Allelic Series of Four Powdery Mildew Resistance Genes at the Pm3 Locus in Hexaploid Bread Wheat

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At the Pm3 locus in hexaploid wheat (Triticum aestivum), 10 alleles conferring race-specific resistance to powdery mildew (Blumeria graminis f. sp. tritici) are known. A cluster of genes encoding coiled-coil–nucleotide-binding site–leucine-rich repeat proteins spans the Pm3 locus on wheat chromosome 1A, and one member of this gene family has recently been identified as the Pm3b resistance gene. Using molecular markers closely linked to Pm3b, we performed haplotype analysis of 10 lines carrying different Pm3 alleles. All these lines have a conserved genomic region delimited by markers cosegregating with Pm3b and including a structurally conserved Pm3b-like gene. A polymerase chain reaction–based strategy allowed the amplification of one Pm3b-like sequence from lines carrying Pm3a, Pm3d, and Pm3f alleles. These candidate genes for Pm3a, Pm3d, and Pm3f conferred AovP3a, AovP3d, and AovP3f-dependent resistance, respectively, to wheat powdery mildew in a single cell transient transformation assay. A high level of amino acid similarity (97.8%) was found between the PM3A, PM3B, PM3D, and PM3F proteins. The coiled-coil domain was 100% conserved, whereas, in the nucleotide binding site region, sequence exchange was detected, indicating intragenic recombination or gene conversion between alleles. All these results indicate that Pm3a, Pm3b, Pm3d, and Pm3f form a true allelic series. The low level of sequence divergence between the four characterized alleles as well as the finding of a conserved Pm3 haplotype are in agreement with the hypothesis of a recent evolution of Pm3-based resistance, suggesting that some or most of the diversity found at the Pm3 locus in modern wheat has evolved after wheat domestication.

In wheat, 30 Pm genes conferring resistance to the obligate biotrophic powdery mildew fungus Blumeria graminis f. sp. tritici have been genetically characterized (Hsam and Zeller, 2002). Among them, the Pm3 gene was one of the first described loci (Briggle and Sears, 1966), and since then a total of 10 different resistance specificities (Pm3a to Pm3j) have been assigned to the Pm3 locus on the short arm of wheat chromosome 1A (Briggle and Sears, 1966; Zeller and Hsam, 1998). The Pm3 locus has the highest number of identified alleles compared to the other wheat Pm resistance genes. The Pm3 alleles have been found in wheat lines or cultivars originating from the five continents, and a number of them have been widely used in wheat breeding in Europe (Pm3a, Pm3d) and in the United States (Pm3a; Hsam and Zeller, 2002). The Pm3 alleles have been characterized based on classical allelic tests (Zeller et al., 1993; Zeller and Hsam, 1998). There, distinct genes might be misclassified as allelic due to the low genetic recombination between them. Thus, alleles defined by classical genetic analysis might represent two closely linked genes at the molecular level.

Classical genetic studies as well as molecular studies of cloned genes have shown that resistance genes are found in two distinct genomic arrangements. Resistance genes can occur as a single gene with one or more alleles encoding different resistance specificities. At the flax (Linum usitatissimum) L locus, a single gene is found as 11 allelic variants, 10 of which encode different resistance specificities against the flax rust pathogen (Ellis et al., 1997). Resistance genes can also occur as a series of tightly linked genes forming complex loci, such as the flax M locus (Anderson et al., 1997), the tomato (Lycopersicon esculentum) Cf9 loci (Thomas et al., 1997), the RPP1 locus in Arabidopsis (Arabidopsis thaliana; Botella et al., 1998), and the Rp1 locus of maize (Zea mays; Collins et al., 1999; Sun et al., 2001). At these loci, individual genes from a cluster can confer different recognition specificities, conditioning resistance to different pathogen isolates. For example, at the RPP1 locus in the Arabidopsis accession Wassilewskija, three of four tightly linked genes confer specific resistance to distinct isolates of the biotrophic oomycete Peronospora parasitica (Botella et al., 1998). Resistance gene loci may also carry related but highly divergent sequences, such as the Mla powdery mildew resistance locus of barley (Hordeum vulgare) where three families of resistance gene homologs, RGH1, RGH2, and RGH3, have been identified (Wei et al., 1999). Approximately 30 distinct resistance specificities have been described at the Mla locus on barley chromosome 1H, and all cloned Mla genes (Mla1, Mla6, Mla7, Mla10, Mla12, and Mla13) are true alleles of the same member of the RGH1 gene family (Jorgensen, 1994; Halterman et al., 2001; Zhou et al.,

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Resistance genes encode proteins that directly or indirectly recognize pathogen-specific avirulence (Avr) gene products and trigger a resistance response (Dangl and Jones, 2001). This resistance response is often characterized by cell death at attempted penetration sites. The most abundant class of cloned resistance genes encodes intracellular proteins with a coiled-coil (CC) or Toll-interleukin receptor-like domain fused to a nucleotide-binding domain (NBS) and a C-terminal region that contains Leu-rich repeats (LRR). The LRR is the candidate recognition domain and the N-terminal regions, including the NBS, are possibly the major signaling domains (Jones and Jones, 1997; Ellis et al., 2000). Evidence of diversifying selection was found in regions of the LRR domain that are predicted to be solvent exposed, as would be expected if these amino acids were involved in ligand binding (Parniske et al., 1997; McDowell et al., 1998; Meyers et al., 1998; Noel et al., 1999). Evidence for direct physical interaction between an NBS-LRR protein and an avirulence gene product was rarely shown (Jia et al., 2000; Deslandes et al., 2003). Therefore, the guard hypothesis was proposed where NBS-LRR proteins could function as members of multiprotein complexes and detect the interaction of the avirulence gene product with other proteins of the complex (Mackey et al., 2002; Belkhdar et al., 2004).

The powdery mildew resistance gene Pm3b was isolated from the hexaploid (Triticum aestivum) wheat genome by positional cloning (Yahiaoui et al., 2004). Pm3b encodes a CC-NBS-LRR protein and is a member of a large family of resistance-gene-like (RGL) genes spread over approximately 1 Mb on the short arm of chromosome 1A. This gene family is also present on wheat homoeologous chromosomes 1B and 1D. A number of the Pm3b paralogous genes are expressed and could potentially encode functional resistance genes (Yahiaoui et al., 2004; N. Yahiaoui, unpublished data). Thus, genetically characterized Pm3 alleles could represent a true allelic series but could also be derived from different, closely linked paralogous genes on wheat chromosome 1A. Here, we report the cloning of the Pm3a, Pm3d, and Pm3f alleles of the wheat powdery mildew resistance locus Pm3. Haplotype analysis of wheat lines carrying different Pm3 alleles showed that all these lines show a conserved haplotype at the Pm3 locus. A gene structurally very similar to Pm3b was isolated from line Asosan/8*Chancellor (Pm3a), cultivar Kolibri (Pm3d), and line Michigan Amber/8*CC (Pm3f). Transient expression analysis showed that the Pm3a, Pm3d, and Pm3f candidate genes conferred AvrPm3a-, AvrPm3d-, and AvrPm3f-dependent resistance to wheat powdery mildew, respectively. Haplotype and gene structure conservation as well as sequence analysis strongly support the hypothesis that Pm3a, Pm3b, Pm3d, and Pm3f are true alleles at the Pm3 locus.

RESULTS
Haplotype Analyses of Wheat Lines and Cultivars Carrying Pm3 Alleles

Haplotype studies were carried out on 10 hexaploid wheat lines and cultivars with different Pm3 specificities, and on five lines susceptible to powdery mildew (Fig. 1). Nine markers (six RFLP probes, one microsatellite, and two sequence-tagged-site [STS] markers) linked to the cloned Pm3b gene (Fig. 1) were used for these studies. These markers were developed during positional cloning of Pm3b where the high-resolution genetic mapping was made in hexaploid wheat and physical mapping in diploid Triticum monococcum and tetraploid Triticum durum (Yahiaoui et al., 2004).

Markers cosegregating with Pm3b were used first (Fig. 1B). Probes derived from the coding region of Pm3b generated complex and polymorphic patterns in the tested lines, in agreement with the presence of a large family of Pm3-related genes in wheat (Yahiaoui et al., 2004). The low-copy sequences TmRGL-1pro, 3’UTR-Pm3, and 294DD1-31 (Fig. 1, A and B) were more useful to characterize the haplotype in the Pm3 lines. In all Pm3 lines, these noncoding sequences showed an identical hybridization pattern of the fragments segregating with Pm3b (Figs. 1B and 2). The TmRGL-1pro probe from the 5’ noncoding region of Pm3b, 4 kb upstream of the start codon (Fig. 1A), was originally identified in the orthologous 5’ noncoding region of the TmRGL-1 gene on T. monococcum bacterial artificial chromosome (BAC) 45SN11 (Fig. 1B; Yahiaoui et al., 2004). TmRGL-1pro was found at two loci in hexaploid wheat, one locus on chromosome 1A where Pm3b is present, and one on chromosome 1B. In the line Chul (Pm3b), TmRGL-1pro and the 3’ noncoding region of Pm3b (3’UTR-Pm3) both hybridize to a BamHI restriction fragment of a similar size of more than 9 kb (Fig. 2, A and B). This hybridization pattern is consistent with the unique BamHI restriction site found in the Pm3b sequence (Fig. 1A). Therefore, these probes specifically identify a restriction fragment containing the Pm3b gene sequence. If the haplotype is conserved in the Pm3 lines, then these markers should enable the identification of a Pm3b-like gene in these lines. Indeed, similarly to the pattern in Chul (Pm3b), TmRGL-1pro hybridized to two fragments in all Pm3 lines, one on chromosome 1A and one on chromosome 1B (Fig. 2A). Both fragments are absent in the susceptible tetraploid cultivar Langdon, and only the chromosome 1B locus is present in the susceptible lines Chancellor and Frisel (Fig. 2A). The chromosome 1A band corresponds to a BamHI restriction fragment of more than 9 kb in all lines carrying Pm3 alleles, and the 3’ noncoding sequence of Pm3b (3’UTR-Pm3) also hybridized to this fragment (Fig. 2B). Probe Chul8kb corresponding to 4 kb of 5’ noncoding sequence and 4 kb of Pm3b coding sequence (Fig. 1A) also identified a similar BamHI fragment of more than 9 kb in all lines carrying Pm3 alleles (data not shown). The presence of
a similar fragment or possibly even identical fragment on chromosome 1A of all Pm3 lines indicates that a genomic region containing a Pm3b-like gene is well conserved and present in all the lines carrying known Pm3 alleles. Interestingly, among the five susceptible lines not known to carry a Pm3 gene, the two lines Kanzler and Chinese Spring showed the same hybridization pattern as the resistant Pm3 lines (Fig. 2).

Markers flanking the Pm3b locus on the genetic map were then used to check the extent of haplotype

Figure 1. Haplotype analysis at the Pm3 locus in different wheat lines. A, Position of RFLP probes TmRGL-1pro, CH8kb, and 3’UTR-Pm3 and of the primer pairs used for haplotype analysis and Pm3 gene cloning. A unique BamHI restriction site is present at 189 bp on the 9,150-bp-long genomic fragment containing Pm3b. B, Results of the haplotype analysis on 10 lines/cultivars carrying different Pm3 alleles (Asosan/8*Chancellor [Pm3a], Chul/8*Chancellor [Pm3b], Triticale/8*Chancellor [Pm3c], Koliibri [Pm3d], W150 [Pm3e], Michigan Amber/8*Chancellor [Pm3f], Aristide [Pm3g], Abessi [Pm3h], N324 [Pm3i], GUS122 [Pm3j], and five powdery mildew–susceptible lines [Kanzler; CS, Chinese Spring; T. durum cv Langdon; Frisal; and Chancellor]). The Pm3b genetic map and the physical contigs established using BAC clones of diploid wheat T. monococcum and tetraploid wheat T. durum cv Langdon are given at the top (Yahiaoui et al., 2004). RFLP markers SFR159, TmRGL-1pro, CH8kb, 3’UTR-Pm3, 294D11-31, 294D11-22; STS markers for XTaRGL-9 and XTaRGL-12; and microsatellite marker Psp2999 indicated on the genetic map were used for the haplotype analysis. Markers SFR159 and 294D11-22 flanking Pm3b delimit a genetic interval of 0.29 cM corresponding to approximately 260 kb on the T. durum BAC contig spanning the Pm3 locus (Yahiaoui et al., 2004). Haplotypes identical to the Pm3b haplotype are indicated in black. White boxes indicate the absence of the band linked to Pm3b. Crossed boxes indicate polymorphic loci on chromosome 1AS. LMW glutenin, low-Mr glutenin.
conservation among the Pm3 lines. STS markers for loci XTaRGL-9 and XTaRGL-12 (Fig. 1B) amplify two different members of the RGL gene family on Chul (Pm3b) chromosome 1A (0.15 cM and 0.55 cM proximal to Pm3b, respectively; Yahiaoui et al., 2004). These markers revealed null alleles in several Pm3 lines, indicating variability in the RGL gene cluster. Low-copy RFLP probe 294D11-22, located 0.2 cM proximal to Pm3b, revealed a polymorphic profile on lines Kolibri (Pm3d) and GUS122 (Pm3j; data not shown), while RFLP probe SFR159 and microsatellite Psp2999 (Glu-3)-1A that map at 0.07 cM distal to Pm3b both showed a null allele in Triticale/8*Chancellor (Pm3c; Figs. 1 and 2D). Microsatellite Psp2999 generated a total of three different patterns among Pm3 lines as shown in Figure 2D. Thus, the markers flanking the Pm3b locus reveal a high level of polymorphism among Pm3 lines, indicating a physically small region of haplotype conservation among Pm3 lines.

We conclude that the region of haplotype conservation between Pm3 lines and also the two susceptible lines Chinese Spring and Kanzler includes a 4.3 kb noncoding region upstream of the Pm3 gene up to the 294D11-31 region. Based on the T. durum physical map (Fig. 1B) and assuming conservation of distances between the RGL-1 genes and the 294D11-31 sequence in hexaploid and tetraploid wheat, the size of haplotype conservation is estimated to be approximately 100 kb.

Cloning of Pm3a, Pm3d, and Pm3f Candidate Alleles from Lines Asosan/8*Chancellor, Kolibri, and Michigan Amber/8*Chancellor

In the absence of large insert libraries from lines carrying Pm3 alleles and based on the hypothesis that at least some of the genetically identified Pm3 genes are true alleles, a PCR-based strategy was used to clone candidate genes for the Pm3a, Pm3d, and Pm3f resistance alleles. The first step was to test if the genomic sequence that contains a Pm3b-like gene and is conserved among Pm3 lines corresponds to a single-copy region on chromosome 1A. To test this, primer pair U3P3B/ChulFF1R (Fig. 1A) that amplifies a 5.6-kb fragment containing the TmRGL-1pro region and covering 4,359 bp upstream of Pm3b and 1,251 bp of coding sequence (up to the NBS domain) was used for amplification on Pm3a, Pm3d, and Pm3f lines. Two PCR fragments of different size were generated from each line and mapped to chromosomes 1A and 1B, respectively, using nullitetrasomic lines of Chinese Spring (Fig. 3). The chromosome 1A fragment was cloned and

![Figure 2. RFLP and microsatellite analysis at the Pm3 locus of 10 lines carrying Pm3 alleles and of five powdery mildew-susceptible lines. RFLP analysis of markers TmRGL-1pro (A), 3′UTR-Pm3 (B), and 294D11-31 (C) cosegregating with Pm3b using BanHI-digested wheat genomic DNA is shown. The Pm3 lines Asosan/8*Chancellor (Pm3a), Chul/8*Chancellor (Pm3b), Triticale/8*Chancellor (Pm3c), Kolibri (Pm3d), W150 (Pm3e), Michigan Amber/8*Chancellor (Pm3f), Aristide (Pm3g), Abessi (Pm3h), N124 (Pm3i), and GUS122 (Pm3j) were used. The arrowheads indicate the conserved fragments on chromosome 1A in all 10 Pm3 lines/cultivars as well as in the susceptible lines Kanzler and Chinese Spring (CS). N1A/T1B is a nullitetrasomic line of Chinese Spring and Kanzler, and Chinese Spring (CS). N1A/T1B, indicating that this marker is specific for chromosome 1A of wheat. Absence of amplification from Triticale/8*Chancellor (Pm3c) confirms a different haplotype also indicated by the SFR159 RFLP probe (Fig. 1B).]

![Figure 3. Characterization of the Pm3 haplotype in lines with Pm3a (Asosan/8*Chancellor), Pm3b (Chul/8*Chancellor), Pm3d (Kolibri), and Pm3f (Michigan Amber/8*Chancellor) alleles based on specific primers from the Pm3b gene sequence. The amplification of two genomic fragments is indicative of a Pm3b-like gene structure on chromosomes 1A and 1B of hexaploid wheat lines. Primer UP3B, 4.36 kb upstream of Pm3b, and primer ChulFF1R from the NBS region of Pm3b were used. CS, Hexaploid wheat line Chinese Spring; N1A/T1B, N1B/T1A, N1D/T1B are nullitetrasomic lines of Chinese Spring. Arrows indicate bands missing in the aneuploid lines. The bands missing in N1A/T1B and in N1B/T1D are attributed to chromosome 1A and to chromosome 1B of Chinese Spring, respectively.]

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sequenced. Three cloned fragments per line showed identical sequences, indicating the presence of a single-copy sequence. Sequence comparison of the amplified 5.6-kb fragment showed a very high degree of conservation between the Pm3 lines, particularly in the 5’ noncoding region (99% sequence identity). In this region, the Pm3d line sequence (4,359 bp of 5’ noncoding region) was identical to Pm3b, whereas sequences from the Pm3a and Pm3f lines (4,360 bp of 5’ noncoding region) were identical to each other but showed one single nucleotide InDel compared to Pm3b.

In a second step, expression of the Pm3b-like gene was tested. Using the Pm3b LRR-derived primers 3’GSP1 and consLRR3 (Fig. 1A) in a reverse transcription (RT)–PCR experiment, a fragment of approximately 450 bp was amplified from lines carrying Pm3a, Pm3d, and Pm3f alleles (data not shown). The specific primer 3’GSP1 was then used in a 3’ RACE-PCR experiment to obtain the noncoding 3’ end of the expressed genes. Fragments of approximately 576 bp (Pm3a), 759 bp (Pm3d), and 572 bp (Pm3f) were obtained and consisted of a coding region of 357 bp and different sizes of the 3’ noncoding region of 219 bp (Pm3a), 402 bp (Pm3d), and 215 bp (Pm3f). Three clones were sequenced for each line, and only one type of sequence was found from each of them. RACE-PCR sequence comparison with the corresponding region of Pm3d showed 97% (Pm3a), 99% (Pm3d), and 97% (Pm3f) identity in the coding part of the RACE products. The 3’ noncoding regions showed more differences (an average of 83% identity in overlapping sequences). The 3’ untranslated region (3’UTR) products from the Pm3a and Pm3f lines were the most similar to Pm3b (99.5% identity) compared to the 3’ UTR from Kolibri (Pm3d) with 68% sequence identity.

Sequence alignment of the fragments amplified by primer pair UP3B/ChulFF1R from lines Asosan/8*Chancellor (Pm3a), Kolibri (Pm3d), and Michigan Amber/8*Chancellor (Pm3f) showed that the primer sequence UP6 at 300 bp upstream of the start codon (Fig. 1A) was conserved. A second primer (N3’SP3R; Fig. 1A) was designed based on a conserved region in the 3’ noncoding RACE-PCR sequences from these lines. These primers were used in nested PCR to amplify the entire coding region of Pm3b-like genes from Asosan/8*Chancellor (Pm3a), Kolibri (Pm3d), and Michigan Amber/8*Chancellor (Pm3f). A fragment of approximately 4.5 kb was amplified from both lines. For each gene, the sequence comparison with respective UP3B/ChulFF1R and RACE-PCR overlapping sequences revealed 100% identity. Sequence comparison with Pm3b showed that the Pm3a, Pm3d, and Pm3f candidate genes have an identical gene structure as Pm3b, with two exons and one intron of 200 bp at 86 bp upstream from the predicted stop codon. The size of exon 1 of the Pm3d candidate gene slightly differs by two InDel triplets, resulting in a size of 4,156 bp compared to 4,162 bp for the Pm3a candidate and Pm3b. The exon 1 of the Pm3f candidate gene shares one triplet InDel with Pm3d, resulting in a size of 4,159 bp. The alignment of 200 bp of intron sequence showed 100% identity. The coding nucleotide sequences among the four genes share, on average, 97.7% identity. We conclude that we have identified and cloned, from a region of highly conserved Pm3 haplotype, an expressed gene with a genomic structure identical to Pm3b in three lines carrying different Pm3 alleles. Therefore, these genes were good candidates for being Pm3 alleles.

Functional Analysis of Pm3a, Pm3d, and Pm3f Candidate Genes

To functionally test the Pm3a, Pm3d, and Pm3f candidate genes, we used a transient single cell transformation assay (Schweizer et al., 1999; Yahiaoui et al., 2004). Leaf segments of the powdery mildew–susceptible wheat variety Chancellor were cocultivated with plasmid constructs containing either the test gene or the empty PGY1 vector as a control together with the β-glucuronidase (GUS) reporter gene. The leaf segments were then inoculated with wheat powdery mildew isolates. In the control experiments, after infection with the different isolates, fully developed haustoria were observed in 63% to 67% of the cells expressing the GUS reporter gene and attacked by a germinating fungal spore (Table I). In contrast, when the Pm3a, Pm3d, and Pm3f candidate genes were used instead of the PGY1 vector, a significant decrease of haustorial growth was observed after infection with avirulent isolates. Only 4%, 17%, and 16% of cells showing both GUS expression and haustorial growth were observed after infection with fungal isolates showing avirulence on Pm3a, Pm3d, and Pm3f, respectively.

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<td>GUS cells with interaction^a</td>
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^aGenomic sequences of Pm3a, Pm3d, and Pm3f candidate genes (test genes) were under control of the 35S CaMV promoter. PGY1, Empty vector (control). ^bGUS-expressing cells attacked by one germinating fungal spore were scored. Data were pooled from three independent experiments. *Significant differences at P = 0.05 compared to the control experiments.
In addition, the number of detectable GUS-expressing cells showing an interaction with a fungal spore showed a 30% to 50% decrease when compared with the control experiment. It is likely that the number of interacting cells is lower because of a hypersensitive reaction leading to early cell death.

No decrease in the number of compatible interactions was observed after infection with isolate 97019, virulent on Pm3d and avirulent on Pm3a on leaves bombarded with the Pm3d candidate gene, demonstrating that the Pm3d candidate gene induced a resistance reaction specific to the isolate avirulent on wheat lines was the key to the identification of additional Pm3 alleles. The isolation of Pm3 alleles might have been complicated by haplotype variation in the different Pm3 lines. RGL genes of the Pm3 family are spread over a large genomic region of approximately 1 Mb on wheat chromosome 1A (Yahiaoui et al., 2004), and many of them are expressed and could potentially encode functional resistance genes. These genes show variability in the different Pm3 lines, as indicated by the absence of amplification of STS markers for two of them in some of these lines. Key markers that allowed the identification of a conserved Pm3 haplotype based on RFLP profiles were derived from the 5′ noncoding region and the 3′ UTR sequence of Pm3b. Conservation of these markers was confirmed at the sequence level and indicates that no large rearrangements have affected the locus in Pm3 lines since the divergence

Resistance Protein Comparison and Substitution Rate Analysis of Pm3a, Pm3b, Pm3d, and Pm3f Alleles

The protein sequences encoded by the Pm3a, Pm3b, Pm3d, and Pm3f genes share, on average, 96.5% identity and 97.8% similarity over the entire sequence. A comparison of the four protein sequences revealed a complete conservation of the CC domain (100% identity). Differences were found in the NBS-LRR domains (Fig. 4). In the NBS region up to the first LRR, different blocks of conserved and nonconserved regions are found between the four alleles. PM3A, PM3D, and PM3F are identical between amino acid 267 and amino acid 402, whereas PM3B is different in this region. Between amino acid 405 and amino acid 543, PM3A and PM3B are identical, whereas PM3D and PM3F show a different sequence (Fig. 4). Finally, between amino acids 544 and 589, PM3A, PM3B, and PM3F share a common sequence different from PM3D (Fig. 4). This indicates the occurrence of gene conversion or intragenic recombination events in the evolution of the allelic sequences. Based on the sequence identity patterns, the PM3A and PM3F sequences of the NBS domain up to the first LRR could result from recombination or gene conversion events between the PM3B and PM3D corresponding sequences (Fig. 4).

In the LRR domain, the number of LRR repeats is identical between the four proteins, and most of the LRR residues were conserved. Amino acid variation occurs mostly in the predicted solvent-exposed residues in the xxLxLxx motif. An overall proportion of 11% (15 of 140) of the predicted solvent-exposed LRR residues shows differences, whereas only 2.4% (17 of 705) of the amino acid residues are different in the rest of the LRR. Among the four alleles, PM3A shows the highest variability in the LRR with 12 different solvent-exposed residues in four LRR repeats, whereas PM3B, PM3D, and PM3F show fewer differences (4–5 different solvent-exposed residues; Fig. 4). Comparison of the LRR domain between PM3A and PM3F reveals an identical structure interrupted by a block of 72 amino acids in PM3A where polymorphism is found (between amino acids 1,087 and 1,158; Fig. 4). This sequence block could originate from a recombination or gene conversion event with an unknown gene as a donor. Almost all of the differences in solvent-exposed residues occur in the last one-third of the LRR domain of the four proteins.

Nonsynonymous (Ka) and synonymous (Ks) nucleotide substitution rates of the CC-NLS domain, LRR domain, structural LRR residues, and predicted solvent-exposed LRR residues of the PM3A, PM3B, PM3D and PM3F proteins were calculated, and Ka/Ks ratios were compared (Table II). Indication for diversifying selection (Ka/Ks > 1) was observed only for the predicted solvent-exposed LRR residues, where an average Ka/Ks ratio of 3.4 was obtained. This average ratio mostly reflects the divergence between PM3A/PM3F and PM3B/PM3D, whereas the pairwise value of Ka/Ks between PM3B and PM3D was much lower due to very few differences in the LRR sequence of these genes. The rest of the coding sequences did not show statistically significant differences of Ka to Ks (Table II).

DISCUSSION

Identification of a Pm3 Haplotype in Different Wheat Lines/Cultivars

The first cloned Pm3 allele, Pm3b, was identified using a combination of genetic mapping in hexaploid wheat and physical mapping in lower ploidy relatives (T. monococcum cv DV92 and T. durum cv Langdon; Yahiaoui et al., 2004). None of these relatives showed complete haplotype conservation with the Pm3b line Chul, and different haplotypes were also found in hexaploid wheat between the lines Chul (Pm3b) and Frisal (susceptible; Yahiaoui et al., 2004). The conservation of a characteristic Pm3 haplotype in all Pm3 lines was the key to the identification of additional Pm3 alleles. The isolation of Pm3 alleles might have been complicated by haplotype variation in the different Pm3 lines. RGL genes of the Pm3 family are spread over a large genomic region of approximately 1 Mb on wheat chromosome 1A (Yahiaoui et al., 2004), and many of them are expressed and could potentially encode functional resistance genes. These genes show variability in the different Pm3 lines, as indicated by the absence of amplification of STS markers for two of them in some of these lines. Key markers that allowed the identification of a conserved Pm3 haplotype based on RFLP profiles were derived from the 5′ noncoding region and the 3′ UTR sequence of Pm3b. Conservation of these markers was confirmed at the sequence level and indicates that no large rearrangements have affected the locus in Pm3 lines since the divergence...
Figure 4. Comparison of amino acid sequences of the proteins encoded by the Pm3a, Pm3b, Pm3d, and Pm3f genes. The CC domain is underlined. NBS and LRR domains are indicated. Conserved motifs characteristic of the NBS domain of NBS-LRR proteins (in the following order: P-loop, Kinase 2, RNBS-B, GLPL, MHD) are underlined. Color-shaded regions in the NBS domain represent blocks of sequences that are indicative of recombination/gene conversion events. Polymorphic amino acids in the XXLXLXX motif (where X represents predicted solvent-exposed residues in the LRR domain, and L is a Leu or another aliphatic amino acid) are in red.
of the identified Pm3 genes. Based on the T. durum BAC contig spanning the Pm3 locus, the size of the conserved haplotype in Pm3 lines could be as large as 100 kb. However, the extent of conservation at the sequence level in the Pm3 lines is yet unknown. This haplotype conservation suggests that the Pm3 region is relatively stable meiotically in contrast to other cereal disease resistance loci, such as rpl1 in maize (Hulbert, 1997).

Interestingly, the two wheat lines Kanzler and Chinese Spring also show a Pm3 haplotype. Chinese Spring and Kanzler are generally susceptible to powdery mildew, and powdery mildew isolates are regularly propagated on line Kanzler (Zeller et al., 1993). If functional Pm3 alleles are present in these two lines, these genes were possibly overcome by virulent pathogen races and are no longer efficient against any of the tested isolates. Susceptibility in these lines could also be due to a mutation in these genes or to a defect in signal transduction downstream of PM3.

The Pm3a, Pm3b, Pm3d, and Pm3f Alleles Form a True Allelic Series

During the different cloning steps of Pm3a, Pm3d, and Pm3f, specific probes and primers detected only one copy of a gene showing an identical structure to Pm3b on wheat chromosome 1A. An almost complete sequence conservation was found in the 5’ noncoding region of 4.36 kb associated to the cloned Pm3 alleles where only one nucleotide InDel polymorphism allowed distinguishing of the Pm3a/Pm3f sequences from the Pm3b/Pm3d sequences. This region was only present as a single copy in the A genome of the Pm3 lines used in this study. Sequence analysis and comparison of the Pm3a, Pm3b, Pm3d, and Pm3f genes revealed an identical gene structure and a high level of sequence identity over the entire sequence, in agreement with the overall haplotype conservation. These data, together with the functional analysis, strongly suggest that Pm3a, Pm3b, Pm3d, and Pm3f are true alleles at the Pm3 resistance locus. Up to now, a total of four loci encoding allelic series of resistance genes have been characterized at the molecular level. In two cases, the flax rust resistance gene L and the P. parasitica resistance gene RPP13 in Arabidopsis, the locus consisted of one single gene encoding different alleles in different lines or accessions (Ellis et al., 1999; Bittner-Eddy et al., 2000). The Pm3 locus is more similar in structure to the other two loci, the P flax rust resistance locus and the Mla barley powdery mildew resistance locus, which are complex loci consisting of several paralogous NBS-LRR genes. P locus genes encoding P and P2 rust resistance specificities in flax have been shown to probably be allelic (Dodds et al., 2001), and the cloned Mla alleles form a true allelic series where all variants are descendants of one ancestral RGH1 family member (Halteman et al., 2001; Zhou et al., 2001; Wei et al., 2002; Shen et al., 2003; Halterman and Wise, 2004). Similarly, our data indicate that the Pm3 locus is defined by a single RGL gene that is a member of a large gene family. This would imply that all other genetically defined Pm3 alleles are encoded by the same gene, a hypothesis that remains to be tested.

Diversifying Selection on the Pm3a-, Pm3b-, Pm3d-, and Pm3f-Encoded Proteins

The comparison of the proteins encoded by the four Pm3 alleles revealed differences in the NBS-LRR domain. Our data of the Ka/Ks ratios indicated that the predicted solvent-exposed LRR residues of the Pm3A, Pm3B, Pm3D, and Pm3F proteins are under diversifying selection and are likely to play a role in resistance specificity. These data are consistent with the majority of LRR-containing plant disease resistance proteins, where diversifying selection is generally most noticeable on amino acids predicted to be solvent exposed (Parniske et al., 1997; Botella et al., 1998; Meyers et al., 1998; Noel et al., 1999; Halterman et al., 2001; Sun et al., 2001; Rose et al., 2004). Diversifying selection was also shown to act on avirulence gene products from flax rust Melampsora linii (Dodds et al., 2004) and from downy mildew P. parasitica (Allen et al., 2004), indicating that hosts and pathogens are locked in a coevolutionary arms race. Specificity determination can be controlled by few amino acids changes at LRR solvent-exposed residues. This has been shown between the P and P2 alleles of flax rust resistance, where six amino acids changes in the LRR β-strand/β-turn motif determined the difference between the P and P2 rust resistance specificities (Dodds et al., 2001). Given that the majority of polymorphic solvent-exposed residues in PM3 proteins are present in the last one-third of the LRR domain, we speculate that the determination of specificity mediated by PM3 proteins lies in this region.

In the NBS domain of PM3 proteins, no evidence for diversifying selection was found, although variability was detected. NBS domains typically do not show evidence of diversifying selection. Exceptions are the N-terminal Toll-interleukin receptor-like domain of

<table>
<thead>
<tr>
<th>Allele</th>
<th>Complete</th>
<th>CC-NBS</th>
<th>LRR</th>
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<td></td>
<td>Gene</td>
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<td>2.1</td>
<td>1.3</td>
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<td>1.2</td>
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</tr>
<tr>
<td>Ka/Ks</td>
<td>0.6</td>
<td>0.4</td>
<td>1.2</td>
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*aSignificant differences at P = 0.02 were found between synonymous and nonsynonymous changes.*

### Table II. Average rate of nucleotide substitutions per 100 sites among four Pm3 alleles (Pm3a, Pm3b, Pm3d, and Pm3f)

The coding sequences for the NC-NBS (nucleotides 1–1,710), LRR (nucleotides 1,711–4,245), structural LRR residues (2,115 nucleotides), and predicted solvent-exposed LRR residues (420 nucleotides coding for 140 X residues in the XX(XX)XX motif) were analyzed separately.
the L protein (Ellis et al., 1999; Luck et al., 2000) and the N-terminal CC-NBS domain of the Lr10 gene product (Feuillet et al., 2003). Comparison among the proteins encoded by the four Pm3 alleles also revealed a higher conservation of the N-terminal region containing the CC domain (100% identity over 190 amino acids). This is similar to the Mla1, Mla6, Mla7, and Mla12 alleles, which have 100% identity over 90 amino acids in the CC domain (Haltermann et al., 2001; Zhou et al., 2001; Shen et al., 2003; Haltermann and Wise, 2004). This sequence conservation indicates a highly conserved function of the CC domain both in MLA and PM3 proteins. Domain swapping experiments between alleles will be very valuable to learn how the different regions of the Pm3 gene products function together to result both in specific pathogen recognition and in signal transduction. As long as the nature of the recognition targets of the PM3 proteins remains unclear, such an improved understanding will be limited to the plant-encoded partner of the interaction. Recently, the first avirulence gene products from biotrophic fungi have been cloned from flax rust M. linii (Dodds et al., 2004) and were shown to be expressed in rust haustoria. No avirulence gene has yet been isolated from wheat powdery mildew, and it is currently not possible to test for direct or indirect interaction between PM3 proteins and avirulence gene products.

Evolution of the Pm3 Locus

High sequence conservation (more than 97% sequence identity) is observed between the Pm3a, Pm3b, Pm3d, and Pm3f genes, including the 5′ noncoding region of 4.36 kb. This is different from the situation at the Mla locus in barley, where different retroelements that inserted less than 2 kb from the start codon of Mla alleles allowed the identification of at least two evolutionary pathways leading to the generation of functional Mla alleles (Haltermann and Wise, 2004). No such events could be detected in the available Pm3 allelic sequences. Another difference between Mla and Pm3 genes is illustrated by differences within intron sequences among Mla genes (Shen et al., 2003), whereas in the Pm3 genes the single intron sequence is identical. Due to recombination suppression at the Mla locus (Wei et al., 1999, 2002), evolution of Mla allelic sequences was shown to be mostly due to insertion/deletion events and point mutations. Pm3 alleles 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. In the LRR region, PM3A and PM3F differ only by one sequence block that could have resulted from recombination or gene conversion, whereas the few changes between PM3B and PM3D could be the result of point mutations. Four types of NBS domain sequences found in the PM3 proteins probably resulted from a limited number of sequence exchanges between the allelic sequences. This suggests that sequence evolution generating new Pm3 alleles is based on recent events of mutation and intragenic recombination or gene conversion occurring most probably between allelic sequences, and that the Pm3 alleles could have diverged more recently than the Mla alleles.

Many functional resistance alleles at the barley powdery mildew resistance locus Mla have been described in the wild progenitor of cultivated barley (Hordeum spontaneum; Jahoor and Fischbeck, 1993). This indicates that Mla specificities could have been introgressed into barley from its wild relative. With the exception of Pm3h, which might have originated from a cultivated T. durum Ethiopian line, the active Pm3 alleles have all been found in hexaploid bread wheat lines (Hsam and Zeller, 2002). In addition, the alleles were first characterized in lines from different gene pools originating from all continents where wheat is grown (Briggle and Sears, 1966; Zeller et al., 1993). This suggests that they have evolved independently from each other in different environments and breeding conditions. The finding of a conserved Pm3 haplotype in the lines with an active Pm3 allele is in agreement with a recent evolution of new alleles derived from a common progenitor haplotype. Based on all these data, we propose that the Pm3 alleles have a very recent evolutionary origin, and possibly have evolved only after wheat domestication. Further work on the remaining Pm3 alleles and on the Pm3 locus in tetraploid wheat species should shed more light on the evolution of this locus in the wild and cultivated wheat species.

MATERIALS AND METHODS

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requester.

Plant Material and Wheat Powdery Mildew Isolates

Seeds of wheat (Triticum aestivum) near-isogenic lines in the Chancellor background Assosan/8′Chancellor (Pm3h), Chul/8′Chancellor (Pm3b), Triticale/8′Chancellor (Pm3c), and Michigan Amber/8′Chancellor (Pm3f) were provided by FAL Reckenholz, Switzerland. Lines Abessi (Pm3h), N324 (Pm3i), and GUS122 (Pm3j) were supplied by F. Zeller, Technische Universität Munich, Germany. Cultivar Kolibri (Pm3l) was provided by J. Enjalbert, INRA Grignon, France. Wheat line W150 (Pm3k) was provided by R. Park, Plant Breeding Institute, Cobbitty, Australia. Wheat powdery mildew (Blumeria graminis f. sp. tritici)–susceptible lines Frisal, Chancellor, Chinese Spring, and Kanzler and tetraploid wheat Triticum durum cv Langdon were used as controls. A set of aneuploid nullitetrasomic lines of Chinese Spring (Sears, 1966) was used for chromosomal assignment of molecular probes. Wheat powdery mildew isolates 97019 (AvrPm3a and avrPm3d), 96229 (avrPm3c and avrPm3f) and 96224 (AvrPm3d and AvrPm3f) were provided by P. Streckeisen, FAL Reckenholz, Switzerland. Isolates were maintained on wheat cv Kanzler by weekly transfer to fresh plants.

Marker Analyses

Isolation of genomic DNA, Southern hybridization, and labeling experiments were performed as previously described (Stein et al., 2000). Markers for
haplotype analysis were chosen from the genetic and physical map at the Pm3b locus (Yahiaoui et al., 2004) and from the 9-kb genomic fragment of Pm3b amplified from Chul (pm3b). Analysis of microsatellite marker Psp2999, associated with low-M, glutenin genes on chromosome 1A, was carried out as described (Devos et al., 1995). RFLP probes were as follows: (1) SFR159, from Triticum monococcum BAC 453N11; (2) TmRL1-1pr, a noncoding 500-bp fragment located 4 kb upstream of Pm3b (This probe partially corresponds to the original TmRL1-1pr, a probe derived from the 5′ noncoding region of the TmRL1-1 sequence on T. monococcum BAC 453N11); (3) Chs3kb, an 8-kb fragment covering 4 kb of noncoding 5′ region and 4 kb of the coding region of the Pm3b gene; (4) 3′ UTR-Pm3, the 3′ noncoding sequence of Pm3b; and (5) 294D11-31 and (6) 294D11-22, low-copy probes identified by low-pass-sequencing of the T. durum BAC clones spanning the Pm3b locus. For SSTS marker analysis, PCR amplification was performed in 25 μL with 0.5 units of Taq DNA polymerase (Sigma), 1x PCR buffer (10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.5 mM MgCl2, and 0.001% gelatin), 0.1 μM dNTPs, 10 μM primers, and 65 ng of genomic DNA template. Amplification was carried out in a PTC-200 thermocycler (M J Research) as follows: 3 min at 94 °C, 34 cycles of 45 s at 94 °C, 0.45 to 2 min at annealing temperature, 0.45 to 2 min at 72 °C, and final extension at 72 °C for 5 min. Amplified products were separated by electrophoresis on a 1% agarose gel.

Expression Analysis by RT-PCR and 3′ RACE-PCR

Total RNA was extracted from leaves of 10-d-old wheat seedlings using the Trizol reagent (Invitrogen). RT-PCR was performed on 2 μg of Poly(A)+ RNA (Quiagen) by using specific primer pairs for 3′ SP1 (5′-GCTGCCTGACCCCTCAAGAGACCTG-3′) and consLR3 (5′-GCTGCCAGCATCRAAGGGAGC-3′) from the LRR region of Pm3b. The GAPc (glycerol aldehyde-3-P dehydrogenase cytoplasmic) gene was used as a control. 3′ RACE-PCR was performed on 500 ng of Poly(A)+ RNA with the Gene Racer kit (Invitrogen) using primer 3′ SP1 and primer 3′ SP2 (5′-GCTGCCTGACCCCTCAAGAGACCTG-3′) as a nested primer.

Cloning of Pm3 Alleles and Test Gene Constructs

Amplification of the UP3B/ChulFIIR fragment was carried out with the Ex Taq polymerase (Takara Bio) using primers UP3B (5′-TGTGTTCCAGACAAATCCCA-3′) and ChulFIIR (5′-TACCTGAACTGAGTATTCGCA-3′). PCR amplification of the Pm3 genes was carried out with the PfuUltra high-fidelity DNA polymerase (Stratagene). Specific primers UP1 (5′-GCCACACAGGAGGAATCTTAGAA-3′) and consLR3 (5′-GCTGCCAGCATCRAAGGGAGC-3′) derived from the 5′ noncoding region, 300 bp upstream of the start codon, and the 3′ noncoding region, respectively, were used in a first step. A second round of amplification was carried out using modified primers at the start and stop codons with restriction sites for BamHI and SalI enzymes (BHI-1, 5′-TATAATGCTCAAACTTGCCAGGCCGCTGC-3′; Sal-1, 5′-CCAGCCCTGCAGGACGTAGGGC-3′). Two independent PCR reactions were carried out for each line. The obtained fragments were directly cloned into the multiple cloning site vector of pPGV1 (Schweizer et al., 1999) between a 540-bp fragment of the cauliflower mosaic virus (CaMV) 35S promoter and the CaMV 35S terminator. Three clones per PCR reaction were sequenced. The reporter-gene (pUBiGUS) construct was obtained as previously described (Schweizer et al., 1999). DNA sequencing was carried out on an Applied Biosystems Sequence model 377.

Single-Cell Transient Assay

Biological bombardment was performed as previously described (Schweizer et al., 1999; Yahiaoui et al., 2004). Leaves were bombarded with a 1.2 (w/w) mixture of pUbGUS and PGV1 empty vector used as control, or with a 1:2 (wt/wt) mixture of pUbGUS and PGV1 containing test gene constructs. Leaf segments were challenged with powdery mildew 4 h after the bombardment. Staining for GUS activity was carried out 48 h postinoculation, followed by staining of fungal structures with Coomassie blue. GUS-expressing cells attacked by a single germinating spore were evaluated by transmission light microscopy. A compatible (resistance) interaction was characterized by the presence of an appressorium. A Student’s t test was performed to analyze significance of the results.

Sequence Analysis, Resistance Protein Comparison, and Substitution Rate Analysis

Sequence assembly was performed with the CAP3 and Phrap assembly engine (version 0.990319; provided by P. Green (Department of Genome Sciences and Howard Hughes Medical Institute, University of Washington)) and available at http://www.phrap.org). Nucleotide sequences were aligned using the ClustalW software. Comparison of the resistance proteins was performed with the GCC sequence analysis software package version 10.1. The substitution rate (Ka/Ks) analysis was carried out on Pm3a, Pm3b, Pm3d, and Pm3f nucleotide sequences aligned with the PILEUP program of the GCC software. The alignment was adjusted manually with the program LINEUP using the amino acid sequence as a guide to keep a codon-by-codon alignment. The rate of nonsynonymous (Ka) versus synonymous (Ks) nucleotide substitutions per 100 sites (Ka/Ks) was computed with the program DIVERGE. A Student’s t test was performed to test for significant differences between the rates of nonsynonymous (Ka) versus synonymous (Ks) nucleotide substitutions. The different domains (CC-NBS, LRR, structural LRR residues, and solvent-exposed LRR residues in the XXLXXX motif) were chosen according to the domains defined by Meyers et al. (2003) for CC-NBS-LRR-encoding genes.

Sequence data from this article have been deposited with the GenBank database under accession numbers AY939880 (Pm3a), AY939881 (Pm3d), DQ071554 (Pm3f), DQ071555 (3′ RACE-Pm3a), AY605285 (3′ RACE-Pm3d), and DQ071556 (3′ RACE-Pm3f).

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