BRIEF COMMUNICATION

Isolation of resistance gene analogues to powdery mildew resistance sequences in hexaploid wheat

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Abstract

This paper reports the characterization of the powdery mildew resistance homologous genes family of *Triticum aestivum*. Using degenerate primer pair for wheat resistance genes, we have cloned seven 3' truncated powdery mildew resistance gene homologous fragments *Tpc5a*, *Tp25a*, *Tp25b*, *Tp3a5a*, *Tp3a5b*, *Tp4b5a* and *Tp4b5b*. These fragments were sequenced. The deduced amino acid sequences showed that six of them have premature stop codons. All these sequences had a very high level of similarity to known *Pm* resistance genes such as *Pm3a*, *Pm3b*, *Pm3d* and *pm3f* in hexaploid wheat. By ignoring the stop codons in the sequences, their deduced protein sequences were of coiled-coil (CC)-nucleotide binding site (NBS)-leucine repeat rich (LRR) structure. These results suggest that there are many powdery mildew resistance gene analogues in both resistant and susceptible wheat. Among them, small insertion/deletion events and point mutations can result in the diversity of wheat *Pm* resistance homologous genes.

Additional key words: *Blumeria graminis, Triticum aestivum*.

Powdery mildew, caused by *Blumeria graminis* (DC.) E.O. Speer f. sp. *tritici* Em. Marchal (syn. *Erysiphe graminis* f. sp. *tritici*), is one of the most severe foliar diseases of common wheat worldwide. This disease has caused severe yield losses ranging from 13 to 34 % (Griffey *et al*. 1993). Up to now, 32 loci (*Pm1* - *Pm32*) with 48 genes/alleles for resistance to powdery mildew have been identified and located on various chromosomes (Huang and Röder 2004). Molecular mapping and cloning of resistance genes in plants will promote the understanding of molecular mechanisms and evolution of disease resistance, and will permit marker-assisted selection in breeding programs (Huang and Röder 2004). The largest group of resistance genes encodes proteins which carry leucine-rich repeats (LRRs) and nucleotide-binding site (NBS) (Hulbert *et al*. 2001). In NBS-LRR type resistance proteins, there are conserved NBS motifs. Based on the presence or absence of a Toll/Interleukin-1 receptor (TIR) domain at the N-terminus of the protein, plant resistance (*R*) genes can be categorized into TIR and nonTIR classes (Meyers *et al*. 1999). It has been reported that most of the nonTIR NBS-LRR resistant proteins contain a coiled-coil (CC) motif or a leucine zipper structure in the N-terminal region (Pan *et al*. 2000). In addition, the single amino acid residue at the final position of the kinase-2 motif, the trytophan (W) and aspartic acid (D) are characteristic of the nonTIR and TIR-type proteins, respectively (Meyers *et al*. 1999). In recent years, the homology-based cloning method has been extensively applied in isolation of resistance gene analogues (RGAs) from total genomic DNA or cDNA of various plant species, such as rice (Leister *et al*. 1999), wheat (Dilbirligi *et al*. 2004), grapevine (Donald *et al*. 2002) and coffee (Noir *et al*. 2001). The combined analysis of genomes from wheat species was used to positional clone *Pm3b* gene (Yahiaoui *et al*. 2004). Then a polymerase chain reaction-based strategy allowed the amplification of the candidate genes *Pm3a*, *Pm3d* and *Pm3f*, which were shown to have high level of amino acid similarity (97.8 %) to *Pm3b* (Srichumpa *et al*. 2005). The cloning of these four powdery mildew resistance genes

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Abbreviations: CC - coiled-coil; EMS - ethylmethane sulfonate; InDels - insertions/deletions; LRR - leucine repeat rich; NIL - near-isogenic line; NBS - nucleotide binding site; *Pm* gene - powdery mildew resistance gene; RGAs - resistance gene analogues; SNPs - single nucleotide polymorphisms; TIR - toll/interleukin-1 receptor.
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open the way to the identification of other *Pm* genes. Using degenerate primers designed on basis of known *Pm* genes and GenBank data of wheat and rice, we have obtained seven 3' truncated powdery mildew resistance homologous genes. The structure and translation character was analysed.

Seedlings of wheat (*Triticum aestivum* L.) near-isogenic line (NIL) in the Chancellor (Cc) background Ulka/XX 194/8*Chancellor (*Pm2*), Asosan/8*Chancellor (*Pm3a*), Armada/8*Chancellor (*Pm4b*) were used in this research. They were all developed through backcrossing for eight generations using Cc as the recurrent parent. Line Chancellor was used as the susceptible control in this research. Using software *Primer Premier 5* and programs in GenBank (http://www.ncbi.nlm.nih.gov) network, a pairs of degenerate primers were designed according to known wheat powdery mildew resistance genes. In designing primers, codon bias of wheat was taken into account. The product of the primer pairs composes the complete N-terminus and NBS sequences and partial LRR region of coding sequence of *Pm3b*. The forward degenerate primer is 5'-TTCCRATGGCAGAG CTGRTG-3' and the reverse primer is 5'-AGTGTAGAG GTGRCRGAGGSMWGTC-3'. Genomic DNA was extracted from leaves of wheat plants about 4 - 6 weeks old, as described by Sambrook *et al*. (1989). In order to reduce the risk of introducing errors into the sequence, a polymerase *LA GC Taq* (*TaKaRa Biotechnology*, Dalian, China) with a buffer for GC-rich template was used in PCR. Thermal cycling conditions were: denaturation at 95 °C for 3 min followed by 36 cycles at 94 °C for 40 s and annealing and extension at 68 °C for 4 min, and finally the samples were kept at 68 °C for 10 min. PCR products were separated in 1.0 % agarose gel. Lambda DNA, double digested with *Hind*Ⅲ/*Eco*R I, was used as a molecular mass standard to estimate the size of the amplified products. Fragments of the appropriate size were excised from the gel and ligated into *pUCm-T* vector (*Sangon*, Shanghai, China) as described previously (Feng *et al*. 2004), then transformed into *Escherichia coli* DH5α competent cells. DNA sequencing using AM3730 sequencer was performed by *Bio-Asia Company* (Shanghai, China). The DNA sequence obtained in this research and their deduced amino acid sequence were compared with those in GenBank database using the *BLAST* search program. Polygenetic analysis was performed with program *DNAman* for Windows, version *4.0* (*Lynnon Biosoft*, USA).

Using degenerated primer pairs, a strong single band of about 2.0 kb was amplified from genomic DNA of different materials. Interestingly, even from the susceptible line Chancellor a similar band was amplified as in the other wheat lines. The bands from those different templates were recovered and inserted into pUCm-T vector. Five clones were randomly picked from recombinant clones of each band obtained above and the inserted fragments were sequenced. Seven different resistence gene analogues (RGAs) were obtained. Of the seven RGAs, one (*TpC5a*) came from Chancellor, two (*Tp25a*, *Tp25b*) from Ulka/XX 194/8*Chancellor (*Pm2*), two (*Tp3a5a*, *Tp3a5b*) from Asosan/8*Chancellor (*Pm3a*) and two (*Tp4b5a*, *Tp4b5b*) from Armada/8*Chancellor (*Pm4b*). These sequence data have been submitted to the GenBank databases under accession number DQ241561 (*Tpc5a*), AY899934 (*Tp25a*), DQ241562 (*Tp25b*), DQ256077 (*Tp3a5a*), DQ287983 (*Tp3a5b*), DQ256078 (*Tp4b5a*) and DQ288248 (*Tp4b5b*). The seven *Pm* homologous fragments were compared with four known *Pm* genes in the GenBank database for DNA sequences and deduced amino acid sequences by the *BLASTN* and *BLASTX*. The results indicated that they were all highly homologous to *Pm* members from wheat *Pm3* locus. However, there are distinctions including some base substitutions and insertions/deletions (InDels) in different sequences. DNA sequence alignment of these genes showed the presence of InDels and single nucleotide polymorphisms (SNPs) that may now be targeted for the development of gene-specific markers useful for identification of corresponding genes in hexaploid wheat and for the analysis of allelic variation at the *Pm* locus. Translation analysis showed that six of them have more than one premature stop codons. The number and position of the premature stop codons were found to be variable in the six fragments. TP3A5A had one stop codon in the N-terminus because of point mutation and six stop codons in the NBS region mainly because of InDels resulting in frameshift. TPC5A had twenty four stop codons in the NBS region mainly because of InDels. TP25B, TP3a5B, TP4B5A and TP4B5B had one stop codon in the NBS region because of point mutation. By ignoring the stop codons, the deduced amino acid sequences appeared to be nonTIR-type RGAs (Fig. 1) based on visual assessment according to the criterion reported by Meyers *et al*. (1999). This was supported by the fact that all the seven sequences contained the RNBS-A-nonTIR motif (FDLxKx WVSVSDDF), a characteristic motif specific to the nonTIR-type plant R proteins (Meyers *et al*. 1999), and further, they contained a tryptophan (W) at the end of the kinase-2 motif (Fig. 1). Comparison of the amino acid sequences between the seven sequences and known PM3 protein from wheat showed that they have similar structure: A coiled-coil domain in the N- terminus, followed by NBS domain which have conserved motifs (P-loop, kinase 2, RNBS-B, GLPL, MHD, *etc*.). Followed them, there was conserved LRR region with the consensus motif LxxLxLxx(C/N/T)xxLxxLxxLP observed in known *Pm3* gene products (Srichumpa *et al*. 2005). Therefore, these *Pm* homologous genes belong to CC-NBS-LRR proteins. Because the frameshift in *Tpc5a* and *Tp3a5a*, some of the conservative motifs of their deduced amino acid sequences were not identical to other sequences (Fig. 1). Phylogenetic tree based on the deduced precursor proteins of seven *Pm* homologous genes and relevant proteins encoded by resistance genes or resistance gene analogues showed that they were homologous. The cloned seven *Pm* homologous sequences can be divided into three groups.

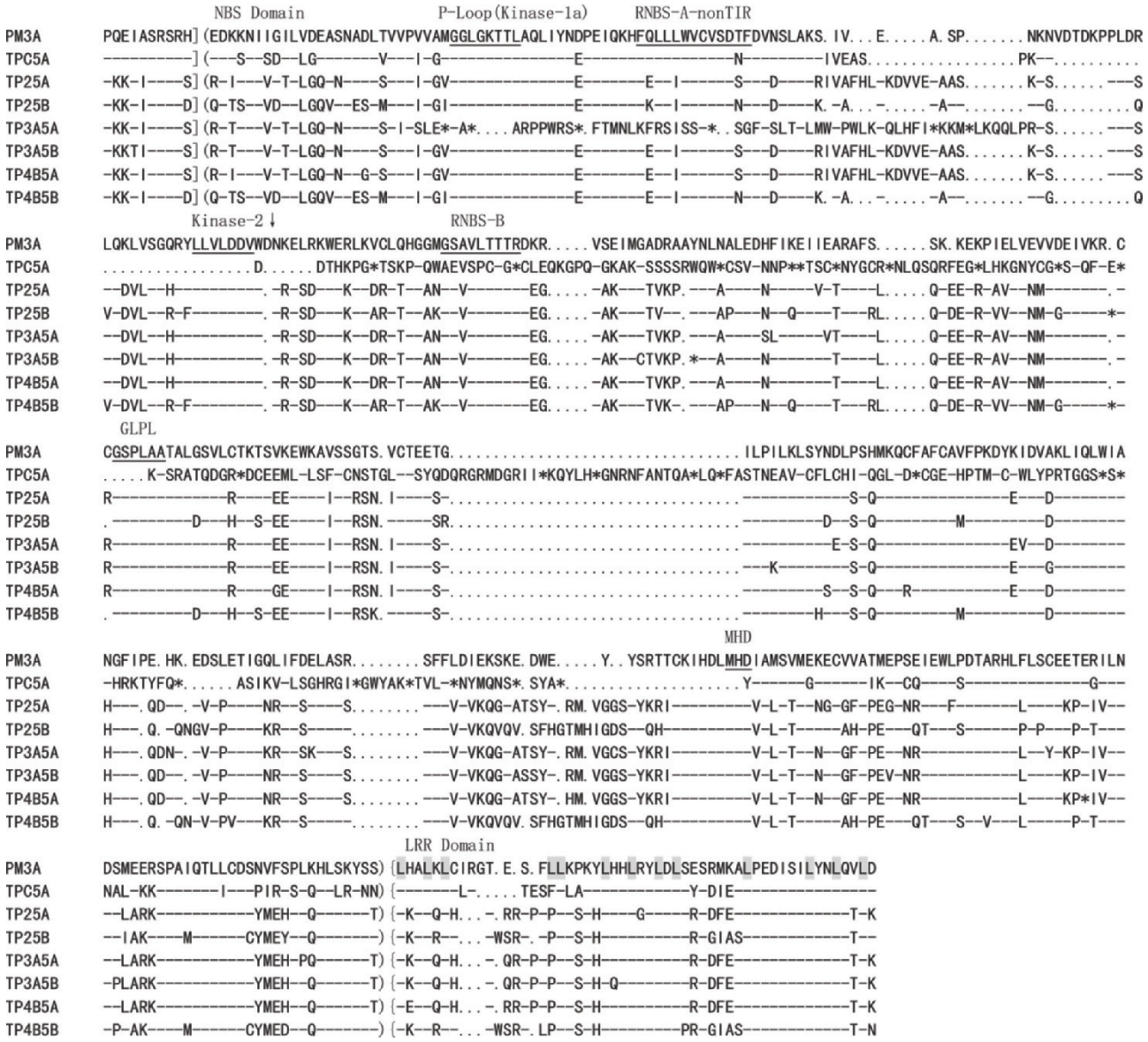

Fig. 1. Partial sequence alignment of deduced precursor proteins of seven *Pm* homologous genes (pseudogenes) and known *Pm3* genes. Positions of premature termination codons are indicated by asterisks and deleted residues by dots, respectively. *Short bars* indicate the identical peptides. The N-terminus, NBS and LRR domain are indicated by [], (),{}, respectively. Conserved motifs characteristic of the NBS domain (in the following order: P-loop, RNBS-A-nonTIR, Kinase 2, RNBS-B, GLPL, MHD) are *underlined* and shown above the previously isolated resistance genes. The tryptophan (W) residue at the final position of kinase-2 motif is indicated by *arrow*. The leucines (L) in LRR domain are *shaded*.

The first group included *Tp25a*, *Tp4b5a*, *Tp3a5a* and *Tp3a5b*, which differ from each other by few nucleotides substitution. The second group included *Tp25b* and *Tp4b5b*, also differ from each other by few nucleotides. The third group consists of only *Tpc5a* (Fig. 2).

Plant disease resistance is often controlled by Mendelian genes and follows a gene-for-gene relationship in many plant species and their pathogens (Flor 1971). According to this theory, there are many resistance (R) genes in a plant species against each of its pathogens and there is a corresponding avirulence gene in the pathogen population for every R gene in the host plant. Although Chancellor was a susceptible line to wheat powdery mildew, homologous sequences which have similar structure to known *Pm* genes were obtained. Two susceptible line Kanzler and Chinese Spring not known to carry a *Pm3* gene showed the same hybridization pattern as the resistant *Pm3* lines (Srichumpa *et al*. 2005). Many pathogenesis related proteins were induced by glycoprotein elicitor in resistant and susceptible cultivars of sugarcane (Ramesh Sundar *et al*. 2008). This paper confirmed that there were many homologous sequences to

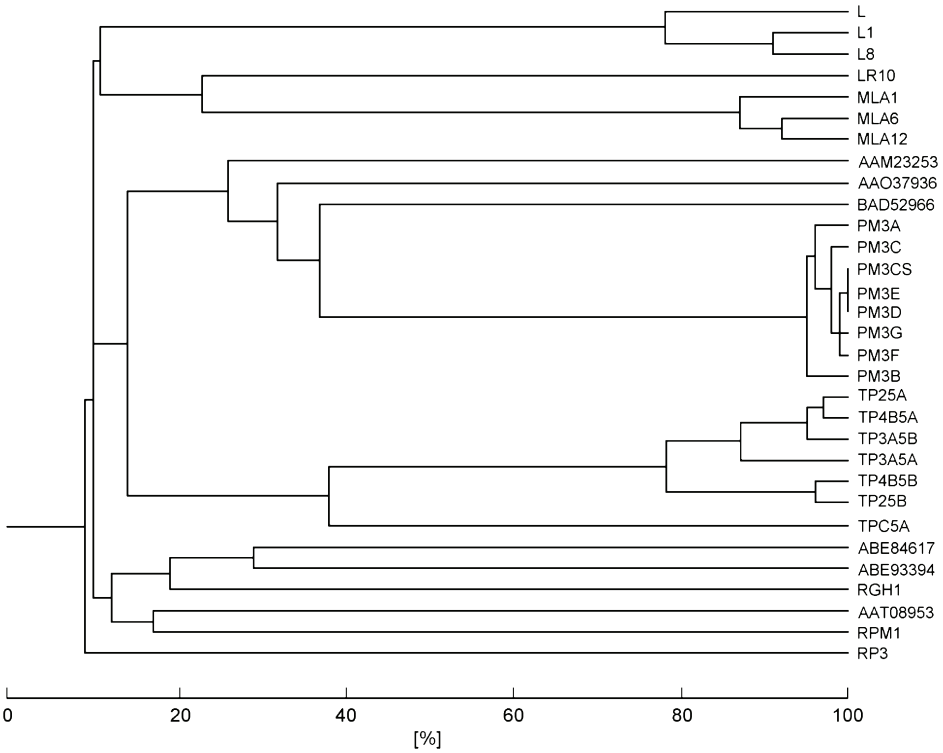

Fig. 2. Phylogenetic tree based on the deduced precursor proteins of seven *Pm* homologous sequences and relevant proteins encoded by resistance genes or resistance gene analogs. The following proteins showing high homology using *BLAST* analysis were included: wheat powdery mildew resistance proteins PM3A (AAY21626.1), PM3B (AAQ96158.1), PM3C (ABB78077.1), PM3D (AAY21627.1), PM3E (ABB78078.1), PM3F (AAZ23113.1), PM3G (ABB78079.1) and PM3CS (ABB78080.1); rice disease resistance-like proteins AAO37936.1, AAO37948.1, BAD52966.1, AAM23253.1 and BAD08985.1; *Medicago truncatula* disease resistance proteins ABE84617.1 and ABE93394.1; *Helianthus annuus* CC-NBS-LRR resistance protein AAT08953.1. Additional included proteins are as followed: maize RP3 (AAN23081) protein, flax proteins L (AAD25965.1), L1 (AAD25966) and L8 (AAD25974), wheat leaf rust resistance protein LR10 (AAQ01784), barley powdery mildew resistance proteins MLA1 (AAG37354.1), MLA6 (CAC29242.1), MLA12 (AAO43441.1) and *Arabidopsis* disease resistance protein RPM1 (CAA61131).

Pm genes in susceptible and resistant lines. They differed mainly because of point mutations and InDels. At some mutation site, the premature stop codons result in the failure of translation. Monosi *et al*. (2004) found that over 100 of NBS-LRR genes were predicated to be pseudogenes in the rice cv. Nipponbare. Perhaps they originated from the evolution of functional powdery mildew resistance genes. DNA sequence alignment of these genes showed the presence of InDels and SNPs that may now be targeted for the development of genetic-specific markers. These gene-specific markers will be useful for identification of the corresponding genes in hexaploid wheat. High sequence conservation (more than 97 % sequence identity) is observed between the *Pm3a*, *Pm3b*, *Pm3d*, and *Pm3f* genes. They show few differences due to small InDels and point mutations in their coding sequences, but also show evidence for sequence exchange by recombination or gene conversion events (Srichumpa *et al*. 2005). Four of the new sequences *Tp25a*, *Tp3a5a, Tp3a5b,* and *Tp4b5a* presented in this paper showed high sequence conservation too, *Tp*25b and *Tp4b5b* had high identity to each other, but the sequence *Tp*c5a showed lower conservation with the other six sequences. Sequence evolution generating new alleles is based on recent events of mutation and intragenic recombination or gene conversion occurring most probably between allelic sequences (Srichumpa *et al*. 2005). The degenerate primer pair used in this paper could not obtain the *Pm3a* gene from cultivar Asosan/8*Chancellor (*Pm3a*) genomic DNA. The reason for this may be that the forward degenerate primer does not match *Pm3a* sequence precisely. Further work on the cloning of other *Pm* alleles analogue in hexaploid wheat and closely related species should shed more light on the evolution of these genes in the wild and cultivated wheat species and near species.

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