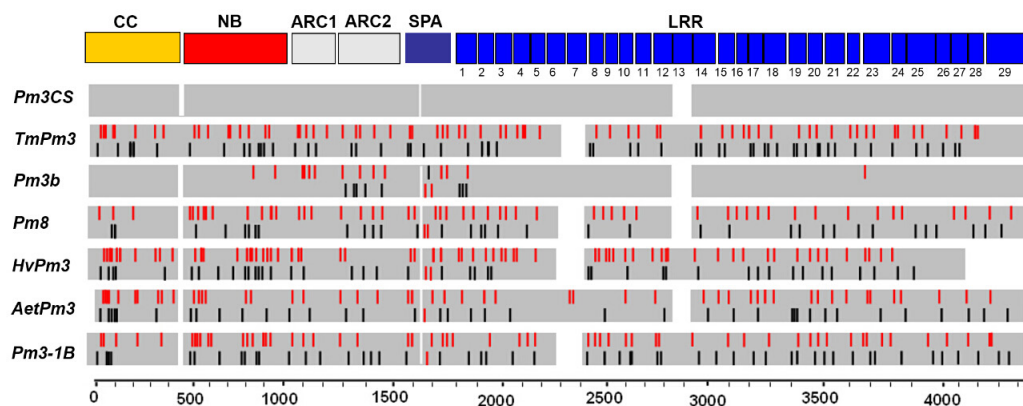


Figure 2. Multiple-sequence alignment (exons) of the newly identified *TmPm3* with its homologs from different sub-genomes. *Pm3CS* (DQ251490) and *Pm3b* (AY325736) come from chromosome 1AS of common wheat (*Triticum aestivum* L.), *Pm3-1B* (KF572031) come from chromosome 1BS of common wheat. *Pm8* (KF572030) come from the 1RS chromosome arm of rye. *AetPm3* (JX295577) and *HvPm3* (AK371992) represented the homolog genes come from *Aegilops tauschii* and *Hordeum vulgare*, respectively. *Pm3CS* is the consensus as well as the reference sequence. Red bars and black bars represent the non-synonymous and synonymous mutations, respectively, in relation to *Pm3CS*.

Phylogenetic analysis and sequence alignment of *Pm3* homolog proteins

A neighbor-joining phylogenetic tree was produced in MEGA 6.0, based on the deduced protein sequences of *TmPm3* and *Pm3* homolog proteins (Figure S3). The layout obtained evidenced a cluster containing the *Pm3CS* and *Pm3a-g* proteins (the core group) from which the other proteins radiated; *AetPm3* was the closest relative to the core group. This phylogenetic tree suggested that the newly identified *TmPm3* from *T. monococcum* (from A^m genome) was closer to *Pm3CS* and *Pm3a-g* alleles (from A^u genome) than to the *Pm3-1B* (from B genome). Compare to *TmPm3*, *AetPm3* (from D genome) and *Pm8* (from R genome) are closer to the core group, where the *Pm3* proteins come from the common wheat.

Functional studies of *TmPm3*

The transient single-cell assay showed that pMDC32-*TmPm3* had a lower number of GUS-expressing cells than the pMDC32 control (148 and 178, respectively). Statistical analysis demonstrated that the HI of pMDC32-*TmPm3* cells was significantly lower than that of control cells (41.03 and 81.88%, respectively; Figure 3). The reduction of HI in the GUS-expressing cells of pMDC32-*TmPm3* indicated that *TmPm3* was involved in wheat's resistance to powdery mildew.

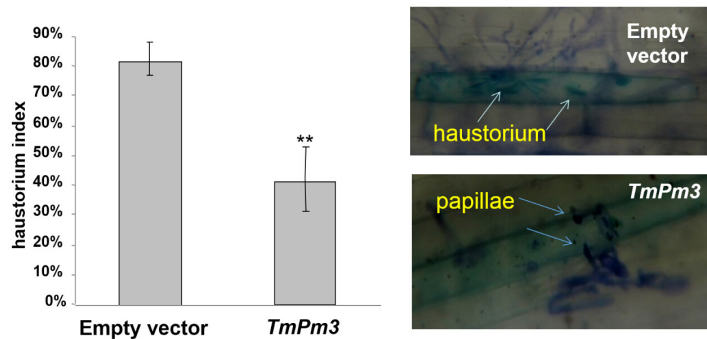


Figure 3. Transient expression assay of *TmPm3* in wheat epidermal cells. The haustorium index was significantly lower in the leaves of the susceptible wheat cultivar Xuezaobao bombarded with *TmPm3* and infected with E09 than in the empty vector control (Student *t*-test, ** $P < 0.01$). Error bars indicate \pm SE obtained from three biological replicates.

DISCUSSION

Homology-based cloning is a useful strategy to isolate R genes

Wheat is one of the most important crops in the world and it is frequently threatened by pathogens, including powdery mildew. Resistance breeding is an efficient means to control plant diseases. *Pm3* was the first powdery mildew-resistance gene to be cloned and, to date, more than 17 functional *Pm3* alleles have been isolated, all sharing high nucleotide sequence identity, especially in their flanking sequences (Tommasini et al., 2006). *Pm3* homolog genes have been identified in both wild and domesticated close relatives of wheat, including wild tetraploid *T. dicoccoides* as well as domesticated tetraploid *T. dicoccum* (Schrank) Schubl. and *T. durum* Desf. (Yahiaoui et al., 2009), suggesting there should be a *Pm3* homolog gene in *T. monococcum*. In the present study, a series of primers were designed, according the sequences of known *Pm3* alleles, and a functional *Pm3* homolog gene, *TmPm3*, was successfully isolated from *T. monococcum*, further supporting homology-based cloning as a useful approach to clone resistance genes. Contrarily to positional-cloning approaches, homology-based cloning does not require the screening of very large segregating populations, a high number of markers, a high-density genetic map, nor extensive chromosome walking. Thus, homology-based cloning can be useful for isolating agronomically important genes with known DNA sequences, particularly R genes with conserved domains.

Sequence alignment indicates the evolution of the *Pm3* locus of wheat

To counter bacterium, fungus, and virus infections, plants have evolved sophisticated defense mechanisms to protect themselves. Resistance genes recognize the pathogens, and trigger strong defense responses that are sometimes accompanied by the programmed cell death of the host (Jones and Dangl, 2006). The NBS-LRR gene family is the largest class of genes known to be related to disease resistance. To date, numerous NBS-LRR genes have been cloned from plants: *Arabidopsis* *RPS2* and *RPS5* genes (Bent et al., 1994; Warren et al., 1998); rice blast-disease *Pi9*-, *Pi36*-, and *Pi5*-resistance genes (Qu et al., 2006; Liu et al., 2007; Lee et al., 2009); and wheat leaf-rust *Lr10*- (Feuillet et al., 2003) and *Lr21*-resistance genes (Huang

et al., 2003). Plant NBS-LRR genes can be divided into the TIR (Toll/interleukin receptor)-NBS-LRR and non-TIR-NBS-LRR groups, according to their N-terminal regions. LRR motifs are involved in protein-protein interactions and in the determination of the specificity of these interactions, which are thought to be crucial for specific pathogen recognition (Torii, 2004). Thus, it is interesting to analyze the sequences and the genetic diversity of the LRR domains of R genes. Typically, the solvent-exposed residues of LRR domains have undergone strong purifying selection during evolution, particularly in the xxLxLxx motifs (Hurni et al., 2013). In the present study, 29 different LRR motifs were found in Pm3 homolog proteins (Figure 2). Sequence alignment showed that leucine was conserved, whereas the other amino acids in the core motif of the LRR units varied (Figure S2). Among the 29 LRR motifs, LRR7 was nearly identical and specific to Pm3b and AetPm3. LRR6 and LRR7 motifs of Pm3 alleles are considered as conserved and might have originated from a duplication event (Wicker et al., 2007b). Here, LRR6 and LRR7 were also conserved in Pm3b and AetPm3 (Figure S4); in addition LRR13 was absent in Pm3 (A genome) and AetPm3 (D genome), but present in TmPm3 (Am genome), HvPm3 (H genome), and Pm3-1B (B genome) (Figure S2). The common LRR motifs between A and D genomes supported the hypothesis of multiple rounds of hybrid speciation, suggesting that the D genome of bread wheat emerged from the speciation of the A x B genomes homoploid hybrid that occurred about 1 to 2 million years ago (Marcussen et al., 2014). In that homoploid hybrid speciation event, the *Pm3* locus of the A genome was inherited and latter passed on to the D genome in *AetPm3* (Figure 4). The present findings suggest that the LRR6 and LRR7 duplication events might have occurred after the divergence of *T. monococcum* (A^m genome) from the *T. turgidum*/*T. aestivum* lineage (or A^u genome), about 1.3 to 3 million years ago, but before the D genome emerged. These dates imply that the A^m and the A genomes (or A^u genome) might have diverged before the D genome emerged.

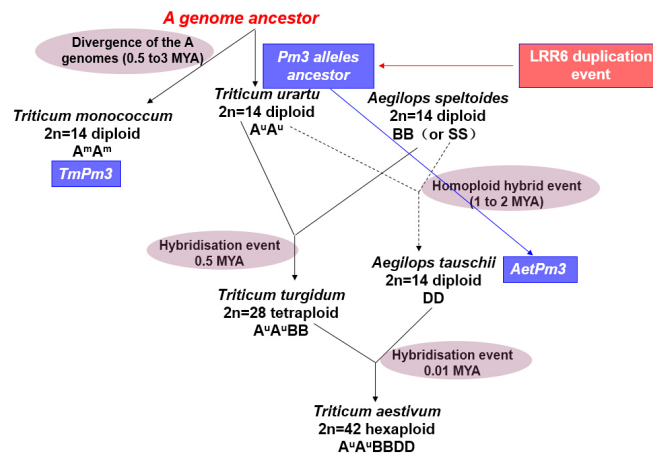


Figure 4. Evolution of *Pm3* homolog genes. The common LRR domains in A and D genomes support the hypothesis of multiple rounds of hybrid speciation in which the D genome of bread wheat would have emerged through an A x B homoploid hybrid speciation about 1 to 2 million years ago (MYA). In that homoploid hybrid speciation event, the *Pm3* locus of the A genome was inherited, implying that the ancient *Pm3* locus of the A genome was the donor of *AetPm3* to the D genome. Our findings also suggested that the LRR6 and LRR7 duplication event might have occurred after the divergence of *T. monococcum* from the *T. turgidum*/*T. aestivum* lineage about 1.3 to 3 MYA, but before the D genome emerged. These dates imply that the divergence of the A^m genome and the A genome (or A^u genome) might have occurred before the D genome emergence.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Figure S1. Multiple-sequence alignment of *TmPm3* with *Pm3-CS* and *TmRGL-1*. Bases highlighted in black are identical in, at least, two of the sequences.

Figure S2. Polymorphism in the LRR domains of *Pm3* genes. Amino acids highlighted in black are identical in, at least, two of the sequences.

Figure S3. Phylogenetic tree of the *Pm3* homolog proteins considered in this study.

Figure S4. Sequence alignment of the LRR6 and LRR7 domains of *Pm3* genes.

Table S1. Wheat lines used in this study.