A diverse panel of 755 bread wheat accessions harbors untapped genetic diversity in landraces and reveals novel genetic regions conferring powdery mildew resistance

Novel sources of powdery mildew resistance in a diverse wheat panel

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Abstract

Wheat breeding for disease resistance relies on the availability and use of diverse genetic resources. More than 800,000 wheat accessions are globally conserved in gene banks, but they are mostly uncharacterized for the presence of resistance genes and their potential for agriculture. Based on the selective reduction of previously assembled collections for allele mining, we assembled a trait-customized panel of 755 geographically diverse bread wheat accessions with a focus on landraces, called the LandracePLUS panel. Population structure analysis of this panel based on the TaBW35K SNP array revealed an increased genetic diversity compared to high-quality sequenced wheat accessions and 632 landraces of an earlier study. The additional diversity mostly originated from Turkish, Iranian and Pakistani landraces. We characterized the LandracePLUS panel for resistance to ten diverse isolates of the fungal pathogen powdery mildew. Performing genome-wide association studies and dividing the panel further by a targeted sub-setting approach for accessions with unique resistance patterns and distinct geographical origin, we detected several known and already cloned genes, including the *Pm2a* gene. In addition, we identified 17 putatively novel powdery mildew resistance loci that represent useful sources for resistance breeding and for research on the mildew-wheat pathosystem. Our study shows the value of assembling trait-customized collections and utilizing a diverse range of pathogen races, to detect novel loci that can be integrated in breeding programs. It further highlights the importance of integrating landraces of different geographical origin into future diversity studies.

Author Summary

Wheat breeding relies on the availability of wheat lines with diverse agricultural traits, based on their genetic differences. While over 800,000 wheat lines are conserved in gene banks, their value for agriculture is mostly not characterized. We assembled a collection of 755 wheat lines that focuses on

older wheat lines, which are known to have broader genetic diversity than modern cultivars. Population genetic analysis of this collection showed a broad genetic diversity compared to previous studies on older varieties and wheat reference genomes. Wheat lines from Turkey, Iran and Pakistan were most distinct in this regard. We tested the symptom severity of powdery mildew infections on all wheat plants from this collection. Combining the genomic data with this disease assessment gave insight on the potential geographic origin of previously described resistance genes and revealed 17 associated genomic regions in wheat that contribute to resistance to powdery mildew that have not been described before. These findings provide a valuable resource for future diversity studies and wheat lines to include in the breeding process to achieve mildew resistant cultivars.

50 **Introduction**

51 Bread wheat (*Triticum aestivum* L.) provides more calories and protein per person than any other crop on
52 Earth (1). This allohexaploid (2*n* = 6*x* = 42, AABBDD) crop species originated from successive hybridization
53 events, where the latest polyploidization is thought to have occurred ~ 8,000 years ago in the Fertile
54 Crescent (2,3). After domestication, wheat was disseminated to Europe and Asia (4), where accessions
55 were selected based on the needs of individual farmers, thus becoming locally adapted traditional
56 accessions, so-called landraces (5,6). With the Green Revolution in the 60s, landraces were systematically
57 replaced by advanced cultivars (7) at the cost of a narrower genetic diversity due to the bottleneck effect
58 of breeding (8,9). As a consequence, modern cultivars are more likely to lack the genetic potential to
59 combat abiotic and biotic stresses, including infection by pathogens. Plant diseases are a big threat to
60 agriculture, with fungal pathogens playing a major role by causing an estimated annual yield loss of 18%
61 in wheat (10). Among those, wheat powdery mildew, which is caused by the obligate biotrophic
62 ascomycete *Blumeria graminis* f.sp. *tritici* (*Bgt*), is a major source of yield loss worldwide (10). Chemical
63 control via pesticides is expensive, detrimental on the surrounding ecosystem (11,12) and the European
64 Commission proposed binding rules to reduce EU pesticide usage by 50% until 2030 (13). Therefore,
65 alternative strategies are needed to control wheat mildew, especially in the context of climate change, as
66 the geographical dispersal of pathogens and the severity of their infections are expected to increase
67 rapidly (14,15).

68 In the Farm-to-fork strategy, the deployment of wheat accessions that harbor disease resistance is
69 proposed as a sustainable and effective way to combat pathogens (13). Such resistance can be conferred
70 by resistance (*R*) genes, which typically encode intracellular nucleotide-binding leucine-rich repeat
71 receptors (NLRs), albeit not always, that recognize pathogen avirulence effector proteins (AVRs) (16–18).
72 *R* gene resistance acts in a race-specific manner, when an R protein recognizes the corresponding AVR
73 (16,19). The over 90 characterized *R* genes against powdery mildew (*Pm* genes) represent a cornerstone

74 of wheat breeding (20). However, race-specific resistance can be rapidly overcome by pathogens by
75 evolving AVRs to evade recognition (21–23). Due to this constant arms-race and the low efficacy of the
76 currently cloned *Pm* genes (24), the identification of new resistance genes is needed for successful wheat
77 breeding programs. It is estimated that more than 7,000 distinct NLR encoding genes are present in the
78 wheat gene pool (25). Based on this genomic analysis, there are possibly hundreds of potentially active,
79 but unknown *Pm* genes in the wheat germplasm. The untapped genetic diversity of wheat landraces and
80 their adaptation to individual environments with high disease pressure makes them promising candidates
81 for containing such genes (5,8,26).

82 Together with modern cultivars and wild relatives, landraces are conserved in gene banks, where to date
83 more than 800,000 *Triticum* accessions are stored (27). However, these remain mostly uncharacterized
84 for their potential in agriculture and their adaptive mechanisms are poorly understood, limiting their use
85 in breeding (8,26). Attempts to unlock this hidden potential have been made using different approaches.
86 For example, Balfourier et al. focused on maximizing the representation of different breeding statuses,
87 dates of registration and geographical origin, to reduce the collection size of the INRAe bread wheat
88 collection from about 12,000 to 4,506 accessions (28). Genotyping these accessions resulted in a better
89 understanding of wheat phylogeography and genetic diversity over time. Another study characterized the
90 genetic diversity of 80,000 accessions, which represented a large part of the CIMMYT and ICARDA
91 germplasm banks, covering not only domesticated hexaploid wheat, but also tetraploids and crop wild
92 relatives (29). This revealed unexplored diversity in landraces and wheat selection footprints. In a third
93 recent example, Schulthess et al. genotyped the IPK winter wheat collection of 7,651 accessions and 325
94 European elite cultivars (30). Later, this collection was phenotyped with a single powdery mildew isolate,
95 resulting in the detection of eleven previously undescribed resistance loci (31).

96 Here, we used a panel based on former bread wheat collections assembled for allele mining (32,33). These
97 former collections included a main panel that had been selected using a focused identification of

98 germplasm strategy (FIGS) approach, revealing accessions with potentially high selection pressure for
99 powdery mildew resistance (32,34). We now reduced these collections, focusing on landraces and
100 maximizing the diversity of geographical origin. The reduced size of the panel allowed us to phenotype it
101 with a diverse set of ten *Bgt* isolates, constituting a trait-customized panel that is ideal to search for
102 powdery mildew resistant accessions and the underlying genes (35). Using genome-wide association
103 studies (GWAS) and a targeted subset approach for accessions of shared geographical origin and isolate-
104 specific resistance patterns, we detected 17 most likely novel genetic regions associated with powdery
105 mildew resistance.

106

107 **Results**

108 We assembled a geographically diverse panel of 755 bread wheat accessions (Fig 1A-C), based on the
109 selective reduction of former collections used for allele mining of the powdery mildew resistance gene
110 *Pm3* (32,33). In the selection process, we focused on landraces and combined data on geographical origin
111 with preliminary phenotypes of powdery mildew resistance. The resulting panel, hereafter LandracePLUS
112 panel, contains 521 winter wheat and 234 spring wheat accessions, including 576 landraces, 162 older
113 cultivars (acquisition date from 1946 to 2003, with the main part from the 60s and 70s), seven research
114 or breeding material lines and eleven unknown accessions (S1 Excel). We used the LandracePLUS panel,
115 which covers a broad geographical distribution, with a focus on accessions originating from the Middle
116 East (Fig 1A-B), to detect genetic loci associated with resistance to the powdery mildew pathogen (Fig 1D).

117 <u>Diversity analysis of the LandracePLUS panel reveals unexplored genetic diversity</u>

118 A total of 29,556 high quality, polymorphic single nucleotide polymorphisms (SNPs) derived from
119 genotyping with the TaBW35K SNP array was used for diversity analysis. All these SNPs had known
120 chromosomal positions based on the Chinese Spring reference genome assembly RefSeq v1.0 (36),

121 including 18,389 regular SNPs and 11,167 off-target variants (OTVs), i.e. markers that detect both
122 presence-absence polymorphisms and nucleotide polymorphisms. The 29,556 markers were distributed
123 across the wheat genome similar to earlier findings (37–39), with a lower coverage of genome D compared
124 to the A and B genomes: 11,528 (39.0%) markers on the A-genome, 13,389 (45.3%) on the B-genome and
125 4,639 (15.7%) on the D-genome. On average, 1,407 markers were assigned per chromosome, resulting in
126 an average density of one marker each 480 Kbp. Least markers were assigned to chromosome 1D and
127 most to chromosome 2B, with 493 and 2,431 markers, respectively.

128 A principal component analysis (PCA) (Fig 2A) and a dendrogram based on hierarchical clustering analysis
129 (Fig 2B) revealed four genetic clusters, which overall correlated with geographical origin. Group 1 was
130 dominated by accessions from Iran and Pakistan, while Group 2 was composed by accessions of mixed
131 origin (Ethiopia, South Africa, USA, Canada, Mexico, Argentina, France, Switzerland, Kazakhstan,
132 Kirgizstan, Tajikistan, Uzbekistan, Armenia and Russia). Group 3 predominantly contained accessions from
133 Turkey and Group 4 was a last group for South, Southwest and East Asia (Iraq, Azerbaijan, India, Nepal,
134 China and Japan). Few outliers were however identified, namely East Asian accessions in Group 2, and
135 African accessions in Group 3. For a better interpretation of the observed genetic diversity within the
136 LandracePLUS panel, wheat accessions with high-quality genome sequences, namely the 10+ wheat
137 reference genomes (25,36), Fielder (40) and Renan (41) were included in the analysis with 27,016 out of
138 the 29,556 SNPs that mapped unambiguously to chromosomes of these genomes. Most of the high-
139 quality sequenced genomes clustered in Group 2 of the LandracePLUS panel, while Norin61 and Chinese
140 Spring were on the edge of Group 4 (Fig 2A).

141 When highlighting the different types of accessions in the PCA, landraces covered almost the full genetic
142 diversity range of the LandracePLUS panel. In contrast, the 162 cultivars clustered mainly with Group 2
143 and partially with Group 4 (S1A Fig). Therefore, landraces were genetically more diverse than cultivars
144 and clustered apart from them. However, this was not always the case, as shown by the example of

145 Ethiopian landraces. These clustered with cultivars in Group 2 (S1B Fig), suggesting that Ethiopian
146 landraces have contributed to breeding programs substantially. The close clustering of accessions from all
147 over the world described above (Fig 2A, Fig 2B) is likely driven by this separation between landraces and
148 cultivars. As most of these geographically diverse accessions clustering together are cultivars, their genetic
149 similarity is not derived from their origin, but rather their breeding status as cultivars. While there was no
150 clear pattern in Group 4 regarding the distribution of winter and spring wheat, clusters that were
151 dominated by landraces (Group 1 and 3) mainly contained winter wheat accessions (S1 Excel). Vice versa,
152 the cultivar dominated Group 2 consisted mainly of winter wheat accessions.

153 We assessed population structure using a kinship matrix and compared it to an Admixture analysis (Fig
154 2C), which reflected the four groups found by hierarchical clustering and the PCA. Estimated ancestral
155 populations K=5 revealed a good fit for the LandracePLUS panel's diversity into four groups. Yet, the
156 kinship matrix revealed additional subdivision within Groups 3 and 4.

157 To assess whether the LandracePLUS panel represents a similar genetic diversity compared to former
158 studies on wheat germplasm, we compared our data with a collection of 632 landraces that were
159 genotyped with the TaBW280K SNP array (42), which contains all markers that were used for the
160 LandracePLUS panel (28), allowing a direct comparison. A PCA with 30,021 high quality, polymorphic SNPs
161 (S2 Fig) showed that the LandracePLUS panel covers the genetic diversity of this collection and further
162 revealed unexplored genetic diversity absent in the study of Balfourier and colleagues (28). This additional
163 diversity was mainly contained in Groups 1 and 3, composed of Turkish, Pakistani and Iranian landraces.

164 Taken together, the LandracePLUS panel is a diverse selection of wheat accessions with a pronounced
165 diversity of landraces compared to cultivars and high-quality sequenced genomes. Furthermore, the panel
166 covers earlier found genetic diversity and additionally expands it, mainly with Turkish, Pakistani and
167 Iranian landraces.

168 A diverse set of ten powdery mildew isolates enables the prediction and detection of known *Pm* genes

169 We aimed at phenotyping the reaction of the LandracePLUS panel to several powdery mildew isolates to
170 maximize the chances of finding novel resistance genes. Consequently, we first tested ten random isolates
171 on 27 so-called differential lines, including near-isogenic lines (NILs) and donors of cloned or genetically
172 described *Pm* genes (Fig 3A). On average, isolates were avirulent on eleven out of 27 differential lines.
173 While isolates CHE_96224, CHE_97266 and CHE_98230 seemed more avirulent, with 16, 17 and 18
174 resistant differential lines, respectively, isolates CHE_19004 and ISR_94 were more virulent, with five and
175 two resistant lines, respectively. There were rare cases of resistant differential lines, despite the absence
176 of an avirulent Avr haplotype. This is likely caused by an additional resistance gene in the genetic
177 background, as it has recently been described in the case of *Pm17* containing differential line Amigo by
178 Müller et al (43). Phenotypic Pearson correlation between the isolates corroborated the diversity of
179 virulence (S3A-B Fig), with an average correlation coefficient of 0.38. The most similar isolates were
180 CHE_96224 and CHE_97266, with a correlation of 0.76, while the two most diverging were CHE_94202
181 and CHE_98230 with a negative correlation of 0.07. The diversity in phenotypic reactions suggests highly
182 different effector contents between the isolates, increasing the chance of detecting potentially novel
183 resistance loci in the LandracePLUS panel with this set of isolates.

184 Each isolate had a distinct virulence pattern, reflecting that the ten chosen mildew isolates represent
185 extensive diversity in virulence. The response of differential lines based on the presence of specific *Pm*
186 genes facilitated the prediction of cognate *Avr* genes in the corresponding isolates. To test the accuracy
187 of this prediction, we compared the suggested presence of seven cloned *Avrs*, *AvrPm1a* (44,45), *AvrPm2*
188 (46), *AvrPm3a/f* (47), *AvrPm3b/c* (48), *AvrPm3d* (48), *AvrPm8* (49) and *AvrPm17* (43) to their presence and
189 haplotype in the genomes of the ten isolates (S1 Table). In the following we show this for the
190 representative case of *Pm2a* and *AvrPm2a* (46). We found the described avirulent haplotype H0 of
191 *AvrPm2a* in *Bgt* CHE_94202, CHE_96224, CHE_97266, CHN_46_30 and ISR_106, fitting the *Pm2a*

192 differential line resistance pattern to these five isolates. The susceptibility of the *Pm2a* differential line
193 towards the remaining five isolates CHE_19004, CHE_97223, CHE_98230, GBR_JIW2 and ISR_94, reflected
194 the absence of *AvrPm2a* in these isolates.

195 The virulence of an isolate on differential lines reflects the known *Pm* genes that can be detected with it
196 in wheat genotypes with unknown gene content. For example, the isolates CHE_94202, CHE_96224,
197 CHE_97266, CHE_98230, CHN_46_30, GBR_JIW2 and ISR_106 enable *Pm12* detection, but none of the
198 isolates would allow the detection of *Pm20* (Fig 3A). Based on the observed resistance reactions, our set
199 of ten powdery mildew isolates can detect 24 of the 27 tested *Pm* genes.

200 We conclude that the resistance patterns of the differential lines based on a phenotypically diverse set of
201 ten powdery mildew isolates facilitate the detection of previously isolated *Pm* genes and allow the
202 prediction of avirulence gene presence in the isolates.

203 <u>The LandracePLUS panel shows resistance variation to ten wheat powdery mildew isolates</u>

204 Response of the 755 wheat accessions to the ten powdery mildew isolates revealed an overall prevalent
205 susceptibility, a smaller portion of intermediate reactions and only few strong resistance interactions,
206 with an average of 84 resistant accessions per isolate (Fig 3B). Considering the response to individual
207 isolates, only 57, 51, 55 and 55 wheat accessions were resistant to CHE_19004, CHE_97223, GBR_JIW2
208 and ISR_94, respectively. This reflected the broad virulence of CHE_19004 and ISR_94 already found in
209 the differential lines (Fig 3A). Similarly, 119 and 115 wheat accessions were resistant to CHE_96224 and
210 CHE_98230, respectively, which were also the most avirulent isolates on the differential lines together
211 with CHE_97266. Instead of CHE_97266, however, here CHE_94202 showed a similar number for the
212 LandracePLUS panel, with 115 resistant wheat accessions (Fig 3B). Pearson correlation between the
213 isolates based on the LandracePLUS panel phenotypes showed patterns resemblant of the above
214 correlation matrix based on the differential line phenotypes (S3A and C Fig). While the overall relation

215 between isolates was similar, correlation coefficients differed notably for ISR_94 (S3C-D Fig). This strong
216 deviation could be caused by the absence of a the *Pm4a* gene in the LandracePLUS panel, compared to
217 the differential set where it is present. The average correlation coefficient was slightly lower, with 0.34,
218 pointing at even more virulence diversity than estimated above with the differential lines.

219 On average, eleven genotypes were resistant to a single isolate, but susceptible or intermediate
220 susceptible to the nine other isolates. 23 wheat accessions were exclusively resistant to CHE_98230 and
221 25 accessions to CHE_94202, two of the more avirulent isolates. Merely four wheat accessions showed
222 resistance to all ten isolates, while 58 wheat accessions were resistant to at least five of the isolates. On
223 average, excluding the 61 accessions that were susceptible to all ten isolates, wheat accessions were
224 resistant to three isolates.

225 We predicted the presence of known *Pm* genes in accessions of the LandracePLUS panel that matched the
226 pattern of the corresponding *Pm*-containing differential line. For example, 32 accessions of the
227 LandracePLUS panel had the same resistance pattern as the *Pm2a* differential line and, hence, are good
228 candidates for containing *Pm2a*. Indeed, 16 of these 32 accessions contained *Pm2* according to haplotype-
229 specific markers (50)(S1 Excel). However, the potential presence of several *Pm* genes in the same
230 accession can mask the resistance pattern of a specific *Pm* gene. For example, when we predict the
231 presence of *Pm1a* in accessions of the panel, the expected resistance pattern could be derived from *Pm1*
232 alleles, but also from *Pm3* alleles, *Pm12*, *Pm17* or *Pm29*. This limits the use of differential lines for analysis
233 of overlapping resistance patterns and the prediction of specific genes. Nevertheless, the information on
234 resistance patterns can be a useful tool to narrow down candidate accessions for the presence of a specific
235 *Pm* gene of interest.

236 <u>Association studies for seedling resistance to wheat powdery mildew in the full LandracePLUS panel reveal</u>
237 <u>previously cloned *Pm* genes as well as possibly novel genes</u>

238 We conducted GWAS with the phenotyping data obtained for each of the ten powdery mildew isolates
239 on all 755 wheat accessions with a minor allele frequency (MAF) of 1%, revealing six genomic regions
240 associated with wheat mildew resistance on chromosomes 1A, 2B, 2D, 5D, 7A and 7B (Table 1, S4 Fig). The
241 most significant peak was located on chromosome 5D and was observed for the *AvrPm2*-containing *Bgt*
242 isolates CHE_94202, CHE_96224, CHE_97266, CHN_46_30 and ISR_106. When focusing on one
243 representative *AvrPm2*-containing isolate, ISR_106, the mildew resistance association spanned the region
244 from 43,201,514 to 45,174,806 bp of the short arm of chromosome 5D of Chinese Spring (Fig 4A-C) and
245 included the *Pm2* resistance gene locus (51). To test if the presence of *Pm2* was responsible for the
246 significant association, we screened the LandracePLUS panel with a *Pm2* haplotype-specific marker (51)
247 and found that out of 102 wheat accessions that were resistant to ISR_106, 39 contained *Pm2*, of which
248 34 were landraces. Most of these 34 accessions were from Turkey, with three landraces from Russia,
249 Pakistan and Tajikistan (Fig 4F). GWAS with a covariate for *Pm2* presence resulted in the loss of the
250 significant peak (Fig 4D), corroborating that the peak was indeed caused by *Pm2*. Sequencing of the
251 amplified *Pm2* locus in four randomly selected landraces that were positive for the haplotype marker,
252 uniformly revealed the presence of the known allele *Pm2a* (51).

253 **Table 1. Powdery mildew associated regions detected in the complete LandracePLUS panel of 755 accessions.**

Chromosome	Position	Isolate	*Pm* genes described on specific chromosome (arm)
1AS	4,062,943 – 6,342,008	CHE_98230	***Pm3***(52), *Pm17*(53), *Pm25*(54)
2BL	707,987,645 – 722,628,991	CHE_96224, CHE_97223	*Pm6*(55), *Pm33*(56), ***Pm51***(57), *Pm52*(58), *Pm57*(59), ***Pm63***(60), *Pm64*(61), ***PmKN0816***(62), ***PmLS5082***(63), ***PmQ***(64), *PmY39*(65), ***pmYN99102***(66), *MlAB10*(67), *Mlzec1*(68)
2DL	639,543,074 – 639,666,878	CHE_98230	*Pm43*(69), *PmSE5785*(70)
5DS	43,201,514 – 45,174,806	CHE_94202, CHE_96224, CHE_97266, CHN_46_30, ISR_106	***Pm2***(51), ***Pm46***(71), *Pm55*(72)
7AL	723,214,600 – 727,232,696	CHE_94202, CHE_97266, CHE_98230, GBR_JIW2	*Pm1*(44), *Pm9*(20), *Pm37*(73), ***Pm59***(74), *Pm60*(75), *PmG16*(76), ***Mlllw72***(77), ***Mlm2033***(78), ***Mlm80***(78), *mlRd30*(79), ***MlUM15***(80), *PmU*(81), *PmTb7A*(82), ***MlIW172***(77), *MlWE18*(83)
7BL	708,382,369 – 708,545,443	CHE_96224, CHE_98230	*Pm5*(84), *pmDHT*(85), *PmSGD*(86), *PmTm4*(87), ***pmHYM***(88), ***pmYBL***(89), *mlxbd*(90), *mljy*(91), *mlsy*(91)

254 Physical positions of GWAS peaks on Chinese Spring and the isolates detecting them. *Pm* genes are listed that were
255 described on the same chromosomal arm or on the same chromosome without specified arm. *Pm* genes that were
256 described in an overlapping genomic interval with the GWAS peak are highlighted in bold.

257 In addition to the *Pm2* gene, we detected a peak on chromosome 1AS for mildew isolate CHE_98230,
258 which spanned the genomic region from 4,062,943 to 6,342,008 bp (Table 1, S4 Fig) and contained the
259 locus of the cloned gene *Pm3*, of which to date 17 alleles have been identified (33). All cloned avirulence
260 genes specifically recognized by *Pm3* alleles, namely *AvrPm3a/f* (47), *AvrPm3b/c* (48) and *AvrPm3d* (48)
261 were present in CHE_98230 (S1 Table) and the *Pm3* differential lines were all resistant to CHE_98230,
262 except for *Pm3g* (Fig 3A). Earlier studies have shown that out of 115 resistant accessions in the

263 LandracePLUS panel, 27 contained functional *Pm3* alleles, with 16 and five accessions containing *Pm3c*
264 and *Pm3b*, respectively (S1 Excel) (32,33,92). We suggest that the peak is caused by these functional *Pm3*
265 alleles.

266 A third, very large region (707,987,645 to 722,628,991 bp) on chromosome 2BL was associated with
267 powdery mildew resistance for isolates CHE_96224 and CHE_97223 (Table 1, S4 Fig). Eight out of nine
268 significantly associated SNPs in this region overlapped for both isolates and showed strong linkage
269 disequilibrium (LD), ranging from r^2 values of 0.76 to 1, with a mean of 0.92. This strong linkage between
270 the markers suggests a region of reduced recombination, which could be caused by an introgression.
271 Indeed, it was shown that an introgression on chromosome 2BL is present in the wheat gene pool, which
272 is most likely derived from the diploid wild relative *Triticum timopheevii* (25,93). This introgression is
273 potentially present in the LandracePLUS panel. The strong LD makes it challenging to discover the causal
274 genes of the association, which could be a single or multiple resistance genes. Indeed, many candidate
275 *Pm* genes have been described in this genomic interval, including *Pm51* (57), *Pm63* (60), *PmKN0816* (62),
276 *PmLS5082* (63), *PmQ* (64) and *pmYN99102* (66). Accessions that likely contained the causal gene were
277 predominantly from Iran and the USA. The same isolates that revealed this association, CHE_96224 and
278 CHE_97223, also showed a peak on chromosome 2DL from 594,546,895 to 595,439,911 bp (Table 1, S4
279 Fig). To test if this association was an echo-like artifact derived from the peak on chromosome 2B, we
280 checked the chromosome-specificity of the two significantly associated markers on chromosome 2D.
281 Blasting against the Chinese Spring genome revealed that marker contig79838_204_BS00100069 mapped
282 with 97% identity on chromosome 2B 723,302 kb, closely distal to the peak, while marker GENE-1908_97
283 mapped with 97% identity and 97% coverage on chromosome 2B within the peak at 722,089 kb. Vice
284 versa, this low marker specificity was only true for two out of eight significantly associated markers of the
285 peak on chromosome 2B. Thus, we regarded the peak on chromosome 2D for CHE_96224 and CHE_97223
286 as an artefact.

287 For the isolate CHE_98230 we detected a peak on chromosome 2DL that was located further distal,
288 between 639,543,074 and 639,666,878 bp (Table 1, Fig 5A). Out of twelve accessions that likely carried
289 the causal gene, eight were Iranian and three Indian landraces suggesting a potential origin of this gene
290 in the Middle East. The genomic region contained only two predicted genes on the reference genome
291 Chinese Spring, putatively encoding an alpha/beta-hydrolase superfamily protein and a transketolase (S2
292 Excel). Two *Pm* genes were described previously on this chromosomal arm, namely *Pm43* and *PmSE5785*,
293 which are both derived from diploid wild relatives (69,94). *Pm43* was introgressed into wheat via partial
294 amphiploids of *Thinopyrum intermedium* (69). Based on the markers that defined the mapping interval,
295 this gene maps between 467 to 577 Mb on Chinese Spring. Thus, *Pm43* is located at least 63 Mb proximal
296 to our associated region, which ranged from 639.5 to 639.6 Mb. *PmSE5785*, on the other hand, was
297 introduced into wheat via synthetic hexaploids from *Aegilops tauschii* and mapped proximal to 513.1 Mb
298 (94), separated by at least 126 Mb from our peak. We conclude that we detected an undescribed powdery
299 mildew resistance-associated region on chromosome 2DL.

300 We further detected a peak on chromosome 7AL for CHE_94202, CHE_97266, CHE_98230 and GBR_JIW2
301 that spanned from 722,307,347 to 727,232,696 bp (Table 1, S4 Fig). Possible candidate genes in this
302 genomic interval include *Pm59* (74), *MlIw72* (95), *Mlm2033* (78), *Mlm80* (78), *MlUM15* (80) and *MlIw172*
303 (77). Alleles of *Pm1* have also been mapped to chromosome 7AL (McIntosh), however, we could not
304 confirm an overlap with our resistance-associated region, because the *Pm1* locus is absent in Chinese
305 Spring (36).

306 Finally, we detected a peak on chromosome 7BL for CHE_96224 and CHE_98230 that spanned from
307 708,382,369 to 708,545,443 bp (Table 1, S4 Fig), possibly caused by *pmHYM* (88) or *pmYBL* (89), which
308 have been mapped to this region earlier. Accessions that likely contained the causal gene were mostly
309 cultivars from diverse countries in Asia and the Americas.

310 *Pm4* alleles are widely present in the LandracePLUS panel but not revealed by association studies

311 Based on the resistance of differential lines *Pm4a* and *Pm4b* to five and four of the ten isolates,
312 respectively, we expected to detect the cloned gene *Pm4* in the LandracePLUS panel (96). *Pm4a* and *Pm4b*
313 were described earlier to confer resistance to CHE_94202 and CHE_96224, but not to GBR_JIW2 (96).
314 Phenotyping revealed 17 accessions which showed the expected resistance pattern for the three
315 diagnostic isolates, while haplotype-specific markers (96) detected the presence of the *Pm4* haplotype in
316 62 accessions, however, no peak was produced at the *Pm4* locus on chromosome 2AL for any isolate. To
317 explain this missing association despite the broad haplotype presence of *Pm4*, we sequenced the locus in
318 all 62 accessions. This revealed 51 and four accessions containing the non-functional alleles *Pm4f* and
319 *Pm4g*, respectively (96), and four accessions with an undescribed allele, hereafter *Pm4_42460*, while the
320 functional alleles *Pm4b* and *Pm4d* only occurred once and twice, respectively. The undescribed allele
321 resembled *Pm4f*, except for one amino acid change in position 421 of splicing variant V1 (421L:P). While
322 allele *Pm4g* was present in accessions of diverse geographical origin, 42 out of 51 accessions carrying *Pm4f*
323 and all four accessions containing *Pm4_42460* were Turkish landraces. Our findings suggests that the non-
324 functional alleles *Pm4_42460* and *Pm4f* originated in Turkey.

325 Taken together, we find the presence of known *Pm* genes with GWAS of the complete LandracePLUS
326 panel, giving insights on their geographic distribution and potential origin. In addition, we discovered at
327 least one genomic region on chromosome 2D which has not been implicated with powdery mildew
328 resistance before.

329 Utilizing subsets of the LandracePLUS panel discovers novel loci that are associated with powdery mildew
330 resistance of distinct geographical origin

331 As for the *Pm2a* gene described above, it is likely that some resistance genes are present only in certain
332 groups of the LandracePLUS panel, e.g., accessions with similar geographical origin or unique phenotype

333 patterns. We first tested if subsets of the panel based on such groups are suitable to detect additional
334 genomic regions associated with powdery mildew resistance. To assess the sensitivity of this approach,
335 we used a re-sampling approach and created 100 random subsets of 300, 200, 150 and 90 accessions from
336 the LandracePLUS panel as an input for GWAS with the *Pm2* avirulent isolate ISR_106. Since the MAF
337 threshold of 1% used above would detect an association already if the respective allele occurs only two
338 times for a subset of 200 to 299 accessions, we decided to adjust the MAF threshold to 5% for all subsets
339 below a sample size of 300. We found the *Pm2*-associated peak in 98%, 95%, 75% and 48% of cases for
340 the four subset sizes, respectively. We therefore considered subsets of a minimum of 150 to 200
341 accessions suitable for the detection of loci associated with major resistance genes in the LandracePLUS
342 panel.

343 In a first sub-setting approach, we created sets of accessions based on their geographical origins. A subset
344 of all 173 landraces from Pakistan and Iran revealed four additional peaks compared to the full
345 LandracePLUS panel, located on chromosomes 1A, 2A, 4A and 6A (Table 2, S5 Fig, Fig 5B). While the peak
346 on chromosome 2A was located in a genomic region that has been described to contain the resistance
347 gene *Ml92145E8-9* (97), the other regions have not been associated with powdery mildew resistance
348 before (Table 2). The resistance-associated region on chromosome 6A spanned the region from
349 47,543,345 to 48,168,130 bp and occurred for isolates CHE_98230 and CHE_97266 (Fig 5B). In this region,
350 ten genes were annotated on Chinese Spring, including genes that putatively encode five leucine-rich
351 repeat (LRR)-domain-containing proteins and a serine/threonine kinase (S2 Excel). On chromosome arm
352 6AS, only *Pm21* and *Pm56* have been described. *Pm21* originated and was cloned from the diploid grass
353 *Dasypyrum villosum* (98,99). The gene was introduced into the 17exaploidy gene pool in China through a
354 translocation line T6AL.6VS in the late 1980s (100). The translocated arm from *D. villosum*, however, does
355 not recombine with wheat homeologs (101). We would therefore expect to see a broad association
356 covering the short arm of chromosome 6A in case of *Pm21* detection. Further, *Pm21* was described to

357 confer broad-spectrum resistance (101), whereas we observed the association for only one out of ten
358 isolates. Therefore, we suggest that the causal gene in our resistance-associated region is different from
359 *Pm21*. Similarly, *Pm56* was only recently introduced as a translocation line 6AL.6RS from rye (102), several
360 years after we obtained and utilized the seeds of the initial collection. We conclude that this is a not
361 previously described resistance-associated region on chromosome 6AS.

362 **Table2. Powdery mildew associated regions detected in subsets of the LandracePLUS panel.**

Chromosome	Position	Subset	Isolate	***Pm* genes described on specific chromosome (arm)**
1AS	12,174,235 – 12,208,992	Pakistan/Iran (173)	CHN_46_30	*Pm3*(52), *Pm17*(53), *Pm25*(54)
1AS	29,107,020 – 31,254,548	Turkey (231)	CHE_98230	*Pm3*(52), *Pm17*(53), *Pm25*(54)
1AL	545,768,653 – 549,614,262	Turkey (231)	CHE_98230	*Pm25*(54)
1BL	455,211,699 – 459,929,298	Turkey (231)	CHE_96224	*Pm39*(103)
1BL	666,035,407 – 666,819,820	Turkey (231)	CHE_96224	*Pm39*(103)
1DL	458,298,562 – 470,343,380	CHE_98230 (367)	CHE_98230	*Pm10*(104)
2AL	731,541,214 – 731,543,212	Pakistan/Iran (173)	CHE_97266	*Pm4*(96), *Pm50*(105), *Pm65*(106), *pmX*(107), *PmHo*(108), ***Ml92145E8-9***(97), *PmHNK54*(109), *PmLK906*(110), *PmYm66*(111), *PmSN15218*(112)
3AL	575,148,089 – 624,384,371	CHE_98230 (367)	CHE_98230	-
3AL	649,955,366 – 649,957,392	Turkey (231)	GBR_JIW2	-
3BL	669,757,199 – 671,287,569	CHE_98230 (367)	CHE_98230	*Pm41*(113), *PmHNK*(114)
4AL	621,274,251 – 621,311,627	Pakistan/Iran (173)	CHE_19004	*Pm16*(115), *Pm61*(116), *MlIW30*(117), *MlNFS10*(118), *PmHHXM*(119), *PmPBDH*(120)
4DL	508,250,632 – 508,270,523	Turkey (231)	CHE_97223	-
5AL	661,231,600 – 663,214,964	CHE_98230 (367)	CHE_98230	***pm2026***(121)
5AL	582,889,996 – 585,435,631	Turkey (231)	ISR_106	*pm2026*(121)

5BL	701,148,712 – 701,177,854	CHE_94202 (171)	CHE_94202	*Pm36*(122), *Pm53*(123), *PmAS846*(124), *Ml3D232*(125)
6AS	47,543,345 – 48,168,130	Pakistan/Iran (173)	CHE_98230, CHE_97266	*Pm21*(98,99), *Pm56*(102)
6AS	1,949,085 – 1,960,836	Turkey (231)	CHE_97223	*Pm21*(98,99), *Pm56*(102)
7BL	702,850,716 – 704,268,424	Turkey (231)	CHE_98230	*Pm5*(84), *pmDHT*(85), ***PmSGD***(86), *PmTm4*(87), ***pmHYM***(88), *pmYBL*(89), *mlxbd*(90), *mljy*(91), *mlsy*(91)

Physical positions of GWAS peaks on Chinese Spring, subset description with number of accessions in brackets and the isolates detecting them. *Pm* genes are listed that were described on the same chromosomal arm or on the same chromosome without specified arm. *Pm* genes that were described in an overlapping genomic interval with the GWAS peak are highlighted in bold.

Finally, we used the same geographical approach to investigate a subset of the 231 Turkish landraces, which resulted in the discovery of nine additional peaks on chromosomes 1A, 1B, 3A, 4D, 5A, 6A and 7B (Table 2, S6 Fig), of which only the peak on 7B has been implicated in seedling resistance before. Taken together, subsets based on geographical origin revealed eleven genomic regions that have not previously been associated with powdery mildew resistance, suggesting that these genes arose in the respective countries Pakistan, India and Turkey.

<u>Association studies of further LandracePLUS panel subsets reveal undescribed isolate-specific resistance loci</u>

In a second targeted sub-setting approach, we tested if accessions with resistance to single isolates would reveal novel associated genomic loci. Accordingly, we created subsets for CHE_94202 and CHE_98230, which showed a substantial number of wheat accessions uniquely resistant to them. Complementing these resistant accessions with susceptible ones, we obtained a subset of 171 accessions for CHE_94202, which revealed one new peak compared to GWAS of the complete panel. This association was located on chromosome 5BL, spanning the region from 701,148,712 to 701,177,854 bp (Fig 5C, Table 2), which has

381 not been described in powdery mildew resistance. In this genomic interval, only four genes were
382 annotated in Chinese Spring, including a gene encoding a putative NLR (S2 Excel). Accessions that likely
383 contained the causal gene were mostly Iranian (17) and Turkish (17) landraces, representing distinct
384 clusters from the diversity analysis.

385 Another isolate-specific subset of 367 accessions for CHE_98230 revealed four new peaks on the long
386 arms of chromosomes 1D, 3A, 3B, and 5A (Table 2, S7 Fig, Fig 5D). While we will discuss the latter, most
387 likely novel peak with promising candidate genes, the other three genomic regions have also not been
388 associated with powdery mildew resistance before (Table 2). The peak on chromosome 5AL spanned the
389 region from 661,231,600 to 663,214,964 bp (Fig 5D) and contained 23 annotated genes in the Chinese
390 Spring reference genome. Among these were genes putatively encoding three C2H2-type Zinc fingers, an
391 MLO-related protein, a Serine/threonine kinase and an NLR (S2 Excel). Accessions that likely contained
392 the causal resistance gene were predominantly landraces from India (six) and Nepal (eight). So far, two
393 powdery mildew resistance loci were described on chromosome 5AL. A recent study on 8,316 wheat
394 accessions found a powdery mildew resistance associated region, called MTA10, on chromosome 5AL
395 pointing to a candidate gene which is located at 454.77 Mb, thus, more than 200 Mb proximal to our peak
396 (31). Additionally, one of the temporarily designated genes, *pm2026*, was located on chromosome $5A^{m}L$
397 of the diploid wheat ancestor *Triticum monococcum* accession TA2026 (121). The authors created
398 tetraploid wheat that contained this gene using emmer crosses and started the transfer into hexaploid
399 wheat by backcrossing with an unknown elite cultivar in 2008. Due to the long process required after this
400 pre-breeding step, it is unlikely that our panel contains accessions with the transferred *pm2026* locus.
401 Although we cannot exclude a natural transfer of the locus during wheat hybridization, *pm2026* was never
402 identified outside of the accession TA2026. Conversely, if our association was caused by *pm2026*, we
403 narrowed down the interval of the locus based on Chinese Spring mapping from 10.2 Mb to less than 2
404 Mb and present candidate genes.

405 In summary, subsets for isolate-specific resistance facilitated the discovery of five additional, previously
406 unknown genetic loci that are represented in accessions from few, distinct geographical regions.

407

408 **Discussion**

409 The LandracePLUS panel harbors untapped genetic diversity originating mainly from Turkish, Pakistani
410 and Iranian landraces

411 We assembled a diverse panel of 755 bread wheat accessions with a focus on landraces. Here, a FIGS
412 approach laid the foundation for a trait-customized collection of accessions with potentially high selection
413 pressure for powdery mildew resistance. A second step of selective reduction based on geographical
414 origin resulted in the LandracePLUS panel, which facilitated the detection of novel *Pm* genes using GWAS.

415 The LandracePLUS panel revealed untapped genetic diversity in Turkish, Iranian and Pakistani landraces.
416 It showed four main genetic clusters that correlate with the geographical origin, similar to a study which
417 has shown that wheat accessions from the Caucasus region, as well as Central, South and East Asian are
418 more diverse compared to other regions in the world (28). Landraces covered almost the full diversity of
419 the LandracePLUS panel, whereas cultivars were limited to one cluster. This cluster also included the
420 bread wheat accessions with high-quality sequenced genomes, with the exception of the two landraces
421 Chinese Spring and Norin61 (25,36,40,41). This observation highlights the need to include landraces in
422 breeding programs to enlarge the genetic base of elite wheat varieties (126,127). We propose that future
423 diversity studies of wheat should have a focus on landraces, in particular from regions with untapped
424 genetic diversity, such as Turkey, Pakistan and Iran.

425 Pathogen virulence characterization guides *Pm* gene discovery

426 To discover novel *R* genes against powdery mildew in the LandracePLUS panel, we used a set of ten *Bgt*
427 isolates that showed highly diverse virulence patterns when tested on differential lines with single, known
428 *Pm* genes. As we observed avirulence for almost all differential lines, using these isolates should reveal
429 the presence of most *Pm* genes for which differential lines are available.

430 The tested wheat accessions were rarely resistant to more than three isolates, in line with characteristic
431 *R* gene-based race-specific resistance (19). This implies that the observed single resistance genes would
432 be of limited agricultural use, depending on the isolates present in an agricultural area of interest. To
433 broaden the resistance spectrum and prevent fast evolution of pathogen virulence, such genes should be
434 deployed in a suitable manner, e.g., by gene stacking, gene pyramiding or transgenic overexpression
435 (128,129). On the other hand, the 61 accessions of the LandracePLUS panel that were susceptible to all
436 tested powdery mildew isolates can be assumed to lack any major resistance gene active at the seedling
437 stage. Thus, observed resistance in adult plants in the field would likely be durable, making these
438 accessions worth investigating in field tests.

439 Currently, for the 27 genes represented in the differential set of *Pm* lines, only eight of the corresponding
440 avirulence genes are molecularly known (43–49). Avirulence gene sequence comparison in our ten
441 powdery mildew isolates revealed the presence of recognized haplotypes of seven of the cloned Avrs. This
442 knowledge guided us to determine whether a resistance-associated region in the LandracePLUS panel was
443 derived from a known *Pm* gene or not, as we showed for the cloned gene *Pm2a*. Further, the information
444 of resistance to *Bgt* isolates of distinct geographical origin can be used to suggest an informed deployment
445 of accessions in the respective agricultural areas (43,130). For example, our findings suggest further work
446 on landraces that are resistant to CHN46-30 for potential deployment in China because they must contain
447 effective *R* genes against this isolate of Chinese origin.

448 GWAS of the complete LandracePLUS panel detects known *Pm* genes and reveals an undescribed powdery
449 mildew resistance associated region on chromosome 2DL

450 GWAS on the full LandracePLUS panel and all ten powdery mildew isolates revealed resistance associated
451 regions on six chromosomes. Five of these were in genomic regions with previously described resistance
452 associations. Of these, peaks on chromosomes 1AS and 5DS were likely caused by the cloned genes *Pm3*
453 and *Pm2*, respectively. While *Pm3* was known to be present in several LandracePLUS panel accessions
454 (32,33,92), we did not expect to find *Pm2* widely in the gene pool of landraces. *Pm2* was introduced to
455 the breeding gene pool from the Russian cultivar Ulka (131). It originated from the diploid wheat wild
456 relative *Ae. tauschii* and has eight known alleles, of which only *Pm2a* was detected in hexaploid wheat
457 (50). While *Pm2a* has been identified previously in six wheat landraces (50,132), we found its presence in
458 34, mostly Turkish, landraces. Our findings suggest that *Pm2a* might have entered the wheat gene pool
459 through Turkish landraces. The other three loci were potentially caused by genetically described, but not
460 molecularly known genes. While GWAS did not detect the cloned gene *Pm4* in the LandracePLUS panel,
461 we discovered the large presence of two non-functional alleles in Turkish landraces. Our findings suggests
462 that these alleles originated in Turkey, fitting the origin of *Pm4* from tetraploid wheat, which also arose in
463 Turkey (96,133). Despite the narrow representation of *Pm4b* and *Pm4d* in landraces, a wide presence of
464 these functional alleles compared to *Pm4f* and *Pm4g* has been described in elite germplasm (96). This
465 suggests that the breeding process for *Pm4* mildew resistance was very effective.

466 The sixth detected locus in the full LandracePLUS panel was a 124 Kb region on chromosome 2DL, which
467 has not been described to be associated with powdery mildew resistance before. This region contained
468 two candidate genes, predicted to encode an alpha/beta-hydrolase superfamily protein and a
469 transketolase. These types of proteins have not been linked to disease resistance earlier, and their
470 potential function in the observed defense response would need to be determined. However, the causal
471 gene for resistance might be absent in the reference genome Chinese Spring. One approach to clarify this

472 issue would be the application of recent sequencing technologies, such as circular consensus sequencing
473 (CCS) (134). Such novel approaches have increased the feasibility of sequencing specific donor accessions
474 to assist in cloning a gene of interest. For example, assembling the Kariega genome with CCS has
475 demonstrated the usefulness of this approach in wheat and has led to the cloning of *Yr27* (135). Another
476 option to identify a gene of interest in a specific genotype depends on the availability of a pangenome,
477 which ideally would capture the entire gene repertoire of a species (136). High-quality sequencing efforts
478 have recently resulted in 18 wheat genomes, of which 13 have reference genome quality
479 (25,36,40,41,137). Genome analysis has revealed structural rearrangements, introgressions and
480 differences in gene content (25). A successful example of using this resource is the cloning of *Lr14a*, which
481 was based on the reference genome ArinaLrFor (138). However, despite the recent advances in
482 pangenome projects, the close clustering of the high-quality sequenced genomes compared to the
483 LandracePLUS panel suggests that the currently available pangenome includes only a fraction of the
484 diversity present in landraces, including resistance loci. In fact, Walkowiak and colleagues have shown
485 that ten genotypes capture about 90% of the NLR complement of wheat (25). Thus, it is essential to include
486 more diverse wheat accessions, specifically landraces, in future work to increase the extent of the
487 pangenome, particularly for NLR loci. We propose to assemble high-quality genomes of several landraces
488 from Turkey, Pakistan and Iran. Furthermore, contrasting phenotypes should be included when setting up
489 pangenome consortia. This diversified selection could provide a resource that guides various trait-
490 genotype associations.

491 <u>Targeted GWAS subsets reveal 16 potentially novel resistance-associated loci</u>

492 Utilizing targeted subsets, we discovered 16 peaks on ten chromosomes that are most likely novel (Table
493 2). GWAS are expected to be more powerful when conducted on large datasets where individuals are
494 drawn randomly from the population (139). However, SNPs important at a small regional scale might yet
495 be diluted in species-wide panels and not detected by GWAS, which are typically lacking the power to

496 detect associations with rare alleles (140). Pending sufficient phenotypic and genetic variation, GWAS in
497 local panels have proven to be very effective in *Arabidopsis thaliana* to characterize the genetic
498 architecture of defensive metabolite diversity (141). For this reason, resistance genes which may have
499 been selected at small regional scales might be more efficiently detected in subsets of accessions from
500 the same geographical origin.

501 We therefore focused on accessions originating from distinct countries that harbored novel genetic
502 diversity, namely Pakistan, Iran and Turkey. With a subset of accessions that are exclusively from Pakistan
503 or Iran, we discovered a region on chromosome 6AS associated with mildew resistance. The region
504 contains genes putatively encoding a serine/threonine kinase and F-box like proteins with LRR domains.
505 The latter have been described to facilitate hypersensitive cell death response in tobacco and tomato
506 (142) and shown to be involved in defense response to stripe rust in wheat (143). Serine/threonine kinases
507 have been25escryibed to play a role in defense response, including *Pto*, which confers resistance to
508 bacterial speck disease in tomato (144,145).

509 In the same manner, subsets of accessions that were uniquely resistant to a single isolate revealed a region
510 on chromosome 5BL containing a candidate gene encoding a putative NLR. Another associated region was
511 detected on chromosome 5AL contained candidate genes putatively encoding C2H2-type Zinc fingers, an
512 MLO-related protein, a serine/threonine kinase and an NLR. Zinc fingers of the C2H2-type were linked to
513 plant defense response (146–149), where some cases have shown that the transcriptional repression
514 activity of the zinc finger was the mechanism behind this association (150,151). *Mlo* is a well-known player
515 in powdery mildew resistance in barley, with rice and wheat homologs shown to have a conserved
516 function (152,153). *Mlo* mutants that confer resistance are recessive, as is the only previously described
517 candidate gene in this 5AL genomic region, *pm2026* (121,152). Thus, the next step would be genetic
518 analyses via crosses to test if the resistant phenotype is inherited in a recessive ratio. A dominant ratio

519 would corroborate the strong likelihood described above that the candidate gene is not *pm2026* and guide
520 the focus of further investigations away from the *Mlo*-related candidate.

521 While we present these novel regions and gene candidates, the molecular nature of the observed
522 resistance must be identified in future studies. We suggest choosing a donor accession with confirmed
523 resistance for each peak and sequence the resistance-associated genome using CCS. This will reveal which
524 gene candidates are present in the specific accessions and guide their molecular cloning and validation,
525 e.g., via virus-induced gene silencing (154).

526 The subset-based association analysis done in this work of the genetically diverse LandracePLUS panel
527 challenged with ten *Bgt* isolates unraveled 17 potentially novel powdery mildew resistance genes.
528 Therefore, this study can be used as an example for future work on similar collections in search of other
529 traits of interest. Once a diversity panel is assembled, instead of focusing on single pathogen races, we
530 propose phenotyping with diverse isolates from geographically defined agricultural regions. Followed by
531 subset-based analyses for unique resistance and origin of accessions, this can reveal more resistance loci
532 than studies done with single pathogen isolates in entire collections.

533

534 **Materials and Methods**

535 Plant material and growth conditions

536 As starting material from which we selected our working panel, we utilized a formerly assembled bread
537 wheat collection of 1,320 landraces that had been selected based on FIGS of accessions with potentially
538 high selection pressure for powdery mildew resistance (32). Later, this collection had been complemented
539 with 733 accessions of diverse geographical origin (33). Assessment of accession type, i.e., landrace,
540 cultivar, breeding/research material or unknown, was based on passport data, which can be found at

541 genesys-pgr.org. When no GPS data was available for accession collection sites, we projected GPS using
542 https://maps.google.com from the most detailed description available, i.e., given villages up to countries.
543 Accessions from the two combined wheat collections were phenotyped for powdery mildew resistance at
544 seedling stage with the six *Bgt* isolates CHE_94202, CHE_96224, CHE_97223, CHE_97266, CHE_98230 and
545 GBR_JIW2. The infection phenotype was used to create a reduced panel of wheat accessions, consisting
546 of approximately 50% that showed complete resistance (0%) to one or more isolates or moderate
547 resistance (<= 20%) to at least two isolates. The remaining 50% were susceptible to all six isolates and had
548 the same ratio of spring wheat to winter wheat as the resistant part of the panel. Additionally, we used
549 wheat accession origin as a proxy for relatedness, choosing a geographically close susceptible counterpart
550 for each resistant accession. The resulting diverse LandracePLUS panel of 755 bread wheat accessions (S1
551 Excel) with a focus around the fertile crescent was infected with four additional *Bgt* isolates at seedling
552 stage, i.e., CHE_19004, CHN_46_30, ISR_106 and ISR_94.

553 Differential lines that were used to assess virulence patterns for 27 different *Pm* genes are shown in S2
554 Table, including NILs and accessions containing the designated gene. NILs had been backcrossed multiple
555 times with susceptible accessions "Federation" or "Chancellor", depicted by /x*Accession, where x is the
556 number of backcrosses to the designated accession (20). Other differential lines were used as original
557 seeds from the USDA ARS (https://npgsweb.ars-grin.gov/gringlobal/search), or propagated using isolation
558 bags per single spikes.

559 Seeds that were used for infection tests were obtained by propagating accessions in the field using single
560 rows per genotype without isolation. Seedlings for infection tests were grown in 40-wells plastic trays in
561 a growth chamber cycled at 20°C/16°C, 16/8h photoperiod with 80% relative humidity.

562 Powdery mildew isolates and infections

563 We used previously sampled and sequenced *Bgt* isolates CHE_94202, CHE_96224, CHE_97223,
564 CHE_97266, CHE_98230, CHN_46_30, GBR_JIW2, ISR_94 and ISR_106, which are described by
565 Sotiropolous (155). We sampled chasmothecia of one additional isolate, CHE_19004, in 2019 from a wheat
566 field at Reckenholz, Affoltern, Switzerland, which was revived and sequenced as previously described
567 (155).

568 Powdery mildew infection tests of the differential lines and the LandracePLUS panel accessions were
569 carried out on the first leaves of ten-to-15-day-old seedlings grown under the conditions mentioned
570 above. Leaf segments were placed with their adaxial side up in Petri dishes filled with 0.5% Phyto agar
571 containing 30 ppm benzimidazole. Fresh conidiospores were dispersed using 5 ml Pasteur glass pipettes
572 in a settling tower (156). Petri dishes with detached leaf segments were incubated for six to nine days at
573 20°C, 80% relative humidity with a 16 h light/8 h dark cycle and 50 µmol $m^{-2}s^{-1}$ photon flux density. Disease
574 levels were assessed seven to nine days after inoculation using a discrete quantitative scale with a score
575 from 0 to 100 for the percentage of leaf area covered by sporulating mildew colonies, as described earlier
576 by Kaur (157). When necessary to define resistance categories, i.e., for visualizations and defining
577 overlapping or unique resistance, the following thresholds apply: 0-30% = resistant, > 30-60% =
578 intermediate and > 60% = susceptible. We used mildew susceptible accession Kanzler as a control for a
579 proper mildew infection for all infection tests and Chancellor and Federation as additional susceptible
580 controls for tests on differential lines. Disease levels of differential lines were directly scored as resistant,
581 intermediate and susceptible, following the above thresholds. For Pearson correlation, these values were
582 transformed to 0, 50 and 100.

583 Infections were done with four biological replicates per wheat accession and the mean was taken as the
584 final phenotype. Outliers with a standard deviation > 1.1 were removed. Inconclusive phenotypes, e.g.,
585 50% resistant and 50% susceptible against the same isolate due to possible seed contamination, were
586 excluded for further analyses.

587 Data analysis

588 Unless indicated otherwise, analyses were done using R version 3.6.3 (158), including data handling with
589 Tidyverse (159) and visualizations with R package ggplot2 version 3.3.6 (160). Dendrogram formation and
590 hierarchical clustering were performed using the stats R package version 3.6.3 (158). Pearson correlation
591 between the isolate phenotypes was calculated using package Hmisc version 5.0.1 (161) and visualized
592 with the package corrplot version 0.92 (162). False Discovery Rates were calculated using package hdi 0.1-
593 9 (163).

594 Wheat *Pm* gene sequence assembly and alignment was done with CLC Genomics Workbench version
595 20.0.4 (Qiagen Bioinformatics, https://digitalinsights.qiagen.com/). Pathogen *Avr* gene sequence
596 alignment was done using IGV version 2.15.4 (164).

597 DNA extraction and genotyping

598 DNA extraction of plant material was performed as previously described (165). DNA quality was assessed
599 via agarose gels and genotyped using the TaBW35K SNP array (166). The SNP call dataset included marker
600 positions and flanking sequences based on RefSeq v1.0 of Chinese Spring (36). We filtered for
601 "PolyHighResolution" or OTVs and markers with known chromosomal position in the reference genome
602 Chinese Spring. Absent haplotypes of an OTV were translated to NA to facilitate their inclusion in
603 downstream analyses. Additional thresholds of 25% heterozygosity, 25% missing data per wheat accession
604 and 5% missing data per marker resulted in 29,556 polymorphic markers. These were brought into a
605 Hapmap format with the R package dplyr version 1.0.9 (159) and then transformed into variant call format
606 (VCF) file using the software TASSEL version 5.0 (167). This file was used as an input to generate plink files
607 using vcftools version 0.1.16 (168), which were then transformed together with phenotyping data into
608 .bed, .bim and .fam files using PLINK v1.07 (169) for the diversity and association analyses.

609 *In silico* genotyping of TaBW35K array SNP variants in high-quality sequenced wheat genomes

610 For the comparison of genetic diversity, Fielder (40), Renan (41) and the 10+ wheat reference genomes
611 ArinaLrFor, Chinese Spring, Claire, Cadenza, Jagger, Julius, Landmark, Lancer, Mace, Paragon, Norin61,
612 Robigus, Stanley, SYMattis and Weebill (25,36) were added. Flanking sequences of SNP array markers
613 were queried using BLASTN searches against the publicly available wheat genomes. Blast results were
614 filtered for hits with at least 60bp alignment and 96% shared identity, thereby allowing no more than
615 three mismatched nucleotides. Positions of the SNPs were then extracted from BLASTN alignments on the
616 respective genomes. Merging the resulting dataset with the 29,556 previously used markers resulted in
617 an overlap of 27,016 SNPs that were used for PCAs and hierarchical cluster analysis.

618 Diversity Analysis via PCA and hierarchical clustering

619 PCAs for the LandracePLUS panel and high-quality sequenced genomes were done based on the 27,016
620 SNP set using the R package SNPRelate version 1.18.1 and gdsfmt version 1.20.0 (170). For the PCA for the
621 comparison to the 632 landraces from the INRAe study (28), we first cleaned the genotyping data of the
622 combined datasets provided by INRAe in the same way as we did for the LandracePLUS panel, resulting in
623 30,021 polymorphic SNPs. Because no creation of a Hapmap or VCF file was necessary for further
624 downstream analysis, this dataset could directly be used for PCA using the prcomp function.

625 Hierarchical clustering analysis was performed for the 27,016 SNP set including high-quality sequenced
626 genomes by transforming the VCF into a GT format using vcftools as an input for the functions hclust
627 (method = ward.D2) and dist (method = euclidean). We visualized the dendrogram and built the four
628 groups of clustering genotypes using the package dendextend version 1.16.0 (171).

629 Admixture kinship analysis and comparative visualization

630 We used the .bed file of 29,556 SNPs (not including the chromosome-assembled genomes) as an input to
631 assess population structure using ADMIXTURE version 1.3.0 (172), where the most likely number of
632 founder populations K can be estimated via running the model over a series of values of K and then

633 choosing K around the lowest occurring cross-validation (CV) error. We ran the model with a MAF of 1%,
634 resulting in 28,545 SNPs, for K = 2 to K = 20. However, the CV error steadily dropped with increasing K (S8
635 Fig). We therefore regarded Ks after the largest CV error drops of 31% in total as appropriate estimations
636 for population structure, i.e., K = 4 to K = 6. A bootstrap of 500 and CV of 10 was used for this analysis.

637 A kinship matrix for the visualization was made with the same 29,556 SNP set using GAPIT version 3 (173).
638 For the comparative dendrogram, the kinship matrix was used as an input for hierarchical clustering using
639 standard hclust and dist functions. This dendrogram was visualized using the R package ggdendro version
640 0.1.23 (174). Finally, these two plots and the admixture barplot were merged using R package patchwork
641 version 1.1.1 (175).

642 *Pm* gene screening via polymerase chain reactions (PCRs) and sequencing

643 PCR analysis was performed using *Pm4* (96) and *Pm2* haplotype-specific markers (51). Four random
644 landraces that were identified to carry *Pm2* were then used for long-range and a following nested PCR to
645 amplify the gene for Sanger sequencing as previously described by Sanchez-Martin (51).

646 Genome-wide association analysis (GWAS)

647 GWAS was performed using the software GEMMA (176). For the full LandracePLUS panel and each subset,
648 .bed, .bim and .fam files were created as described above. .fam files were used as an input for creating
649 the kinship matrix in GEMMA using the options -gk 1 and -miss 1. This matrix was then integrated into the
650 univariate linear mixed model with option -miss 1 (176). Phenotypic data for the association studies was
651 added to the .fam files with the dplyr package in R.

652 To test if the peak on chromosome 5D was derived from *Pm2*, we included a covariate (option –c) for
653 binary information on the presence of *Pm2* based on haplotype-specific PCR screening. MAF was set to
654 1% for the full LandracePLUS panel and all subsets above 300 accessions in size, while for the other runs

655 the MAF was set to 5%. P-values were based on the likelihood ratio test and -log10 transformed for
656 Manhattan plots. We used two thresholds to account for multiple testing, namely the False Discovery Rate
657 (FDR) and the more conservative Bonferroni correction. However, we regarded SNPs that passed the FDR
658 test as significant.

659 Random subsets were produced using R package tibble 3.1.8 (177). Subsets of geographical origin were
660 based on countries of origin. Subsets for unique resistance to a single *Bgt* isolate were created by
661 considering accessions that were resistant (<= 30% leaf coverage by mildew) to the isolate of interest, but
662 not resistant (>30%) to the other nine isolates. To complement the resulting accessions with susceptible
663 ones, we used a threshold of >80% for CHE_98230, while all other isolates could have any resistance type.
664 For CHE_94202, we used a different approach, where CHE_94202 >70% and all other isolates >50%.

665 Accessions that most likely carry the causal genes of a resistance associated region were determined by
666 1) showing at least 50% of the effect alleles within an associated region and 2) being resistant to the
667 respective isolate (<=30% leaf coverage). SNP LD was calculated using PLINK --ld for pairwise comparison.
668 The physical position of previously described *Pm* genes was either taken from the corresponding
669 publication when available or estimated by blasting the flanking markers using BLASTN against Chinese
670 Spring RefSeq v1.0.

671

672 **Acknowledgments**

673 We would like to thank Nikolaos Minadakis, Zoe Bernasconi, Alexandros Sotiropoulos, Thomas Wicker and
674 Bruno Studer for their input and guidance in acquiring necessary techniques for this study. This project
675 was financially supported by the University of Zurich, Swiss National Science Foundation grants
676 310030_204165 and 310030B_182833 to B.K. JSM is recipient of the grant “Ramon y Cajal” Fellowship
677 RYC2021-032699-I funded by MCIN/AEI/10.13039/501100011033 and by the “European Union

NextGenerationEU/PRTR". JSM acknowledges the support of the Junta de Castilla y León through the projects "Escalera de Excelencia CLU-2018-04, and CL-EI-2021-04 support to the internationalization of AGRIENVIRONMENT - Unidad Producción Agrícola y Medioambiente" of the University of Salamanca, both co-financed by the European Regional Development Fund (ERDF "Europe drives our growth").

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Contributions

J.S.M. and B.K. conceived the project. E.J. and R.L. performed seed propagation. R.L., V.W. and J.S.M. performed infection tests. R.L. performed DNA extraction, PCR screens and allele detection. E.P. performed SNP array genotyping. R.L. and M.H. performed bioinformatics analysis. A.C.R. provided

697 theoretical contributions to the project. R.L., J.S.M. and B.K. analyzed the data and wrote the manuscript.

698 All authors revised the manuscript.

699 Competing Interests

700 The authors declare no competing interests.

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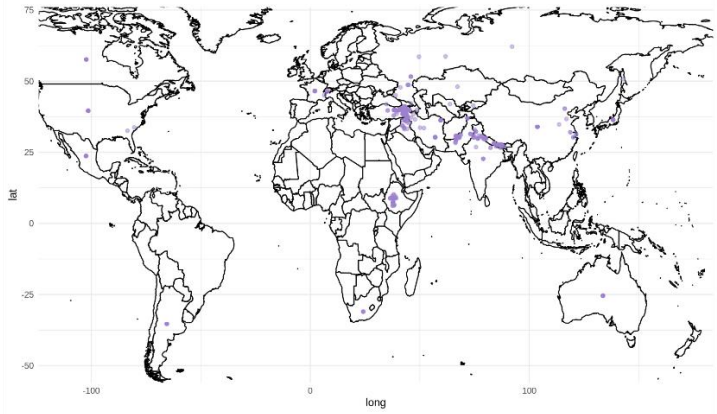
A



B

Country of origin	Number of accessions
Argentina	12
Armenia	8
Australia	14
Azerbaijan	1
Canada	8
Switzerland	4
China	45
Ethiopia	38
France	30
India	40
Iran	131
Iraq	19
Japan	18
Kazakhstan	1
Kyrgyzstan	1
Mexico	20
Nepal	40
Pakistan	42
Russia	13
Tajikistan	4
Turkey	231
USA	19
Uzbekistan	1
South Africa	4
unknown	11

C

D

Figure 1. LandracePLUS panel diversity and powdery mildew symptoms.

A World map with semitransparent purple dots representing the origin of each of the 755 wheat accessions of the LandracePLUS panel. **B** Number of accessions from the LandracePLUS panel per country of origin. **C** Selection of diverse wheat ears from the LandracePLUS panel. Scale bar, 5 cm. **D** Leaf of wheat cultivar “Kanzler” with powdery mildew symptoms. Scale bar, 1cm.

1178

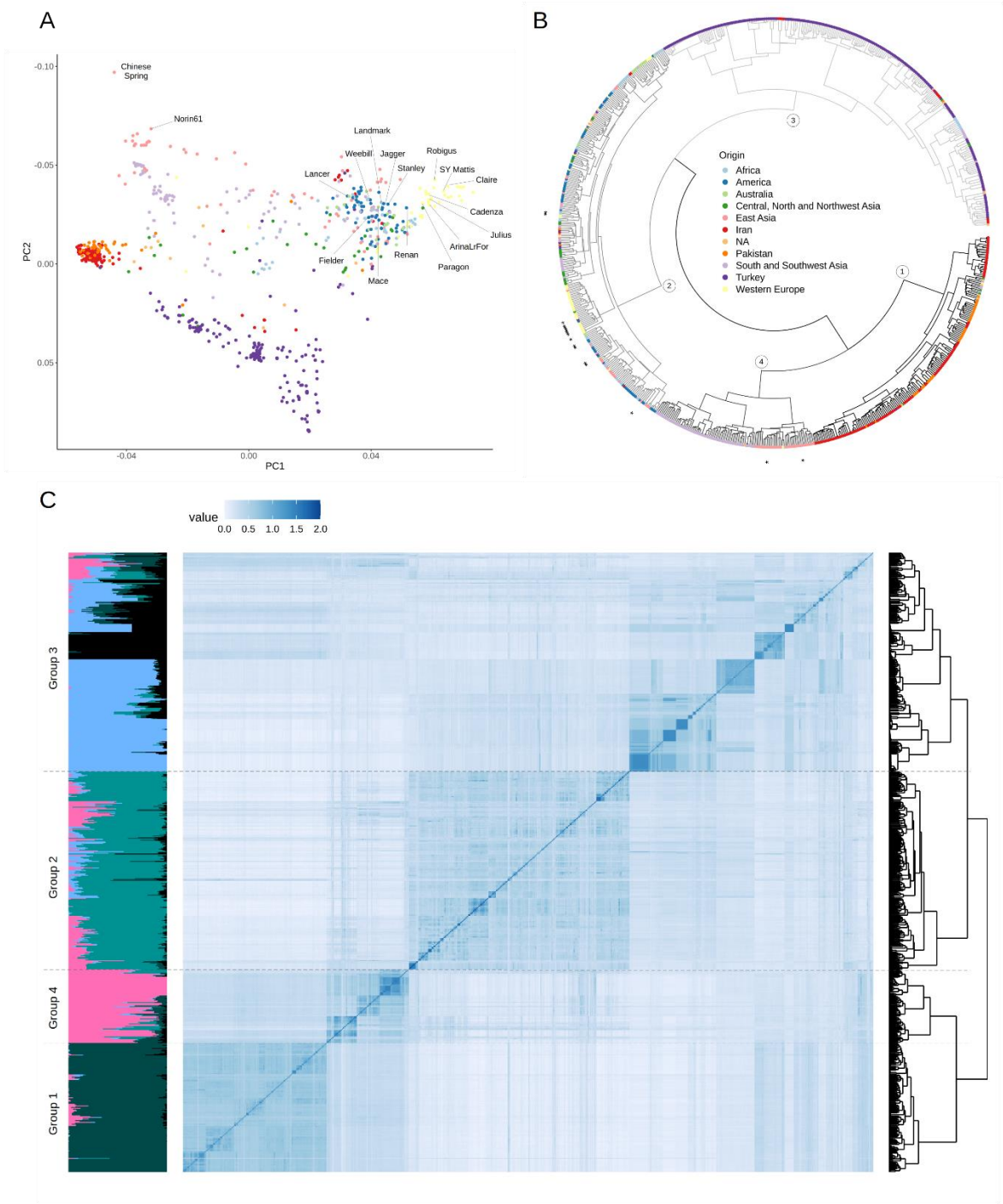


Figure 2. Genetic diversity and kinship analysis of the LandracePLUS panel.

A PCA from 27,016 SNPs including high-quality sequenced wheat accessions that are labelled in the figure. PC1 = 8.5%, PC2 = 5.1%. Colors refer to geographical origin of accessions and are indicated in B.

B Dendrogram of a hierarchical clustering analysis from 27,016 including high-quality sequenced accessions that are indicated with stars. Colors represent the geographical origin of wheat accessions. Circled numbers on nodes refer to Groups 1 to 4 when dividing into four clusters. **C** Alignment of Admixture plot, kinship matrix and dendrogram for the 755 wheat accessions, only, with a MAF of 1%, resulting in 28,545 SNPs. The admixture plot shows K=5, where colors represent ancestral populations. The four groups based on hierarchical clustering are separated by dashed lines.

1179

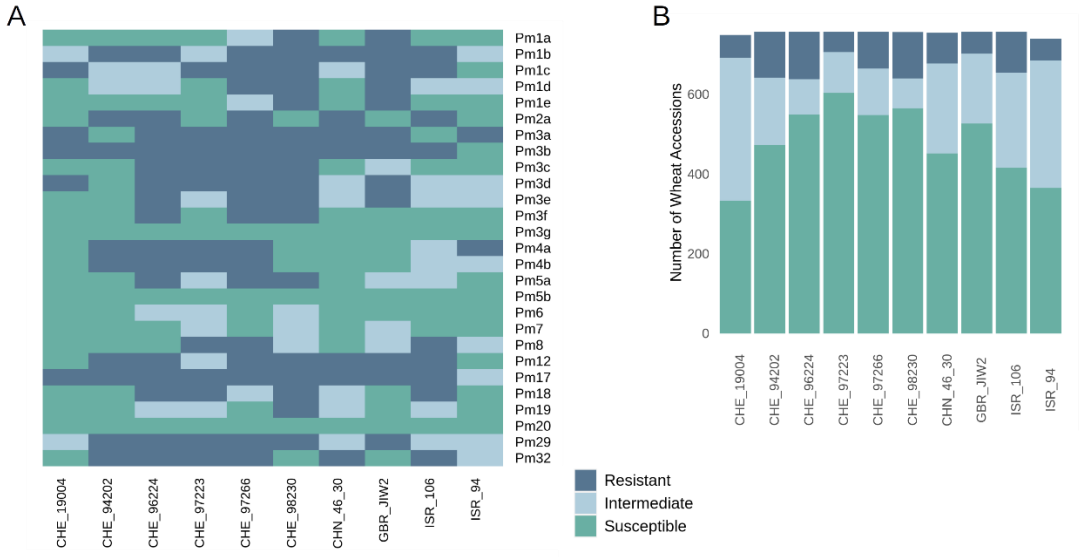


Figure 3. Virulence patterns of powdery mildew isolates and resistance distribution in the LandracePLUS panel.

A Virulence pattern of the ten powdery mildew isolates on differential lines with known *Pm* genes. Differential lines for the 27 *Pm* genes are shown in Suppl. Table 2. **B** Virulence pattern of the ten powdery mildew isolates on the LandracePLUS panel. Inconclusive phenotypes based on possible heterozygosity are not shown. Colors represent host symptoms at seedling stage in the three categories resistant, intermediate and susceptible, as defined in Materials and Methods.

1180

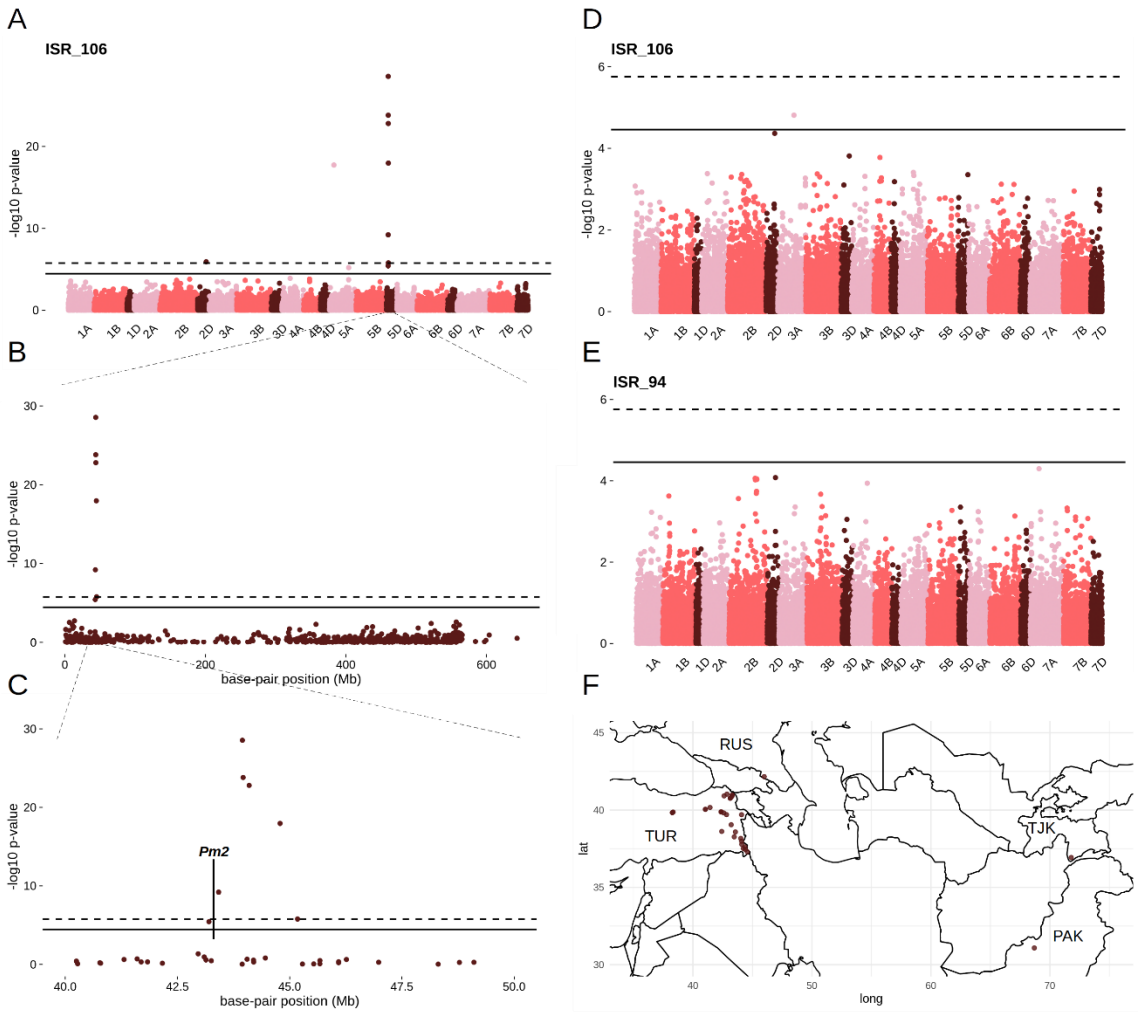


Figure 4. Analysis of the region associated with powdery mildew resistance on chromosome 5D. Manhattan plot for GWAS with all 755 wheat accessions infected with isolate ISR_106 showing **A** all 21 chromosomes, **B** chromosome 5D and **C** the region of 40 to 50 Mb on chromosome 5D. The locus of the Chinese Spring version of *Pm2* is indicated with a line. **D** Manhattan plot for GWAS with ISR_106 when adding a covariate for *Pm2* presence in wheat accessions. **E** Manhattan plot for the *Pm2* virulent isolate ISR_94. Solid lines represent the threshold for False discovery rate and dashed lines for Bonferroni correction. **F** Map with semitransparent brown dots depicting the origin of landraces that contain *Pm2*. Countries of origin are abbreviated with the three-letter country code of ISO 3166.

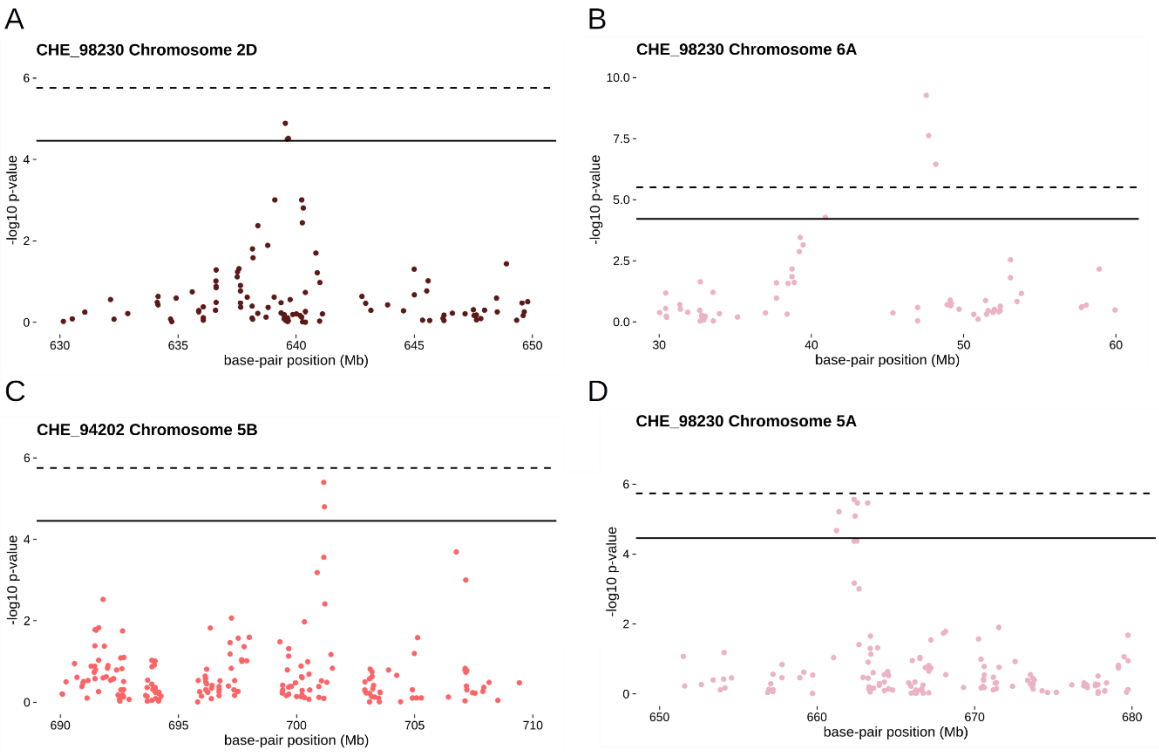


Figure 5. Manhattan plots of novel resistance associated regions.
A Full LandracePLUS panel of 755 accessions. **B** Subset of accessions from Pakistan and Iran. Isolates and chromosomes are depicted in the top-left corner. **C** Subset of accessions that were exclusively resistant to CHE_94202. **D** Subset of accessions that were exclusively resistant to CHE_98230. Solid lines represent the threshold for False discovery rate and dashed lines for Bonferroni correction.

Supporting information captions

S1 Figure. PCAs from 27,016 SNPs of the LandracePLUS panel including high-quality sequenced genomes with PC1 = 8.5% and PC2 = 5.1%. A Types of wheat accessions are highlighted in different colors. **B** Ethiopian landraces are highlighted by color in comparison to accessions of other origin.

S2 Figure. Genetic diversity of the LandracePLUS panel compared to 632 landraces from an INRAe study that have been chosen to represent the original pool of worldwide hexaploid diversity (Balfourier). PCA from 30,021 polymorphic SNPs that were present in SNP arrays of both studies. PC1 = 8.9%, PC2 = 3.4%. Accessions from the LandracePLUS panel are shown in orange, while INRAe landraces are colored in green.

1191 **S3 Figure. Correlation matrix showing Pearson correlation of virulence between the ten isolates used for**
1192 **phenotyping. A** Correlation coefficients based on phenotypic variation of the differential lines and the **B**
1193 Corresponding p-values. **C** Correlation coefficients based on phenotypic variation in the LandracePLUS panel and
1194 the **D** corresponding p-values. Square size and color depend on the specific correlation.

1195 **S4 Figure. Manhattan Plots for GWAS with nine powdery mildew isolates and 755 wheat accessions.** Isolate
1196 names are depicted in the top-left corner. Solid lines represent the threshold for False discovery rate and dashed
1197 lines for Bonferroni correction.

1198 **S5 Figure. Manhattan Plots of resistance associated loci of accessions from Pakistan and Iran.** Isolates and
1199 chromosomes are depicted in the top-left corner. Solid lines represent the threshold for False discovery rate and
1200 dashed lines for Bonferroni correction.

1201 **S6 Figure. Manhattan Plots of resistance associated loci of Turkish accessions.** Isolates and chromosomes are
1202 depicted in the top-left corner. Solid lines represent the threshold for False discovery rate and dashed lines for
1203 Bonferroni correction.

1204 **S7 Figure. Manhattan Plots of resistance associated loci of accessions that were exclusively resistant to**
1205 **CHE_98230.** Isolates and chromosomes are depicted in the top-left corner. Solid lines represent the threshold for
1206 False discovery rate and dashed lines for Bonferroni correction.

1207 **S8 Figure. Cross-validation error of Admixture kinship analysis for K=2 to K=20 for 28,545 SNPs.**
1208
1209 **S1 Table. Molecularly known Avr and Svr haplotypes of the ten powdery mildew isolates used for phenotyping.**
1210 H0 = published avirulent haplotype, H1 = published virulent haplotype, H2 = unpublished haplotype. Based on the
1211 phenotype of the differential lines containing cognate Pm genes, we propose that H2 of AvrPm1a and AvrPm3a/f
1212 are virulent, while H2 of AvrPm3d is proposed to be avirulent. H2 of AvrPm17 is likely virulent, due to the
1213 resemblance of H1, with just one additional amino acid change. The resistant phenotype of differential line Pm17
1214 is suggested to be due to the presence of an additional Pm gene in Amigo.
1215 Amino acid (AA) changes are given in reference to H0 and depicted with position original:new.
1216
1217 **S2 Table. Differential lines used for determining powdery mildew isolate virulence spectra and the respective**
1218 **Pm genes they contain.**
1219 NILs had been backcrossed multiple times with susceptible accessions “Federation” or “Chancellor”, depicted by
1220 /x*Accession, where x is the number of backcrosses to the designated accession (20).
1221

1222 **S1 Excel. Information on accessions of the LandracePlus panel.** Alleles with asterics are non-functional.
1223
1224 **S2 Excel. Gene candidates and annotations in Chinese Spring for regions associated to powdery mildew**
1225 **resistance.**
1226
1227 **S3 Excel. Raw phenotype data of the LandracePLUS panel infected with ten powdery mildew isolates.**
1228
1229 **S1 Data. Raw genotyping data of the LandracePLUS panel with the TaBW35K SNP array.** Empty cells are labelled
1230 "vide" and wheat accession IDs are extended by "_cell identifier".
1231
1232 **S2 Data. Sample statistics of the raw genotyping data of the LandracePLUS panel.** Total numbers are labelled
1233 "nb" and percentage is labelled "pc".
1234
1235 **S3 Data. Marker statistics of the raw genotyping data of the LandracePLUS panel.** Total numbers are labelled
1236 "nb" and percentage is labelled "pc".
1237