A highly differentiated region of wheat chromosome 7AL encodes a Pm1a immune receptor that recognizes its corresponding AvrPm1a effector from *Blumeria graminis*

Tim Hewitt1,2*, Marion C. Müller3*, István Molnár4, Martin Mascher5, Katerina Holusová4, Hana Šimková4, Lukas Kunz3, Jianping Zhang1, Jianbo Li2, Dhara Bhatt1, Raghvendra Sharma1, Seraina Schudel4, Guotai Yu6, Burkhard Steuernagel6, Sambasivam Periyannan1, Brande Wulff6, Mick Ayliffe1, Robert McIntosh2, Beat Keller3, Evans Lagudah1,2 and Peng Zhang2

1Agriculture & Food, Commonwealth Scientific & Industrial Research Organization, GPO Box 1700, Canberra, ACT 2601, Australia; 2School of Life and Environmental Sciences, Plant Breeding Institute, University of Sydney, 107 Cobbitty Road, Cobbitty, NSW 2570, Australia; 3Department of Plant and Microbial Biology, University of Zurich, Zollikerstrasse 107, Zurich 8008, Switzerland; 4Centre of the Region Hana for Biotechnological and Agricultural Research, Institute of Experimental Botany of the Czech Academy of Sciences, Šlechtitelu 31, Olomouc 779 00, Czech Republic; 5OT Gatersleben, Leibniz Institute of Plant Genetics and Crop Plant Research, Corrensstr. 3, Stadt Seeland D-06466, Germany; 6John Innes Centre, Norwich, NR4 7UH, UK.

*These authors contributed equally to the work.

**Summary**

- **Pm1a**, the first powdery mildew resistance gene described in wheat, is part of a complex resistance (R) gene cluster located in a distal region of chromosome 7AL that has suppressed genetic recombination.
- A nucleotide-binding, leucine-rich repeat (NLR) immune receptor gene was isolated using mutagenesis and R gene enrichment sequencing (MutRenSeq). Stable transformation confirmed Pm1a identity which induced a strong resistance phenotype in transgenic plants upon challenge with avirulent *Blumeria graminis* (wheat powdery mildew) pathogens.
- A high-density genetic map of a *B. graminis* family segregating for Pm1a avirulence combined with pathogen genome resequencing and RNA sequencing (RNAseq) identified AvrPm1a effector candidate genes. In planta expression identified an effector, with an N terminal Y/FxC motif, that induced a strong hypersensitive response when co-expressed with Pm1a in *Nicotiana benthamiana*.
- Single chromosome enrichment sequencing (ChromSeq) and assembly of chromosome 7A suggested that suppressed recombination around the Pm1a region was due to a rearrangement involving chromosomes 7A, 7B and 7D. The cloning of Pm1a and its identification in a highly rearranged region of chromosome 7A provides insight into the role of chromosomal rearrangements in the evolution of this complex resistance cluster.

**Introduction**

**Pm1**, the first catalogued wheat gene to confer resistance to powdery mildew caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), has attracted research interest for over 60 years due to its complete genetic association with both a leaf rust (caused by *Puccinia triticina*) resistance gene *Lr20* (*Waterhouse, 1952*) and stem rust (caused by *P. graminis* f. sp. *tritici*) resistance gene *Sr15* (*Watson & Luig, 1966*). McIntosh (1977) used ethyl methanesulfonate (EMS) mutagenesis to show that loss of Pm1 resistance occurred independently of both *Lr20* and *Sr15* resistance, but significantly, these latter two resistances mutated together. **Pm1** was later designated **Pm1a** following the identification of four more allelic variants (**Pm1b**–**Pm1e**) at the **Pm1** locus ([https://shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp](https://shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp)). **Pm1a**, **Pm1c** and **Pm1e** (syn. **Pm22**) were each present in common wheat *Triticum aestivum* whereas the **Pm1b** and **Pm1d** alleles were identified in *T. monococcum* and *T. spelta* var. *dubhmelanum*, respectively (Hsam et al., 1998). Further mapping located **Pm1** in the terminal deletion bin 0.99–1.00 of chromosome 7AL, which constitutes approximately 1% of this chromosome arm (Yao et al., 2007; Ouyang et al., 2014; Worthington et al., 2014).

Over 60 loci for **Pm** resistance have been described in wheat and its relatives since the identification of **Pm1**. A small number of these genes have been cloned and most encode nucleotide-binding, leucine-rich repeat (NLR) immune receptors, i.e. **Pm2**
(Sánchez-Martín et al., 2016), the Pm3 allelic series (Brunner et al., 2010), Pm5e (Xie et al., 2020), Pm8 (Hurni et al., 2013), Pm17 (Singh et al., 2018), Pm21 (He et al., 2018; Xing et al., 2018), Pm41 (M. Li et al., 2020) and Pm60 (Zou et al., 2018). However, a tandem kinase-pseudokinase, Pm24 (Lu et al., 2020), also provides powdery mildew resistance as do broad-spectrum adult plant resistance genes Pm38 and Pm46 that each provide partial resistance to powdery mildew and rust diseases in adult plants and encode an ABC transporter and an altered sugar transporter, respectively (Krattinger et al., 2009; Moore et al., 2015).

The corresponding avirulence effectors for several wheat NLR resistance genes, such as Pm2 and the Pm3 allelic series, were recently identified from Bgt. Map-based cloning using haploid F1 populations enabled the identification of AvrPm2, AvrPm3\textsuperscript{v209/2}, AvrPm3\textsuperscript{g63/2} and a suppressor locus of the Pm3 allelic series SvrPm3\textsuperscript{g60} (Bourras et al., 2015, 2019; Praz et al., 2017). These Av proteins show very little sequence similarity to each other or with known proteins. However, these Bgt Av proteins are small (109–130 amino acids) and may, from modelling, share some structural similarities (Praz et al., 2017; Bourras et al., 2019).

Previous attempts to isolate Pm1 and Lr20 by map-based cloning were unsuccessful due to restricted genetic recombination, which may be due to a translocation from an unknown source to the distal region of chromosome 7AL (Neu et al., 2002). In this study, new techniques were employed to overcome this limitation for positional gene isolation. Specifically, the method of mutagenesis and resistance (R) gene enrichment sequencing (MutRenSeq) is a targeted approach that does not depend on recombination but instead utilizes a sequence bait library to enrich NLR encoding sequences from knockout mutants for the target R gene. Comparing random mutations between independent mutants allows the identification of a candidate gene. Herein, we report the successful cloning of Pm1a from common wheat using MutRenSeq, and the corresponding AvrPm1a effector from the wheat powdery mildew fungus Bgt using genetic mapping and RNA sequencing (RNaseq). Additionally, the method of chromosome sequencing (ChromSeq) allowed us to isolate sequences from Pm1a bearing chromosome 7A, showing evidence of rearrangement leading to suppressed recombination.

Materials and Methods

Plant materials

Six M\textsubscript{2} putative point mutations for Pm1a in Chinese Spring\textsuperscript{5/1} Axminster 7A (CS/Ax7A) substitution line carrying the Pm1a locus on chromosome 7A from Axminster (Sears & Briggie, 1969) were used in RenSeq analysis (mutants #396, 404, 428, 435, 446 and 650, see Supporting Information Fig. S1). F\textsubscript{2} RILs used in genetic analysis and confirmation of a diagnostic marker developed from the gene candidate were derived from a combination of three different crosses segregating for Pm1a, namely, CS × CS/Ax7A, CS × Thew and Thew × CS. Additional mutants from Thew, along with cultivars Norka and Schomburgk were also used to test Pm1 markers. Transformable wheat cultivar Fielder, which does not carry Pm1, was used for transgenic complementation. CS/Ax7A was used in flow sorting of chromosome 7A and sequencing.

Screening for powdery mildew response

The avirulent Bgt inoculum used for screening Pm1a was collected from the glasshouse area of the CSIRO Canberra campus (Australia) and maintained on seedlings of susceptible cultivar Morocco for the duration of study. Screening of the mutagenized and genetic populations took place in an isolated glasshouse at the University of Sydney Camden Campus. Additional powdery mildew tests were similarly conducted at CSIRO Canberra with CS/Ax7A and Thew included as resistant controls. Conidiospores were shaken or brushed directly onto 2-week-old seedlings grown in a disease-free glasshouse. CS/Ax7A and CS were used as resistant and susceptible controls, respectively. Phenotyping of seedlings was simply as resistant or susceptible as Pm1a confers a completely immune response (infection type (IT) 0) compared to CS (IT 3+).

MutRenSeq pipeline

Treatment with EMS was performed as described in Sharp & Dong (2014) on 600 and 300 seeds of CS/Ax7A in 0.5% and 0.6% EMS, respectively. Seeds of Thew were also treated with 0.5% and 0.6% EMS using 300 seeds in each. Thus, 572 and 373 M\textsubscript{1} plants of CS/Ax7A and Thew were harvested, respectively. Homozygous CS/Ax7A mutants retaining the closely linked Lr20 and Sr15 from six different M\textsubscript{2} plants were used in subsequent R gene enrichment. DNA was prepared from leaves of uninoculated seedlings of the six mutants and one wild type (WT). DNA extraction, target enrichment using Triticeae RenSeq Bait Library V2 (https://github.com/steuernb/Muta ntHunter), Illumina sequencing, de novo assembly and read mapping were carried out as described in Steuernagel et al. (2016). Single nucleotide polymorphism (SNP) calling and candidate identification were performed using the MuTrigo pipeline with default parameters (https://github.com/TC-Hewitt/MuTrigo).

Pm1a structure confirmation

The partial candidate contig identified by RenSeq (contig #8725) was aligned to the IWGSC Chinese Spring reference RefSeq v.1.0 (CSv1) (IWGSC, 2018) using BLASTN (Zhang et al., 2000) and the top matching gene was aligned to the RenSeq WT assembly using BLASTN. High scoring pairs were filtered using a custom UNIX script and the corresponding candidate contig was chosen based on presence of expected domains and a SNP in the outstanding mutant. Both candidate contigs were confirmed to belong together based on Sanger sequencing of the PCR product from genomic DNA bridging the two sequences. RNA was extracted from leaves of CS/Ax7A using the RNasy Plant Mini Kit (Qiagen, Chadstone Centre, VIC, Australia). Transcript structure, including flanking untranslated regions (UTRs), was obtained by 5’ and 3’ RACE (rapid amplification of complementary DNA ends) using a SMARTer RACE 5’/3’ Kit (Clontech, Mountain View, CA, USA). The upstream promoter region,
intron sequences and downstream terminator region were inferred from a large contig from the Ax7A de novo ChromSeq assembly bearing the Pm1a sequence. Coding sequence and translation were predicted using FGENESH (Solovyev et al., 2006).

Mutant confirmation

EMS-induced SNPs identified in mutants by MutRenSeq were confirmed with Sanger sequencing of PCR-amplified coding regions from mutants. Nonsynonymous amino acid substitutions were confirmed using CODONCODE ALIGNER 8.0 (https://www.codoncode.com/aligner/).

Transformation confirmation of Pm1a

A 9386 bp genomic sequence of Pm1a, including all introns, 2 kb of 5 ’UTR and 1.5 kb of 3 ’UTR encompassing the native promoter and terminator, was synthesized and cloned (Epoch Life Sciences, Missouri City, TX, USA) into Nod1/Spi-digested binary vector vecBarIII. The Pm1a gene was introduced into cv Fielder using the Agrobacterium-transformation protocol (Ishida et al., 2015) and phosphinothricin as the selective agent. Ten independent transgenic plants (T0) carrying the Pm1a gene as well as three nontransgenic sibling control lines were recovered. The T0 and sibling controls were acclimatized to glasshouse conditions. After 2 weeks, sibling controls, WT controls and T0 plants were inoculated with Bgt as described earlier. Then, 10 cm leaf samples were taken from the second leaf at 10 d post-inoculation (dpi) and again from the third or fourth leaves at 14 dpi. Leaves were scanned using an Epson Perfection V600 Photo scanner immediately after sampling. For quantification of fungal biomass, 10 and 14 dpi leaf samples were processed and stained for chitin as described by Ayliffe et al. (2013). Relative fluorescence was measured on a FLUOstar Omega spectrophotometer (BMG Labtech, Mornington, VIC, Australia) with excitation filter 485-12 and emission filter Em520.

AvRPm1a QTL mapping and fine mapping

Crossing, genotyping and genetic map calculations for bi-parental population 96224 × THUN-12 were described in Müller et al. (2019). Phenotyping was performed as described in Bourras et al. (2019) on wheat cultivar Aixminster/8*Chancellor. Susceptible cultivars Chancellor and Kanzler were used as infection controls. Virulence scoring was performed after 10 d on at least six independent detached leaf segments according to the following scoring system: 0 (0–10% leaf coverage (LC)), 0.25 (10–40% LC), 0.5 (40–60% LC) and 1 (60–100% LC). Quantitative trait locus (QTL) analysis was done with the R/QTL v1.46.2 (https://rqt.org/) in Rstudio (RStudio-Team, 2018). The code used to conduct the analysis as well as the input files were deposited to Github (https://github.com/MarionCMueller/QTL).

Genetic fine mapping was performed manually based on nine recombinant progeny in the QTL interval (defined by flanking markers snp64149 and snp64379) that showed virulence scores of 0 or 1. Four recombinant progeny showing intermediate phenotypes were excluded. A single recombinant progeny did not fit into our proposed high-resolution map and would place AvRPm1a upstream of marker snp64348. However, this recombinant would exclude any of the candidates in the QTL interval. Given the partially quantitative nature of the AvRPm1a phenotype this recombinant was tentatively excluded from the analysis.

Candidate AvRPm1a identification

AvRPm1a candidates were identified by manual curation of gene models in the candidate interval based on assembly, annotation and candidate effector definition of Bgt Genome_v3.16 reported in Müller et al. (2019). To verify the annotation, RNAseq data of both parental isolates were mapped against the reference genome with STAR (v.2.6.0a) (Dobin et al., 2013) as described in Praz et al. (2018) and visualized with IGV (v.2.8.0) (Robinson et al., 2011). The RNAseq dataset was generated previously, from susceptible cv Chinese Spring and triticale cv Timbo infected with 96224 or THUN-12, respectively (Menardo et al., 2016; Praz et al., 2018). Infected leaf samples were collected at 2 dpi during Bgt haustorium formation. The final set of candidate genes in the interval are listed in Supporting Information Table S1.

Protein domains of candidate effectors were predicted using Pfam (v.33.1) (https://pfam.xfam.org/). Signal peptide prediction was based on SignalP (v.3.0) (Bendtsen et al., 2004) with default settings. Alignment of the E004 candidate effector family was done with the Clustal algorithm of MEGA X (Kumar et al., 2018) with default parameters. For expression analysis and differential gene expression, RNAseq reads were aligned against the Bgt 96224 CDS (Müller et al., 2019) with Salmon (v.0.12.0) (Patro et al., 2017) as described in Praz et al. (2018). Subsequent expression and differential expression analysis were done using the edgeR (v.3.11) (Robinson et al., 2010) package in Rstudio (RStudio-Team, 2018) as described in Praz et al. (2018).

Transient co-expression assay in Nicotiana benthamiana

Effector candidate genes were codon-optimized for expression in Nicotiana benthamiana using the online tool of Integrated DNA Technologies (https://eu.idtdna.com/CodonOpt). Signal peptides were predicted using the SignalP algorithm (http://www.cbs.dtu.dk/services/SignalP-3.0/) and subsequently replaced with a start codon. For hemagglutinin (HA)-epitope tagged effectors the corresponding sequence was added at the C-terminus directly upstream of the stop codon. The resulting sequences predicted to encode the mature peptide, including flanking attL gateway sites, were synthesized by BioCat GmbH (https://www.biocat.com). Sequences for all synthesized DNA fragments are described in Supporting Information Dataset S1. The synthesized effector genes were subsequently cloned into the binary vector pIPKb004
(Himmelbach et al., 2007) using Gateway LR clonase II (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

The Pm1a resistance gene was synthesized in two overlapping fragments (Dataset S1) with flanking sequences matching the binary vector pIPKb004 (Himmelbach et al., 2007) by BioCat GmbH. For the HA-epitope tagged version the HA coding sequence was introduced by two subsequent rounds of PCR with the primers listed in Table S2. The two resulting fragments were cloned by In-Fusion Cloning (Takara, Tokyo, Japan) into a modified pIPKb004 plasmid (Himmelbach et al., 2007) in which the gateway cassette was removed using the restriction enzymes HindIII and BsrGI. All pIPKb004-based binary constructs were verified by Sanger sequencing and transformed into Agrobacterium tumefaciens strain GV3101 using a freeze–thaw transformation protocol (Weigel & Glazebrook, 2006). Agrobacterium mediated expression in N. benthamiana and hypersensitive response (HR) assessment was performed 5 d after Agrobacterium infiltration according to the protocol described in Bourras et al. (2019).

Protein detection

To assess whether the effector and R protein were transiently expressed in transformed N. benthamiana leaves we followed the protocol for protein extraction, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting in Bourras et al. (2019). For HA detection we used a peroxidase-conjugated antibody, anti-HA-HRP (rat monoclonal 3F10, Roche, Basel, Switzerland) at a dilution factor of 1 : 3000 in the presence of Supersignal Western blot enhancer solution (Thermo Scientific, Waltham, MA, USA) and a Fusion FX Imaging System (Vilber Lourmat, Eberhardzell, Germany) with default settings.

Microscopy

Infected leaf segments (3 cm) were taken at 10 and 14 dpi and treated by submersion in 1 M potassium hydroxide (KOH) and autoclaved. Treated leaves were washed and resuspended in 50 mM Tris-HCl pH 7.5. Samples were stained with WGA-FITC and aniline blue. Fluorescence microscopy was carried out with an Axiosimager Epifluorescence Widesfield Microscope (Zeiss, Oberkochen, Germany) fitted with FITC, GFP and DAPI filters.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was carried out with oligo probes (Oligo-pSc119.2 and Oligo-pTa535 producing green and red signals, respectively) on CS and Lr20-carrying lines (CS/Ax7A, Kenya W744, Thew, Norka) according to J. Li et al. (2020). Chromosome 7AL in Lr20-carrying lines had a weak pSc119 signal at the distal region, whereas in CS this signal was absent. Using this karyotype, flow-sorted chromosomes were confirmed to be 7A.

ChromSeq and de novo assembly

Chromosome flow-sorting and sequencing was performed as described in Dracatos et al. (2019). Briefly, 50 000 flow-sorted chromosomes enriched for 7A (72% of the sorted fraction) were treated with proteinase K and purified on Microcon YM-100 columns (Millipore, Bedford, MA, USA). Sequencing library, prepared from 20 ng chromosomal DNA using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA), was pair-end sequenced on the Illumina NovaSeq platform, yielding 69 Gb 2 x 250 bp pair-end reads. Ax7A sequence assemblies were generated using the contig assembly method of the Tritex pipeline (Monat et al., 2019). Paired-end reads were merged with BBMerge (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbmerge-guide/) and corrected with BFC (https://github.com/lh3/bfc). Contig assembly was executed with MINIA3 (https://github.com/GATB/minia3) iteratively using k-mer sizes 100, 150, 200, 250, 300, 350, 400, 450 and 500. The k = 500 assembly was used for further analyses.

Axminster 7A assembly comparison to the Chinese Spring reference

After trimming with cutadapt (https://pypi.org/project/cutadapt/), Ax7A ChromSeq reads were aligned to the CSv1 reference sequence assembly using MINIMAP2 (https://github.com/lh3/minimap2). Alignment records were sorted with NOVOSORT (http://www.novocraft.com/products/novosort/) and converted to BAM format with SAMTOOLS (Li et al., 2009). Read counts in nonoverlapping 1 Mb windows along the genome were determined using SAMTOOLS and standard UNIX tools. Binned read counts were imported into R and plotted along the genome using standard R functions. SNP calling was done with BCFTOOLS (https://samtools.github.io/bcftools/). SNP positions were imported into R and binned in 5 Mb windows. SNP densities were plotted along chromosome 7A using standard R functions. The functions used in this analysis are available at https://bitbucket.org/ipk_dg_public/pm1a.

Plot of alignment scores of CSv1 chromosome 7A genes against the Ax7A assembly was created by first extracting gene sequences from CSv1 using BEDTOOLS (https://github.com/arq5x/bedtools2) and a GFF file of CSv1 gene annotations. Genes from only chromosome 7A were aligned to the Ax7A assembly using BLASTN. Outputs were filtered for top alignment over 1000 bp per query based on bit score using BLAST_FILTER2.PYC (https://github.com/TCHewitt/Misc.NGS). Bit scores were normalized to bit score/kb alignment length and alignments were ordered by physical position using a custom UNIX script. Output was imported into EXCEL and plotted. CS IWGSC REFSEQ v.2.0 (CSv2) (www.wheatgenome.org) was used for coordinate-based physical comparisons due to improved assembly accuracy over CSv1 (although annotation data were not yet available). Ax7A
contig mapping to CSv2 chromosomes 2A, 3A, 7A, 7B and 7D was performed in parallel using MASHMAP (Jain et al., 2018) with identity threshold 91%, minimum segment length 5000 bp and filter mode as one-to-one. A custom UNIX script was used to filter for best alignment per query based on the longest alignment length above identity threshold. Dotplots were generated using GENERATEDOTPLOT (https://github.com/TC-Hewitt/MashMap). Putative breakpoints were inferred from coordinate data in filtered MASHMAP outputs.

**Axminster 7A assembly comparison with diploid species**

Decontamination of the Ax7A assembly of likely chromosomes 2A and 3A derived contigs was achieved using previously described MASHMAP outputs to flag contigs having top alignments with ≥95% identity to either 2A or 3A. These contigs were removed from the MASHMAP output which was then used as an index for GET_CONTIGS.PY (https://github.com/TC-Hewitt/Misc_NGS) to extract filtered contigs to a separate FASTA file. A ‘terminal contig set’ was created by filtering the shortlisted MASHMAP output for contigs mapping distally to the respective breakpoints using a custom UNIX script. The output was then used for contig retrieval using GET_CONTIGS.PY. All ‘custom UNIX scripts’ cited throughout the methods can be found at https://github.com/TC-Hewitt/Axminster7A.

Mapping of genotyping-by-sequencing (GBS) data from diploid accessions was performed by first trimming raw reads using TRIMMOMATIC (http://www.usadellab.org/cms/?page=trimmomatic). Each of the 15 species had four accessions which were concatenated into a single file per species. Mapping to the whole Ax7A assembly and ‘terminal contig set’ was performed using BWA (Li & Durbin, 2009). The output was processed with SAMTOOLS to remove duplicate alignments. Uniquely mapped reads were selected based on SAM tag ‘XT:A:U’ and exact matching reads were counted based on SAM tag ‘NM:i:0’. Counts were imported into Excel and plotted as a percentage of total uniquely mapped reads per species to normalize for variation in absolute reads between libraries.

**Phylogenetic tree construction**

R gene protein sequences with an N-terminal coiled-coil domain (CNL class) were taken from the National Centre for Biotechnology Information (NCBI) database. Accession numbers are listed in Table S3. One hundred and twenty-two sequences were imported into Excel and plotted as a percentage of total uniquely mapped reads per species to normalize for variation in absolute reads between libraries.

**Primer design and sequence resources**

The F5 lines from CS × CS/Ax7A were tested with a *Pm1a*-specific dominant sequence-tagged site (STS) marker *Pm1aSTS1*, designed based on the positions of SNPs in the alignment of CSv1 sequences homologous to contig #8725 harbouring the *Pm1a* candidate. Primers used in this study are listed in Table S4.

Genomic and transcriptomic resources used for the identification of *AcerPm1a* were reported previously: genome assembly and annotation of *Bgt* 96224 (Bgt_genome_v3_16) in Müller et al. (2019), re-sequencing and RNAseq of isolate THUN-12 in Menardo et al. (2016) and RNAseq of isolate 96 224 in Praz et al. (2018).

**Results**

**Cloning of *Pm1a* by MutRenSeq**

To clone *Pm1a*, we identified susceptible EMS-generated mutants in CS/Ax7A background. Six independent mutants together with WT CS/Ax7A were processed using the MutRenSeq pipeline. Captured reads from these six lines and WT were aligned to a *de novo* reference assembly of the WT reads. One contig (#8725) of 3096 bp contained a SNP in five of the six mutants. This contig contained NB-ARC and LRR motifs but no coiled-coil (CC) motifs suggesting it might be a partial NLR sequence. To identify a potentially missing segment, the contig was aligned to the CSv1 reference assembly. The top hit (87.5%) was to the 3′ portion of a high confidence gene (*TracesCS7D01G540500*) predicted on chromosome 7D and functionally annotated as a disease resistance gene. The full sequence of *TracesCS7D01G540500* aligned back to the CS/Ax7A RenSeq WT assembly detected an additional 10 contigs matching the 5′ portion at 81% to 92% identity. Only one of these contigs with 91.8% identity (#3966) had a SNP in the remaining mutant and contained a CC domain. Bridging PCR was used to confirm that both contigs (#8725 and #3966) formed a single NLR gene with SNP mutations in all six mutants (Fig. S1). All SNPs were canonical for EMS mutagenesis with either C/T (one mutant) or G/A (five mutants) polymorphisms. A full transcript sequence was obtained using 5′ and 3′ RACE on RNA extracted from CS/Ax7A. SNPs were confirmed in all mutants by Sanger sequencing and determined to cause either amino acid substitutions or a premature stop (Fig. 1) in the translated protein. The full gene sequence of *Pm1a* was 5.9 kb consisting of three exons and two introns of 1816 and 88 bp. The encoded protein was 964 amino acids and contained N-terminal CC, NB-ARC and C-terminal LRR domains (Fig. 1).

The NLR gene candidate cosegregates with *Pm1a* resistance in RIL populations

A dominant STS marker, *Pm1aSTS1*, was designed from the contig #8725 sequence and amplified only in backgrounds carrying *Pm1a* (Fig. S2). The 3 kb amplicon based on the full-length contig #8725 did not amplify in mutants that had lost both *Pm1* and *Sr15/Lr20* resistance consistent with these mutants (not used for RenSeq) containing deletions (Fig. S3). Seventy-six CS/Ax7A F5 RILs and 157 CS/Thew F5 RILs were then screened with the *Bgt* isolate avirulent to *Pm1a*. Resistant (*n* = 123), segregating (*n* = 6) and susceptible (*n* = 104) plants displayed clear phenotypes (Fig. S4) and the dominant *Pm1aSTS1* marker amplified only from resistant plants.
The Pm1a gene candidate confers powdery mildew resistance in transformed plants

Susceptible cv Fielder was transformed with the Pm1a candidate and 10 independent transgenic lines (T0), with controls, were tested with Bgt. At both 10 and 14 dpi (not shown) all T0 lines showed resistance to Bgt similar to that observed on CS/Ax7A (Fig. 2b). The high-level immunity was confirmed by fungal biomass measurements (Fig. 2a). The T1 progeny from four independent T0 lines were screened with Bgt and showed a resistant : susceptible segregation ratio of 3:1 or greater, indicating the presence of a stable and active Pm1a transgene in one or more loci of the T0 parents. All resistant T1 seedlings cosegregated with marker Pm1aSTS1. These data confirmed that this NLR gene conferred Bgt resistance in wheat and is Pm1a.

The Pm1a phenotype was examined microscopically in Bgt-infected T0 leaf tissue at 10 dpi. Hyphal growth was advanced in susceptible controls (Fig. 3a) and secondary infection was evident by the presence of haustoria within epidermal cells (Fig. 3b). Conversely, on leaves of T0 Pm1a transgensics, only germinated conidiospores that produced appressoria were observed without development of conspicuous haustoria (Fig. 3c,d). Leaves from WT CS/Ax7A plants showed minimal infection similar to that of T0 plants (Fig. 3e), although more advanced haustorium formation was occasionally observed (Fig. 3f). Plant cell autofluorescence, indicative of host cell death (Sánchez-Martín et al., 2011), was frequently associated with Bgt infection sites on T0 plants and resistant WT controls (Fig. 3d,g,h), possibly indicating HR. Autofluorescence was not associated with haustoria and secondary hyphae produced in susceptible controls.

Cloning of AvrPm1a

To identify AvrPm1a we used an existing mapping population with 118 sequenced haploid F1 progeny (Müller et al., 2019) originating from the cross between B. g. triticale isolate THUN-12, exhibiting an avirulence phenotype on Pm1a near-isogenic wheat line Axminster/8*Chancellor, and Bgt isolate 96224, which is virulent to Pm1a (Fig. 5a). Single interval QTL mapping with 118 progeny identified a single QTL on chromosome 6 (Logarithm of the odds (LOD) = 5.8) associated with the avirulence phenotype to Pm1a (Figs 4a, 5c). The genetic confidence interval (1.5 LOD) encompassed 210 266 bp in the published chromosome-level assembly of isolate 96224 (Müller et al., 2019) and harboured a cluster of seven predicted effector genes (Fig. 4b–d; Table S1). Using whole-genome re-sequencing and RNAseq data we determined that all seven candidate effectors were present and expressed in the avirulent isolate THUN-12 (Table S1). The high-density genetic map and nine recombinant F1 progeny with clear avirulence/virulence patterns on Pm1a lines allowed us to further reduce the AvrPm1a interval to 90 562 bp and the number of candidates to two effector genes, BgtE-5612 and BgtE-20015, located between the flanking markers (Fig. 4c, d). Both genes were strongly expressed during early infection stages of B. graminis (2 dpi) and exhibited sequence polymorphisms between the parental isolates THUN-12 and 96224 (Fig. 4d; Table S1). Compared to the reference isolate 96224, BgtE-5612_THUN12 carries five SNP's resulting in four amino acid changes, whereas BgtE-20015_THUN12 carries a single nonsynonymous SNP (Fig. 4d), making BgtE-5612 and BgtE-20015 originating from Pm1a avirulent isolate THUN-12 prime candidates for AvrPm1a.

Previously, Bgt avirulence genes were functionally validated by Agrobacterium-mediated co-expression of NLR genes and Avr candidates in the heterologous N. benthamiana system (Bourras et al., 2015, 2019; Praz et al., 2017). Following a similar approach, we optimized BgtE-5612 and BgtE-20015 cDNAs sequences from both parental isolates for expression in N. benthamiana; synthesized genes lacking the signal peptide were tested for recognition by Pm1a in Nicotiana leaves. Co-expression of BgtE-5612_THUN12 but not BgtE-5612_96224 with Pm1a in Nicotiana resulted in a strong HR (Figs 5a,b, S6a), whereas no such effect was observed for either version of BgtE-20015 (Fig. 5a, S6a). We fused all effector candidates and Pm1a C-terminally with a HA epitope tag and verified protein production of all effector variants and the R protein in Nicotiana by Western blotting (Figs. 5c, S6b,c).

These results demonstrated that BgtE-5612_THUN12 is AvrPm1a, which is consistent with the genetic mapping data predicting the AVR component to originate from the THUN-12 parental isolate. The recognized variant BGTE-5612_THUN12 differs from the virulent version BGTE-5612_96224 by four...
amino acids (K28E, L40F, G47R, C60S) occurring as a cluster in the N-terminal part of the protein, surrounding the Y/FxC motif (Fig. 5d).

Phylogenetic analysis of Pm1a and corresponding avirulence effector AvrPm1a

Comparison of the Pm1a protein sequence with a panel of 122 cloned coiled-coil (CNL class) NLR proteins (Fig. S7) showed that Pm1a is not closely related to other known Pm proteins such as the Mla allelic series or Pm3. In fact, Pm1a is relatively dissimilar to the other NLR proteins in this panel including other wheat NLR proteins. It shows greatest similarity to Pm21 which originates from *Dasypyrum villosum* (He et al., 2017).

AvrPm1a is a member of the effector family E004, one of the 235 previously described *Bgt* avirulence effector proteins (i.e. AVRPM2, AVRPM3A2/E2, AVRPM3B2/C2 and AVRPM3E2) which contain, like AVRPM1A, a signal peptide, a N-terminal Y/FxC motif, a single, conserved cysteine towards the C-terminus while otherwise exhibiting highly divergent amino acid sequences. Interestingly, all known *Bgt* avirulence effectors exhibit a similar exon/intron structure and are predicted to consist of a N-terminal α-helix followed by several β-strands (Bourras et al., 2015, 2019; Praz et al., Müller et al., 2019) (Fig. S13, see later). Interestingly, 22 of the 51 genes that encode E004 family proteins, including *AvrPm1a*, reside within a 900 kb genomic region on chromosome 6. This arrangement is likely to have arisen from tandem duplication and diversifying selection imposed by NLR recognition of effector sequences, such as that seen for *AvrPm1a*. In this gene family *AvrPm1a* is amongst the most highly expressed genes (Fig. S8).

AVRPM1A shows some similarity to several other cloned *Bgt* avirulence effector proteins (i.e. AVRPM2, AVRPM3A2/E2, AVRPM3B2/C2 and AVRPM3E2) which contain, like AVRPM1A, a signal peptide, a N-terminal Y/FxC motif, a single, conserved cysteine towards the C-terminus while otherwise exhibiting highly divergent amino acid sequences. Interestingly, all known *Bgt* avirulence effectors exhibit a similar exon/intron structure and are predicted to consist of a N-terminal α-helix followed by several β-strands (Bourras et al., 2015, 2019; Praz et al.,

**Fig. 2** Leaves of transgenic T0 plants (*Triticum aestivum*) showing resistance to *Blumeria graminis* f. sp. *tritici*. (a) Fungal biomass of sampled leaves in (b) measured as relative fluorescent units (RFUs) of stained chitin with SEs of three technical replicates shown. (b) Powdery mildew responses of 10 T0 plants and controls at 10 d post-inoculation (dpi). PC numbers denote independent transgenic lines. Fielder TC lines are nontransgenic tissue culture sibling controls. Resistant control: CS/Ex7A; susceptible controls: CS (Chinese Spring), Fielder, Morocco.
In the case of AVRPM1A this pattern is extended by the presence of a second α-helix at the amino terminus, partially explaining the larger size of the effector (Figs. 5d, S14a,b, see later). Strikingly, all identified Bgt avirulence proteins, including AVRPM1A, exhibit an RNase-like fold when subjected to structural modelling (Figs. 5d, S14c, see later) (Bourras et al., 2015, 2019; Praz et al., 2017).

Terminal chromosome 7AL is highly diverged due to rearrangement

FISH was conducted on lines CS/Ax7A, Kenya W744, Thew, and Norka carrying Pm1-Sr15-Lr20, and CS. Using Oligo-pSc119.2 (green) and Oligo-pTa535 (red) as probes a weak pSc119 signal was present in the distal region of chromosome 7AL in all four resistant lines but not in CS suggesting the presence of an introgression or translocation (Fig. S9).

ChromSeq was conducted on purified DNA of flow sorted chromosomes 7A of line CS/Ax7A. The chromosome sample purity was estimated to be 72% for the final DNA library with contamination primarily from chromosomes 2A and 3A. The sequencing output was c. 69 Gbp. A de novo Ax7A assembly created from the ChromSeq reads comprised of 383 563 contigs with a combined length of c. 1.82 Gbp and N50 of 7.85 kb.

When trimmed ChromSeq reads were aligned to the entire CSv1 reference assembly and SNP counts were plotted along chromosome 7A, few SNPs were observed in peri-centromeric regions but a distinct increase in SNP density was observed at the distal end of 7A (Fig. S10a). This was mirrored by a discrete drop in alignment scores of CSv1 7A annotated genes aligned to the Ax7A assembly (Fig. S10b). These results indicate a clear differentiation in this distal region between chromosome 7A in the CS assembly and the sequenced Axminster 7A.

Counts of uniquely mapped reads were plotted along the length of each CSv1 chromosome (Fig. 6). Majority of the reads (57%) originated from chromosome 7A, with contamination from chromosomes 2A (21%), 3A (18%) and 4A (2%). The sharp decline in read counts at the distal end of 7AL was accompanied by a concomitant rise in read counts at the distal ends of chromosomes 7BL and 7DL. When Ax7A assembly contigs were mapped to the CSv2 reference, the dot plot of 7A showed a downward inflection near the terminus indicating a decrease in homology. This placed a putative breakpoint at c. 728 Mb relative to CSv2 7A (Fig. 7a). Conversely, upward inflections indicating increased homology occurred at the termini of 7B and 7D, which placed putative breakpoints at c. 660 and 720 Mb and 630 Mb relative to CSv2 7B and 7D, respectively (Fig. 7b,c). These results suggest the terminal part of Axminster chromosome 7AL contains segments that are more related to sequences from terminal 7BL and 7DL. Based on the positions of putative breakpoints relative to CSv2 chromosomes 7A, 7B and 7D, the physical
size of the linkage block on Axminster chromosome 7A may be in the range of 12.9 to 43.7 Mb.

Possible ancestral origins of terminal 7AL

Probable contaminant contigs were removed from the Ax7A assembly based on significant alignments to either chromosome 2A or 3A in CSv2 (Fig. S11). To investigate whether the Pm1-Sr15-Lr20 linkage block has potential origins in diploid wheat relatives, GBS data was sourced from an unrelated study on a collection of diploid accessions (Bernhardt et al., 2019). GBS reads of 15 species were aligned to the decontaminated Ax7A assembly. Unsurprisingly, *T. urartu*, the ancestral donor of the wheat A genome (Salamini et al., 2002) had the highest proportion of exact matching reads. Alignments of the other species were not noteworthy (Fig. S12a). A set of 480 contigs, provisionally representing the terminal portion of Axminster 7A, were grouped based on primary mapping after the putative breakpoints in either CSv2 7A, 7B, or 7D. To determine if any of the diploid species became more represented in the terminal portion of Axminster 7A, GBS reads similarly aligned to this terminal contig set indicated exact matching reads of *D. villosum* were notably higher than in the entire Ax7A alignment and were comparable to *T. urartu* (Fig. S12b). Given that Pm1a was also closest to Pm21 in the phylogeny (Fig. S8), this raised the possibility that portions of terminal Axminster 7A were derived from *Dasypyrum* or a related lineage.

Discussion

In this study, the *R/Avr* gene pair of Pm1a and AvrPm1a was successfully cloned using MutRenSeq and genetic mapping with
RNAseq, respectively. We showed that resistance conferred by Pm1a can be transferred by transgenic complementation to susceptible varieties, inducing an immune reaction (Fig. 2) that limited the formation of mature haustoria in transgenic leaves. Whole cell autofluorescence in response to fungal penetration suggested a rapid onset of HR (Fig. 3). However, more rigorous staining methods are required to confirm this, and future observations of initial infection dynamics will require sampling at earlier timepoints given the rapid development of haustoria (Bushnell & Bergquist, 1974; Kunoh et al., 1982).

Additional Pm genes have been mapped to the same terminal bin as Pm1 from various wheat relatives, notwithstanding its five purported alleles (Hsam et al., 1998; Singrun et al., 2003). These genes include MlAG12, Mlm2033, Mlm80, PmTb7A.2, HSM1, MIUM15, MlW11, MlW2, PmU, Pm59, Pm9, mlRD30 and PmG16 (Tan et al., 2018). Primary mapping studies placed the first 11 genes in the same marker interval, whereas the last three were placed distally (Ouyang et al., 2014). It was suggested that all the genes sharing the marker interval with Pm1 were allelic to Pm1. However, Liang et al. (2016) showed that...

Fig. 5 Functional validation of AvrPm1a in Nicotiana benthamiana. (a, b) Functional validation of AvrPm1a (BgtE-5612_THUN12) in Agrobacterium mediated co-expression assays in N. benthamiana. The effector candidates BgtE-5612_96224 and BgtE-5612_THUN12 and Pm1a were infiltrated alone (bottom) or combined (top, OD = 1.2, ratio 1:4 for R:effector). Hypersensitive response (HR) was assessed by fluorescence imaging (a) Fusion FX imager, see the Materials and Methods section) or by eye (b). Photographed 5 d after infiltration. (c) Western blot detection of BGTE-5612_THUN12 and BgtE-5612_96224 C-terminally fused with a hemagglutinin (HA) epitope tag (upper panel) and Ponceau staining of Rubisco as a loading control (lower panel). HR induction of tagged BgtE-5612 variants are shown in Supporting Information Fig. S6(d). (d) Pairwise alignment of BgtE-5612_THUN12 and BgtE-5612_96224 proteins. The signal peptide (red), conserved Y/FxC motif and C-terminal cysteine (yellow), polymorphic sites (blue), and intron position (black arrow) are indicated.

Fig. 6 Purity of sequence reads from flow-sorted chromosomes. Counts of uniquely mapped reads in 1 Mb bins along the Triticum aestivum IWGSC RefSeq v.1.0 reference genome. The red line is a loess smoothing. The y-axis is shown in log scale. Black arrows point to steep changes in read counts at the distal ends of group 7 chromosomes.
Mlm2033 and Mlm80 were in fact separate loci despite occupying the same interval. Most recently, the cloned gene Pm60 (Zou et al., 2018) was concluded to occupy the same locus as PmU (Zhang et al., 2018), within the same interval as Pm1. However, sequence comparison of Pm1a with Pm60 showed they were quite dissimilar (Fig. S7), so are unlikely to be homologous. This raises the question of whether the five Pm1 alleles (Pm1a–Pm1e) are in fact allelic or distinct. Since the naming of these alleles was based on genetic maps covering a region of suppressed recombination, the likelihood of allelism must be questioned. Now that the Pm1a sequence is known, it would be highly informative to use it to probe various accessions possessing genes within this cluster to determine which are true alleles. Moreover, the cloning of additional Pm1 alleles will allow their specificity towards AvrPm1 to be determined.

In recent years numerous Avr genes have been identified in B. g. hordei (Bgh) and Bgt including the Bgh-derived Avra1, Avra7, Avra9, Avra10, Avra13 and Avra22 recognized by specific alleles of the barley Mla resistance locus (Lu et al., 2016; Saur et al., 2019), and the Bgt-derived AvrPm2, AvrPm3<sup>29/2</sup>, AvrPm3<sup>32/2</sup> and AvrPm3<sup>43</sup> recognized by wheat gene Pm2 and several Pm3 alleles, respectively (Bourras et al., 2015, 2019; Praz et al., 2017). Interestingly, all Blumeria Avr proteins belong to the Blumeria protein superfamily of candidate secreted effector proteins (CSEPs) and exhibit common features such as a generally small size (102–130 amino acids), a signal peptide as well as a N-terminal Y/FxC motif and a C-terminal cysteine at a conserved location. Furthermore, numerous CSEP families, including Avr effectors, have a conserved exon/intron structure, indicating a common phylogenetic origin and were predicted to exhibit a RNase-like structure (Pedersen et al., 2012; Bourras et al., 2016, 2019; Lu et al., 2016; Praz et al., 2017; Spanu, 2017; Saur et al., 2019). For Bgh CSEP BEC1054, a member of the effector family E014, which harbours both Avra13 and AvrPm2, the RNase-like structure was recently confirmed experimentally (Pennington et al., 2019). The newly identified AvrPm1a shares many of the earlier-mentioned features of AVR proteins such as conserved sequence motifs (signal peptide, Y/FxC), a similar exon/intron structure and a predicted RNase-fold (Figs S13, S14a,c). However, with 155 amino acids, it is significantly longer than all previously identified Blumeria AVRs. This difference can be attributed to the presence of an additional α-helix at the N-terminus and a slightly extended β-sheet region towards the C-terminus of the protein (Fig. S14a,b). As to how the larger effector size

![Fig. 7 Dot plots of assembled contigs from flow-sorted Axminster 7A chromosome mapped along group 7 chromosomes in the Triticum aestivum IWGSC RefSeq v.2.0 reference genome. Contigs (y-axis) were mapped along whole chromosome assemblies (x-axis). Dashed blue lines indicate approximate physical position along chromosome where there is a putative breakpoint. (a) Chromosome 7A with terminal region enlarged on the right. (b) Chromosome 7B. (c) Chromosome 7D.](image-url)
might influence the mode-of-recognition by its corresponding NLR resistance gene remains to be determined. All amino-acid polymorphisms between the avirulent AvrPm1a\_THUN12 and the virulent avrPm1a\_96224 variants were located in a short stretch surrounding the Y/FxC motif. In AvrPm3\(_{23,2/2}\) effector family E008, multiple amino acid residues close to the Y/FxC motif were shown to be under diversifying selection, which was attributed to a potential selection pressure imposed by the Pm3a and Pm3f alleles (McNally et al., 2018). Similarly, naturally occurring sequence polymorphisms in gain of virulence alleles of AvrPm3b\(_{21,2/2}\) were located around the Y/FxC motif and the underlying protein region was shown to be crucial for recognition of AvrPm3\(_{23,2/2}\) by its corresponding NLRs Pm3b and Pm3c (Bourras et al., 2019). The extent to which similar principles apply for recognition of AvrPm1a by Pm1a will be subject to future experiments. Cloning of additional Pm1 alleles could lead to the identification of additional AvrPm1 alleles, which may help reveal polymorphisms conferring gain or loss of virulence.

The sequencing of Axminster chromosome 7A is a step closer to understanding the true physical size and makeup of the terminal region of this chromosome in lines with Pm1a and Sr15/Lr20. Lines carrying the Pm1-Sr15-Lr20 linkage block characteristically prohibit recombination in the terminal 7AL region. Neu et al. (2002) suggested this region was an alien segment or rearranged fragment. Marker assays also showed no natural recombinants in the region of interest and Australian wheat breeders were unable to combine resistance to root lesion nematode (Rhn1) with a preferred white flour colour allele due to linkage with the Py-A1 locus also linked with Pm1-Sr15-Lr20 in certain cultivars (Jayatilake et al., 2013). Earlier evidence that this linkage block was due to a chromosomal rearrangement within the 7AL region was inferred from the presence of sequences that appeared to originate from the B genome (Crawford et al., 2011; Jayatilake, 2014). In this study, we clearly show this region to be abundant in not only sequences from 7B but also 7D (Figs 6, 7). However, precise physical ordering and composition of 7A, 7B and 7D sequences was not attainable due to significant divergence from current reference assemblies and notable disruption of synteny encompassing this region (Liang et al., 2016). A higher contiguity is required to achieve accurate physical resolution of Axminster 7AL, either from SMRT or Dovetail sequencing (Moll et al., 2017).

Comparison of terminal sequences of Axminster 7A with sequence data from diploid accessions pointed to a relatively high proportion of exact matching D. villosum reads (Fig. S12). Thus, introgression from Dasypyrum or a related lineage was considered. However, due to the limited number of reads available (ranging 10–60 K uniquely mapping to the terminal set), this could not be confirmed without deeper sequencing analyses or direct comparison with a genome assembly of D. villosum. The high divergence of this terminal region, which appears to be common to genotypes carrying Pm1a and Lr20-Sr15, traces back to unrelated land varieties introduced to Australia and North America in the late 19th century with no clear links between them. Ouyang et al. (2014) did not observe characteristic recombination suppression around MIIW172 in T. turgidum subsp. dicoccoides (tetraploid). Intriguingly, Liang et al. (2016) did observe strong recombination suppression in this region of two T. monococcum accessions (diploid). It could be possible that an ancient introgression(s) predisposed this region to its present structure, which in turn promoted a diversification of NLRs that culminated in the linked resistance cluster carrying Pm1a and Sr15-Lr20.

Acknowledgements

This work was supported by the Grains Research and Development Corporation (GRDC), Australia (CSP00164 and 9176057) and the Swiss National Science Foundation grant 310030B\_182833 to BK. TH is grateful for GRDC for providing a scholarship for his PhD study. MCM is grateful for the University Research Priority Programme (URPP) ‘Evolution in Action’ of the University of Zurich for funding her PhD study. The authors thank Z. Dubška, R. Šperková and H. Tvardíková for assistance with chromosome sorting and sequencing and S. Hoxha for technical assistance. HS was supported from European Regional Development Fund (ERDF) project ‘Plants as a tool for sustainable global development’ (no. CZ.02.1.01/0.0/0/16_019/0000827). BW and BS were supported by the Biotechnology and Biological Sciences Research Council cross-institute strategic programme Designing Future Wheat (BB/P016855/1).

Author contributions

TH, JZ, RM, PZ generation of mutants, disease phenotyping, genotyping, contig assembly, and data analysis; TH, BW, SP, BS, GY, EL MutRenSeq approach; IM, MM, HS, KH chromosome flow sorting, sequencing and de novo assembly; MCM, LK, SS AvrPm1a mapping, functional validation and bioinformatic analysis; JL cytological characterization of cultivars/lines; DB, TH, RS, MA, EL analysis of transgensics; EL, RM, BK, PZ designed and supervised the study; TH, MCM, LK, RM, EL, BK, PK, PZ drafted the manuscript and all co-authors provided edits. TH and MCM contributed equally to this work.

ORCID

Tim Hewitt https://orcid.org/0000-0002-4888-7216
Marion C. Müller https://orcid.org/0000-0001-5594-2319
István Molnár https://orcid.org/0000-0002-7167-9319
Martin Mascher https://orcid.org/0000-0001-6373-6013
Katerina Holušová https://orcid.org/0000-0002-4531-7170
Hana Šimková https://orcid.org/0000-0003-4159-7619
Lukas Kunz https://orcid.org/0000-0002-8155-5408
Jianping Zhang https://orcid.org/0000-0003-2488-5723
Jianbo Li https://orcid.org/0000-0002-1078-8319
Raghvendra Sharma https://orcid.org/0000-0002-7612-6932
Seraina Schudel https://orcid.org/0000-0003-3999-843X
Birkhard Steuernagel https://orcid.org/0000-0002-8284-7728
Sambasivam Periyannan https://orcid.org/0000-0002-5421-2872
Brande Wulff https://orcid.org/0000-0003-4044-4346
Robert McIntosh https://orcid.org/0000-0002-4268-9657
Beat Keller  https://orcid.org/0000-0003-2379-9225  
Evans Lagudah  https://orcid.org/0000-0002-6234-1789  
Peng Zhang  https://orcid.org/0000-0002-4191-1068

Data availability
All high-throughput RenSeq and ChromSeq sequencing data described in this article have been deposited under BioProject PRJEB39498. Pm1a sequence is available under GenBank accession MW531535. [Correction added after online publication 15 December 2020: an accession number was added in the preceding sentence.] AverPm1a variant 96224 and variant THUN-12 sequences are available under GenBank accession nos. MT773601 and MT773602, respectively. GBS sequencing data for diploid accessions came from the study by Bernhardt et al. (2019) and can be accessed at http://dx.doi.org/10.5447/IPK/2019/18.

References
Ayliffe M, Periyannan SK, Feechan A, Dry I, Schumann U, Wang MB, Pryor A, Lagudah E. 2013. A simple method for comparing fungal biomass in infected plant tissues. Molecular Plant–Microbe Interactions 26: 658–667.
Bendtsen JD, Nielsen H, von Heijne G, Brunak S. 2004. Improved prediction of signal peptides: SignalP 3.0. Journal of Molecular Biology 340: 783–795.
Bernhardt N, Brassac J-D, Dong X, Willing EM, Poskar CH, Kilian B, Blattner FR. 2019. Genome-wide sequence information reveals recurrent hybridization among diploid wheat wild relatives. The Plant Journal 102: 493–506.
Bourras S, Kunz L, Xue MF, Praz CR, Muller MC, Calvin K, Schlaffi M, Ackermann P, Fluckiger S, Parlane F et al. 2019. The AverPm3–Pm3 effector-NLR interactions control both race-specific resistance and host-specificity of cereal mildews on wheat. Nature Communications 10: 2292.
Bourras S, McNally KE, Ben-David R, Parlane F, Roffler S, Praz CR, Oberhaensli S, Menardo F, Stirnweis D, Frenkel Z et al. 2015. Multiple avirulence loci and allele-specific effector recognition control the Pm3 race-specific resistance of wheat to powdery mildew. The Plant Cell 27: 2991–3012.
Bourras S, McNally KE, Mueller MC, Wicker T, Keller B. 2016. Avirulence genes in cereal powdery mildews: the gene-for-gene hypothesis 2.0. Frontiers in Plant Science 7: 241.
Brummer S, Hurni S, Streckeisen P, Mayr G, Albrecht M, Yahiaoui N, Keller B. 2010. Intragenic allele pyramiding combines different specificities of wheat Pm3 resistance alleles. The Plant Journal 64: 433–445.
Busnell WR, Bergquist SE. 1974. Aggregation of host cyttoplasm and the formation of papillae and haustoria in powdery mildew of barley. Theoretical and Applied Genetics 38: 445–451.
Crawford AC, Stefanova K, Lambe W, McLean R, Wilson R, Barclay I, Francki MG. 2011. Functional relationships of phytoene synthase 1 alleles on chromosome 7A controlling flower colour variation in selected Australian wheat genotypes. Theoretical and Applied Genetics 123: 95–108.
Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast ultrafast RNA-seq aligner. Bioinformatics 29: 15–21.
Dracatos PM, Bartos J, Elmansour H, Singh D, Karafiatová M, Zhang P, Steunenburg B, Sváčka R, Cobbin JCA, Clark B et al. 2019. The Coiled-Coil NLR Rps11 confers leaf rust resistance in barley cultivar Sudan. Plant Physiology 179: 1362–1372.
He H, Ji Y, Zhu S, Li B, Zhao R, Jiang Z, Bie T. 2017. Genetic, physical and comparative mapping of the powdery mildew resistance gene Pm21 originating from Daucus carota var. Carota. Frontiers in Plant Science 8: 1914.
He H, Zhu S, Zhao R, Jiang Z, Ji Y, Ji, Qiu D, Li H, Bie T. 2018. Pm21, encoding a typical CC-NBS-LRR protein, confers broad-spectrum resistance to wheat powdery mildew disease. Molecular Plant 11: 879–882.
Himmelbach A, Zierold U, Hensel G et al. 2007. A set of modular binary vectors for transformation of cereals. Plant Physiology 145: 1192–1200.
Hsam KSL, Huang QX, Ernst F, Hartl I, Zeller JF. 1998. Chromosomal location of genes for resistance to powdery mildew in common wheat (Triticum aestivum L. em Thell.). 5. Alleles at the Pm1 locus. Theoretical and Applied Genetics 96: 1129–1134.
Hurni S, Brunner S, Buchmann G, Herren G, Jordan T, Krukowksi P, Wicker T, Yahiaoui N, Mago R, Keller B. 2013. Rye Pm8 and wheat Pm3 are orthologous genes and show evolutionary conservation of resistance function against powdery mildew. The Plant Journal 76: 957–969.
International Wheat Genome Sequencing Consortium (IWGSC). 2018. Shifting the limits in wheat research and breeding using a fully annotated reference genome. Science 361: eaar7191.
Ishida Y, Tsunashima M, Hiei Y, Komari T. 2015. Wheat (Triticum aestivum L.) transformation using immature embryos. In Wang K ed. Agrobacterium protocols: vol. 1. New York, NY, USA: Springer, 189–198.
Jain C, Koren S, Dilthey A, Philippp AM, Aluru S. 2018. A fast adaptive algorithm for computing whole-genome homology maps. Bioinformatics 34: 748–756.
Jayatilake DV. 2014. Fine mapping of nematode resistance genes Rlm1 and Cxe8 in wheat (Triticum aestivum). PhD thesis, University of Adelaide, Adelaide, SA, Australia.
Jayatilake DV, Tucker EJ, Bariana H, Kuchel H, Edwards J, McKay AC, Chalmers K, Mather DE. 2013. Genetic mapping and marker development for resistance of wheat against the root lesion nematode Pratylenchus neglectus. BMC Plant Biology 13: 230.
Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter LL, Keller B. 2009. A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science 323: 1360–1363.
Kumar S, Steeger G, Li M, Kuyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35: 1547–1549.
Kunoh H, Yamamori K, Ishizaki H. 1982. Cytological studies of early stages of powdery mildew in barley and wheat. VIII. Autofluorescence at penetration sites of Erysiphe graminis hordei on living barley coleoptiles. Physiological Plant Pathology 21: 373–379.
Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25: 1754–1760.
Li H, Handsaker B, Wysooker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25: 2078–2079.
Li J, Dunas I, Dong C, Li G, Trehowan R, Yang Z, Hoxha S, Zhang P. 2020. Identification and characterization of a new stripe rust resistance gene Sr58 on rye chromosome 6R in wheat. Theoretical and Applied Genetics 133: 1095–1107.
Li M, Dong L, Li B, Wang Z, Xie J, Qiu D, Li Y, Shi W, Yang L, Wu Q et al. 2020. A CNL protein in wild emmer wheat confers powdery mildew resistance. New Phytologist 228: 1027–1037.
Liang JC, Fu BS, Tang WB, Khan NU, Li N, Ma ZQ. 2016. Fine mapping of two wheat powdery mildew resistance genes located at the Pm1 cluster. Plant Genome 9: 1–9.
Lu P, Guo L, Wang Z, Li B, Li J, Li Y, Qiu D, Shi W, Yang L, Wang N et al. 2020. A rare gain of function mutation in a wheat tandem kinase confers resistance to powdery mildew. Nature Communications 11: 680.
Lu XL, Kracher B, Saur IML, Bauer S, Ellwood SR, Wise R, Yaeno T, Maekawa A. 2015. PhD thesis, University of Adelaide, Adelaide, SA, Australia.
McNally KE, Menardo F, Luthi L, Praz CR, Muller MC, Kunz L, Ben-David R, Chandrasekhar K, Dinoor A, Cowger C et al. 2018. Distinct domains of the AverPm3A(C20) avirulence protein from wheat powdery mildew are involved in immune receptor recognition and putative effector function. New Phytologist 218: 681–695.
Menardo F, Praz CR, Wyder S, Ben-David R, Bourras S, Matsumae H, McNally KE, Parlane F, Roffler S et al. 2016. Hybridization of

New Phytologist (2021) 229: 2812–2826
www.newphytologist.com
© 2020 The Authors
New Phytologist © 2020 New Phytologist Foundation
powdery mildew strains gives rise to pathogens on novel agricultural crop species. *Nature Genetics* 48: 201–205.

Moll KM, Zhou P, Ramaraj T, Fajardo D, Devitt NP, Sadowsky MJ, Stupar RM, Tiffin P, Miller JR, Young ND et al. 2017. Strategies for optimizing BioNano and Dovetail explored through a second reference quality assembly for the legume model *Medicago truncatula*. *BMC Genomics* 18: 578.

Monat C, Padmarasu S, Lu T, Wicker T, Gundlach H, Himmelbach A, Ens J, Li C, Muehlbauer GJ, Schulman AH et al. 2019. TRITEX: chromosome-scale sequence assembly of *Triticum* genomes with open-source tools. *Genome Biology* 20: 284.

Moore JW, Herrera-Foessel S, Lan CX, Schnippenkoetter W, Ayliffe M, Moll KM, Zhou P, Ramaraj T, Fajardo D, Devitt NP, Sadowsky MJ, Stupar RM, Tiffin P, Miller JR, Young ND et al. 2017. Strategies for optimizing BioNano and Dovetail explored through a second reference quality assembly for the legume model *Medicago truncatula*. *BMC Genomics* 18: 578.

New Phytologist 2825

Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, Praz CR, Bourras S, Zeng FS, Sanchez-Martin J, Krattinger SG, Wicker T et al. 2016. Rapid gene isolation in barley and wheat by mutant chromosome sequencing. *Genome Biology* 17: 221.

Saur IML, Bauer S, Kracher B, Lu XL, Franzeskakis L, Muller MC, Sabelleck B, Kummel F, Panstruga R, Maekawa T et al. 2019. Multiple pairs of allelic MLA immune receptor-powdery mildew AVR(A) effectors argues for a direct recognition mechanism. *eLife* 8: e44471.

Sears ER, Briggle LW. 1969. Mapping gene *Pm1* for resistance to *Erysiphe graminis f. sp. tritici* on chromosome 7A of wheat. *Crop Science* 9: 96.

Sharp P, Dong C. 2014. TILLING for Plant Breeding. In: Fleury D, Whitford R, eds. *Crop breeding methods and protocols*. New York, NY, USA: Springer Protocols. 155–165.

Singh SP, Hurni S, Rainelli M, Brunner S, Sanchez-Martin J, Krukowski P, Pedrito D, Buchmann G, Zbinden H, Keller B. 2018. Evolutionary divergence of the *Type Pm1* and *Pm8* resistance genes reveals ancient diversity. *Plant Molecular Biology* 98: 249–260.

Singrun C, Hsam SL, Harl L, Zeller FJ, Mohler V. 2003. Powdery mildew resistance gene *Pm22* in cultivar *Virest* is a member of the complex *Pm1* locus in common wheat (*Triticum aestivum* L. em Thell.). *Theoretical and Applied Genetics* 106: 1420–1424.

Solovyev V, Kosarev P, Seledsov I, Vorozyev D. 2006. Automatic annotation of eukaryotic genes, pseudogenes and promoters. *Genome Biology* 7(Suppl. 1): S10.1-S10.12.

Spanu PD. 2017. Cellular immunity against powdery mildews targets RNase-Like Proteins associated with Haustoria (RALPH) effectors evolved from a common ancestral gene. *New Phytologist* 213: 909–971.

Steuer Nagel B, Periyannan SK, Hernandez-Pinzon I, Witek K Rouse MN, Yu G, Hatta A, Ayliife M, Barhia H, Jones JD et al. 2016. Rapid cloning of disease-resistance genes in plants using mutagenesis and sequence capture. *Nature Biotechnology* 34: 652–655.

Tan C, Li G, Cowger C, Carver BF, Xu X. 2018. Characterization of *Pm59*, a novel powdery mildew resistance gene in *Afghanistan* wheat landrace *PI* 181356. *Theoretical and Applied Genetics* 131: 1145–1152.

Waterhouse WL. 1952. Australian rust studies. IX. Physiological race determinations and surveys of cereal rusts. *Proceedings of the Linnean Society of New South Wales* 77: 209–258.

Watson IA, Luig NH. 1966. SR 15 – a new gene for use in the classification of *Puccinia graminis var*. *tritici*. *Euphytica* 15: 239–247.

Weigel D, Glazebrook J. 2006. Transformation of *Agrobacterium* using the freeze–thaw method. *CSIH protocol* 7: pdb.prot6466.

Worthington M, Lyerly J, Petersen S, Brown-Guedria G, Marshall D, Cowger C, Parks R, Murphy JP. 2014. *MlUM15*: an *Agrobisneglecta*-derived powdery mildew resistance gene in common wheat. *Crop Science* 54: 1397–1406.

Xie J, Guo G, Wang Y, Hu T, Wang L, Li J, Qiu D, Li Y, Wu Q, Lu P et al. 2020. A rare single nucleotide variant in *Pm59* confers powdery mildew resistance in common wheat. *New Phytologist* 228: 1011–1026.

Xing L, Hu P, Liu J, Witek K, Zhou S, Xu J, Zhou W, Gao L, Huang Z, Zhang R et al. 2018. *Pm21* from *Hyaloscytus villosus* encodes a CC-NBS-LRR protein conferring powdery mildew resistance in wheat. *Molecular Plant* 11: 874–878.

Yao G, Zhang J, Yang L, Xu H, Jiang Y, Xiong L, Zhang C, Zhang M, Zsorrells ME. 2007. Genetic mapping of two powdery mildew resistance genes in einkorn (*Triticum monococcum* L.) accessions. *Theoretical and Applied Genetics* 114: 351–358.

Zhang L, Zheng XW, Qiao LY, Qiao ZL, Zhao JJ, Wang JM, Zheng J. 2018. Analysis of three types of resistance gene analogs in *PmUreg* region from *Triticum urartu*. *Journal of Integrative Agriculture* 17: 2601–2611.

Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* 7: 203–214.

Zou S, Wang H, Li Y, Kong Z, Tang D. 2018. The *NB-LRR* gene *Pm60* confers powdery mildew resistance in wheat. *New Phytologist* 218: 298–309.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Sequences produced by gene-synthesis used in this study.

Fig. S1 Graphs of R gene enrichment sequencing (RenSeq) mutant read alignments to candidate contigs showing mutation positions.
Fig. S2 Marker Pm1aSTS1 assay showing specificity to Pm1a-containing *Triticum aestivum* lines.

Fig. S3 Amplification of a large product based on the candidate contig from *Triticum aestivum* cv Chinese Spring/Axminster*7A (CS/Ax7A) was absent in double mutants of pm1 and sr15/lr20.

Fig. S4 Clear differentiation of resistant and susceptible *Triticum aestivum* seedlings in recombinant inbred line populations inoculated with *Blumeria graminis* f. sp. tritici (Bgt).

Fig. S5 Bi-parental mapping population *Blumeria graminis* f. sp. tritici (Bgt) 96224 (avrPm1a) × B. g. tritcale THUN-12 (AvrPm1a) segregates on the *Triticum aestivum* near isogenic line (NIL) Axminster/8*Chancellor containing Pm1a.

Fig. S6 *Agrobacterium* mediated transient co-expression of BgtE-5612 or BgtE-20015 with Pm1a and verification of protein presence in *Nicotiana benthamiana*.

Fig. S7 Phylogenetic tree of Pm1a with cloned CNL immune receptors from various plant species.

Fig. S8 Expression of the candidate effector family E004 gene members in the two *Blumeria graminis* isolates B. g. tritcale THUN-12 and B. g. tritici 96224 based on RNAseq data.

Fig. S9 Cytological examination of *Triticum aestivum* chromosome 7A in cv Chinese Spring (CS) and lines carrying Pm1a.

Fig. S10 *Triticum aestivum* cvs Chinese Spring (CS) vs Axminster sequence divergence in distal chromosome 7A.

Fig. S11 Contig length distributions from assembly of *Triticum aestivum* cv Axminster flow-sorted chromosome 7A with and without contaminants.

Fig. S12 Proportions of reads from diploid *Triticaceae* species exactly matching to the flow-sorted chromosome 7A assembly of *Triticum aestivum* cv Axminster.

Fig. S13 Protein alignment of the candidate effector family E004 members in the reference *Blumeria graminis* f. sp. tritici isolate 96224.

Fig. S14 Alignment of *Blumeria graminis* f. sp. tritici AVR proteins and three family members of AVRPM1A.

Table S1 Summary of candidate effector genes and the encoded proteins in the genetic confidence interval underlying the QTL on Bgt_chr-06 in the reference *Blumeria graminis* f. sp. tritici isolate 96224.

Table S2 Primers used to clone gene-synthesized *Pm1a* and *Pm1a-HA* into *Agrobacterium* compatible expression vector pIPKbg004.

Table S3 Accession numbers of immune receptor proteins used to construct the phylogenetic tree in Fig. S7.

Table S4 Primers used for PCR and sequencing of *Pm1a*.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.