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Article

Genome-Wide Association Study Reveals Novel Powdery Mildew Resistance Loci in Bread Wheat

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Abstract: Powdery mildew (PM), caused by the fungal pathogen *Blumeria graminis* f. sp. *tritici* (Bgt), significantly threatens global bread wheat production. Although the use of resistant cultivars is an effective strategy for managing PM, currently available wheat cultivars lack sufficient levels of resistance. To tackle this challenge, we conducted a comprehensive genome-wide association study (GWAS) using a diverse panel of 286 bread wheat genotypes. Over three consecutive years (2020-21, 2021-22, and 2022-23), these genotypes were extensively evaluated for PM severity under field conditions following inoculation with virulent Bgt isolates. The panel was previously genotyped using the Illumina 90K SNP Infinium iSelect SNP assay to obtain genome-wide SNP marker coverage. By applying FarmCPU, a multilocus mixed model, we identified a total of 113 MTAs located on chromosomes 1A, 1B, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6B, 7A, and 7B at a significance level of $p \leq 0.001$. Notably, four novel MTAs on chromosome 6B were consistently detected in 2020-21 and 2021-22 environments. Furthermore, within the confidence intervals of the identified SNPs, we identified 96 candidate genes belonging to different proteins including 12 disease resistance/host-pathogen interactions related protein families. Among these, protein kinases, leucine-rich repeats, and zinc finger proteins, were of particular interest due to their potential roles in PM resistance. These identified loci can serve as targets for breeding programs aimed at developing disease-resistant wheat cultivars.

Keywords: powdery mildew; wheat; GWAS; FarmCPU; MTAs; candidate genes

1. Introduction

Bread wheat (*Triticum aestivum* L.) is a vital source of nutrients and serves as the primary staple food crop for approximately 35% of the global population [1]. In 2022, worldwide wheat consumption increased from 783 million metric tons to 792.69 million metric tons [2]. This rise in wheat consumption is necessary for global food security, but has led to extra burdens to wheat production system which is already challenged by the prevalence of pests and diseases such as powdery mildew (PM), a devastating wheat disease.

PM is caused by the fungal pathogen *Blumeria graminis* (DC.) E.O. Speer f. sp. *tritici* Em. Marchal, (syn. *Erysiphe graminis* f. sp. *tritici*) (Btg). It is the third most destructive disease of wheat, causing yield losses ranging from 13% to 34% under low infestation and 50% to 100% under severe infestation in various wheat-growing regions [3,4]. Environmental factors like temperature and relative humidity significantly influence the development and spread of this disease, contributing to epidemics [5]. In the Himalayan regions of India, PM is a significant factor contributing to reduced grain yield [6]. Breeding approaches have proven effective in developing PM-resistant wheat varieties to overcome yield losses and increase economic production. Using resistant varieties is currently considered an efficient, environmentally safe, and successful strategy to control PM and mitigate production losses.

Major effect genes and minor-to-moderate effects PM resistance genes are generally inherited qualitatively and quantitatively, respectively, and have also been identified in wheat and integrated into wheat resistance breeding programs. These includes over 100 alleles distributed over >60 major loci (*Pm1*–*Pm68*: *Pm8* is allelic to *Pm17*; *Pm18* = *Pm1c*; *Pm22* = *Pm1e*; *Pm23* = *Pm4c*, and *Pm31* = *Pm21*) and many valuable quantitative trait loci (QTLs) distributed across wheat chromosomes [7–25]. Whereas some of the PM resistance genes, such as *Pm38*, *Pm39*, and *Pm46*, are inherited quantitatively [26–28], there are other genes which possesses with many resistance alleles. For instance, *Pm3* exhibits the highest allelic diversity, with 17 variants, followed by *Pm1* and *Pm5*, each having 5 alleles. *Pm4* and *Pm2*, on the other hand, possess 4 and 3 alleles, respectively [11].

The PM resistance genes have primarily been transferred from both domesticated and wild relatives and from other species such as rye (*Secale cereale*) to wheat [29]. However, their utilization in wheat breeding is challenged by linkage drags [15]. For instance, *Pm8* gene, which derived from *S. cereale* and also significantly contributed to PM resistance in wheat breeding during the 1990s, is linked with secalin glycopeptide locus on 1RS chromosome segment which causes a decline in flour quality [30]. Furthermore, many of the identified resistance genes have been overcome by the pathogen in different countries, leaving only a few that are still effective against current *Bgt* isolates in the field [31,32]. Thus, the continuous identification, characterization and deployment of new resistance genes is essential to minimize economic losses.

The declining cost of genotyping and the increasing availability of diverse single nucleotide polymorphisms (SNPs) fingerprinting platforms has furthered the identification and deployment of new resistance genes. Many genetic mapping methods are now available to accommodate different types of traits, mapping populations, and study objectives [33]. These methods can be categorized into three main groups based on the principle and genetic material used: (i) QTL mapping using a biparental population, (ii) association mapping or genome-wide association studies (GWAS) in a diverse set of germplasm, and (iii) genetic mapping utilizing the multi-parent populations. The first two approaches, QTL mapping and GWAS, have successfully identified genes and their markers associated with disease resistance [34–37]. However, the QTL mapping approach, which relies on a segregating population generated from parents exhibiting contrasting trait performances [12], faces limitations in QTL detection power. The scarcity of recombination events and genetic variation in such populations often leads to identifying only a few QTLs. To overcome this limitation, advanced wheat populations such as ‘multiparent advanced generation inter-cross’ (MAGIC) have been introduced to enhance the resolution of traditional mapping [38]. Nevertheless, challenges arise in validating map order and analyzing recombination due to using bi-allelic markers in these populations [39]. Conversely, GWAS circumvents the need for developing populations by conducting analyses on diverse germplasm [40] and offers several advantages such as the utilization of larger sample sizes, which facilitates the exploration of a broader range of genetic variations including those with minor effects and allelic diversity [36,41]. Additionally, GWAS enables higher-resolution mapping, identifying candidate genes associated with the trait of interest [42].

Thus, the objectives of the present study were to identify potentially novel loci that confer effective PM resistance at the adult plant stage utilizing a panel of 286 wheat accessions known as ‘wheat association mapping initiative’ (WAMI) panel created by CIMMYT, Mexico, identify potential PM resistance-associated markers and candidate genes, and compare identified loci with the

previously reported genes/QTLs and identify candidate genes using comparative analyses. Leveraging landmark resources such as the high-density 90K SNP array [43] and the Ensemble plants database [44], we aims to address the aforementioned objectives and shed light on the genetic basis of PM resistance in wheat using a GWAS study.

2. Results

2.1. Phenotypic Analysis

The phenotypic analysis conducted in specific environments showed significant variation for disease severity in the WAMI panel. Table 1 provides a summary of the phenotypic variation for PM disease severity. The broad sense heritability for PM disease severity ranged from 65% to 89% (Table 1). The analysis of variance indicated that both genotypic and environment as well as genotype-environment interactions had highly significant ($P \leq 0.001$) estimated variance components for disease response to PM (Table 2). However, despite environmental variation, significant positive correlations (> 0.6 for each environment) were observed for the disease severity among the three environments (Figure 1A).

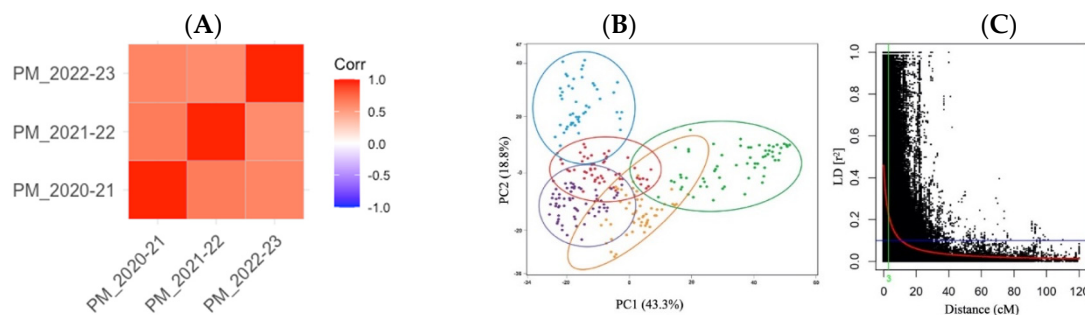


Figure 1. Correlation coefficient, population structure and linkage disequilibrium (LD). **A.** A graphical representation of correlation coefficient among three environmental years for PM disease severity. **B.** Scatterplots showing the results of principal component analysis (PCA) conducted on genotypic data obtained from WAMI panel containing 286 wheat accessions. The figure highlights the population structure of the WAMI panel, as revealed by the first two principal components (PC1 and PC2). **C.** A scatter plot for r^2 values of pairwise markers showing genome-wide linkage disequilibrium decay in 286 genotypes of the WAMI panel assessed using the Hill and Weir formula [45]. The red curved line depicts the fitted model for LD decay (non-linear regression of r^2 against distance), while the blue horizontal line represents a threshold (0.12) for the QTLs. The vertical green line depicts a genetic distance confidence interval of 3 cM at which the LD half-decay ($r^2 = 0.23$, the vertical green line) intersect with the LD decay curve.

Table 1. The descriptive statistics and heritability for the PM disease severity in wheat association mapping initiative (WAMI) panel containing 286 wheat accessions screened at Baru Sahib PM disease nurseries in Himachal Pradesh, India during three field seasons, spanning from 2020 to 2023.

Source	2020-21	2021-22	2022-23
Min	1	2.5	1.5
Max	8.5	8.3	9
Mean	5.3	5.1	5.3
LSD	1.2	1.4	1.3
CV	8.4	12.2	9.0
Heritability	0.89	0.65	0.81

Min: minimum; Max: maximum; LSD: least significant difference; CV: coefficient of variation.

Table 2. Analysis of variance (ANOVA) for PM disease severity in wheat association mapping initiative (WAMI) panel containing 286 wheat accessions.

Source of variation	Df	MSS
Genotypes	285	4.9 **
Environments (years)	2	52.7 **
Replications	1	8.5
Genotype × Environment	570	1.22 **

Df: degrees of freedom; MSS: mean squares significant at **p ≤ 0.001.

2.2. Population Structure Analysis

Principal components analysis indicated the existence of five distinct sub-groups within the WAMI panel (Figure 2B). PC1 accounted for 43.3% of the total variance, while PC2 explained 18.8%. These results suggest that the combination of PC1 and PC2 captures a significant portion of the underlying genetic variation within the panel, allowing for identifying and differentiating the sub-groups present in the WAMI population.

2.3. Genome-wide Marker Trait Association (MTA) Analysis

A scatter plot for r^2 values of pairwise markers showing genome-wide linkage disequilibrium (LD) decay for 286 genotypes of the WAMI panel assessed using the Hill and Weir formula [45] is given in Figure 1C. The average r^2 values of the genome was 0.12. The LD decay curve intersected with the threshold ($r^2 = 0.23$) at 3.0 cM (Figure 1C). This showed the ± 3.0 cM as the genome-wide critical distance to detect linkage and single QTLs as described earlier [46].

The comprehensive genome-wide MTA analysis encompassed three distinct models: GLM, MLM, and FarmCPU; however, the final GWAS analysis exclusively incorporated the best-fitting FarmCPU model. Over the course of the three-year investigation, a total of 19, 85, and 9 MTAs were successfully identified in each respective year (Figure 2 and Table 3). Figure 2 displays the frequency distribution of the best linear unbiased prediction (BLUP) values for PM disease severity for three consecutive years namely, 2020-21, 2021-22, and 2022-23, the Manhattan plot illustrating the results of genome-wide association scans, and the Quantile-Quantile (Q-Q) plot of p-values, comparing the observed -lg (P) for PM resistance to *Bgt* isolates in FarmCPU method.

In the initial year (2020-21), the distribution of MTAs spanned multiple chromosomes, with notable occurrences on the chromosome 1A (2 MTAs), 2B (4 MTAs), 6B (9 MTAs), and 7A (4 MTAs). Notably, a striking concentration of 9 MTAs emerged at a specific genetic position (59-60 cM) on chromosome 6B. This intriguing observation suggests the presence of a potential genomic “hotspot” linked to PM resistance, signifying the collective impact of multiple genetic variants.

Transitioning to the subsequent year (2021-22), the landscape of MTAs extended across various chromosomes: chromosome 2B (1 MTA), 3B (9 MTAs), 4A (19 MTAs), 5A (1 MTA), 5B (2 MTAs), 6B (48 MTAs), and 7B (3 MTAs). Noteworthy is the aggregation of MTAs at 48 cM on chromosome 4A, underscoring another genomic hotspot exerting influence on PM resistance. The 6B chromosome also exhibited remarkable association richness, hosting 49 MTAs. Within this cluster, 8 MTAs were anchored at 59 cM, 77 MTAs at 64 cM, and 3 MTAs at 65 cM, further reinforcing the pivotal role of the 6B chromosome region in contributing to the observed phenotypic variance for PM resistance.

Concluding the study in the final year (2022-23), a reduced yet significant number of MTAs were identified, totaling 9 associations distributed across chromosomes 1B (1 MTA), 3A (1 MTA), 3B (3 associations), 7A (2 MTAs), and 7B (1 MTA).

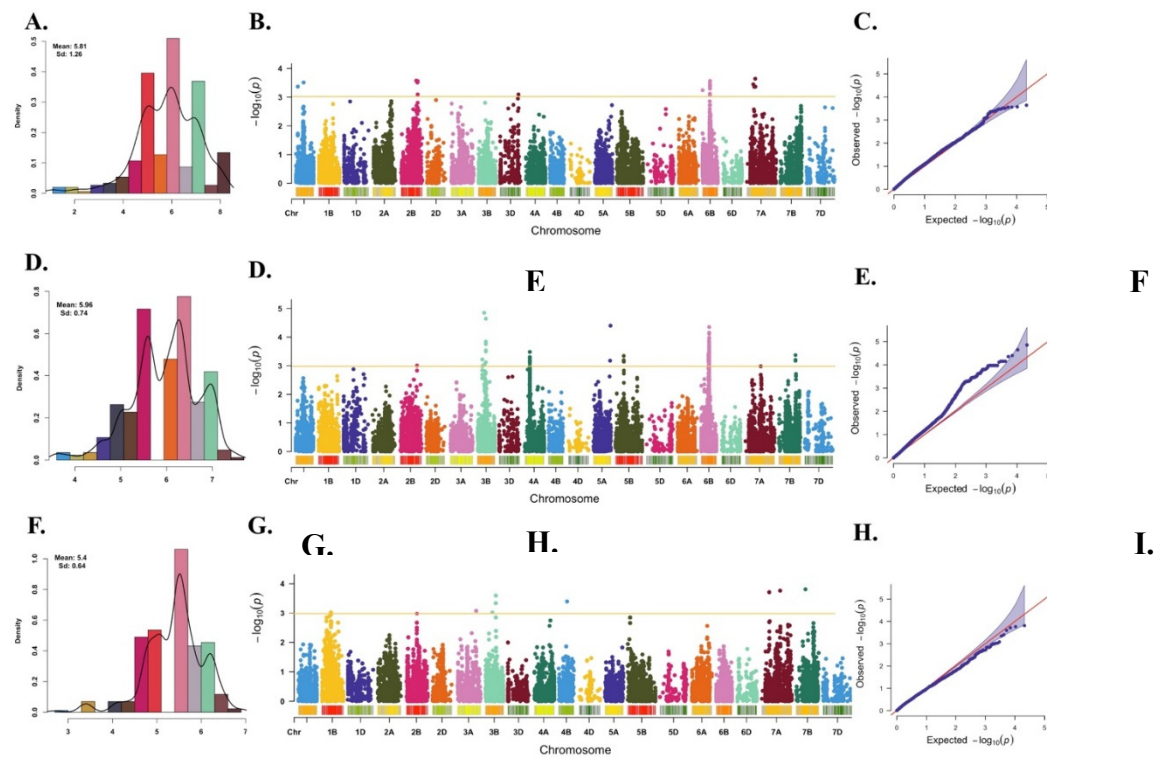


Figure 2. PM disease severity distribution, marker-trait associations and quantile-quantile (Q-Q) plots distributions. Histograms on left (A, D and G) display the frequency distribution of BLUP estimated for PM disease severity for three consecutive years, 2020-21, 2021-22, and 2022-23. Manhattan plots in the middle (B, E, and H) are illustrating the results of genome-wide association scans for PM resistance using BLUP of the 286 lines of WAMI panel across 2020-21, 2021-22, and 2022-23, respectively. The significant thresholds are represented by solid yellow lines, while the number of effective markers is indicated by the yellow lines. The Q-Q plots of p-values on right (C, F, and I) comparing the observed -lg (P) for PM resistance to *Bgt* isolates in FarmCPU analyses to the expected distribution of -lg (P) under a uniform distribution.

Interestingly, four common MTAs were consistently observed in crop seasons 2020-21 and 2021-22 located on chromosome 6B (Table 3). Notably, certain chromosomal regions, such as 2B (132 – 144 cM), 3B (57 – 71 cM), 6B (59 – 65 cM), and 7A (57 – 74 cM), also exhibited repeated associations with PM resistance, indicating their crucial role in the genetic variation of PM resistance. These findings provide valuable insights for future studies aiming to understand the genetic architecture of PM resistance and for targeted breeding efforts to enhance it in commercial cultivars through marker-assisted selection.

Table 3. Details of marker-trait associations (MTAs) detected for resistance against PM in WAMI panel during 2020-21, 2021-22, and 2022-23.

Marker	Chr	Pos (cM) ¹	Pos (bp) ²	Effect	p value
2020-21					
RAC875_c1710_376 ^s	1A	27.0	9103721	-0.55	0.0004
Kukri_c35200_895 ^s	1A	74.0	459514896	-0.59	0.0003
BS00080318_51 ^s	2B	132.0	763842555	0.32	0.0003
Excalibur_c5438_274 ^s	2B	142.0	774958099	0.30	0.0003
Excalibur_rep_c109577_698 ^s	2B	144.0	775368259	-0.27	0.0008
RAC875_rep_c83950_222 ^s	2B	144.0	775053184	-0.29	0.0003
Ra_c22493_190 ^s	6B	0.0	6196794	-0.48	0.0006

Excalibur_rep_c106789_271	6B	59.0	153957925	-0.33	0.0004
RAC875_c25489_1208	6B	59.0	153959656	-0.31	0.0008
wsnp_Ex_c39304_46635517	6B	59.0	151609970	-0.34	0.0003
wsnp_Ex_rep_c102044_87296690	6B	59.0	153959656	-0.32	0.0005
wsnp_Ex_rep_c102044_87297599	6B	59.0	153957925	-0.34	0.0003
wsnp_Ra_c48999_54089942	6B	59.0	153956896	-0.31	0.0008
Excalibur_c3795_198 ^s	6B	60.0	156266046	-0.31	0.0005
Ra_c14852_1487	6B	60.0	156266570	-0.32	0.0003
RAC875_c66770_208	7A	57.0	26879337	-0.58	0.0004
Excalibur_c7538_2718 ^s	7A	65.0	35602223	-0.48	0.0004
wsnp_Ex_c35_77935 ^s	7A	73.0	41960564	-0.36	0.0002
BS00023055_51	7A	74.0	41959666	-0.32	0.0004
2021-22					
Excalibur_c7971_1573	2B	144.0	775371388	0.04	0.0009
BS00011728_51 ^s	3B	57.0	59064340	-0.28	0.0000
BS00022741_51 ^s	3B	61.0	66953509	-0.26	0.0009
Excalibur_c21372_142	3B	61.0	66954023	-0.26	0.0009
Tdurum_contig51993_52 ^s	3B	61.0	66953509	-0.26	0.0009
BS00011869_51	3B	71.0	426452313	-0.23	0.0003
Excalibur_c80041_400 ^s	3B	71.0	429610611	-0.26	0.0000
Kukri_c21818_519 ^s	3B	71.0	556059780	-0.23	0.0002
RAC875_c58159_989	3B	71.0	569425136	-0.19	0.0008
wsnp_Ku_c21818_31604716 ^s	3B	71.0	556059565	-0.24	0.0001
BobWhite_c27944_234 ^s	4A	48.0	109443802	-0.23	0.0007
Ex_c17894_1159 ^s	4A	48.0	654418148	-0.22	0.0005
Excalibur_rep_c66815_273	4A	48.0	104375678	-0.22	0.0005
GENE-2637_94 ^s	4A	48.0	164633479	-0.23	0.0008
IAAV3906 ^s	4A	48.0	113854919	-0.22	0.0006
IAAV8784	4A	48.0	135358615	-0.22	0.0005
IACX1896 ^s	4A	48.0	109431746	-0.22	0.0007
Kukri_c44469_1240	4A	48.0	136432865	-0.22	0.0005
Kukri_c48155_158 ^s	4A	48.0	120605031	-0.22	0.0005
RAC875_c22562_429 ^s	4A	48.0	111292238	-0.23	0.0003
RAC875_rep_c74695_101 ^s	4A	48.0	164632278	-0.23	0.0008
tplb0035b22_184	4A	48.0	135614180	-0.22	0.0005
wsnp_BE442869A-Ta_2_1	4A	48.0	140134106	-0.22	0.0005
wsnp_Ex_c10527_17198865 ^s	4A	48.0	115570013	-0.22	0.0005
wsnp_Ex_c1387_2659020 ^s	4A	48.0	115912754	-0.22	0.0005
wsnp_Ex_c14529_22547438	4A	48.0	115913618	-0.22	0.0005
wsnp_Ex_c1865_3515470 ^s	4A	48.0	136894495	-0.22	0.0005
wsnp_Ex_c36141_44153175 ^s	4A	48.0	111091261	-0.23	0.0007
wsnp_Ex_c4286_7734046	4A	48.0	114744375	-0.22	0.0006
wsnp_Ex_c43734_49968808 ^s	4A	48.0	211000047	-0.24	0.0005
wsnp_Ex_rep_c69890_68851948 ^s	4A	48.0	105249095	-0.24	0.0005
Tdurum_contig51134_191 ^s	5A	144.0	698501228	-0.36	0.0000
RAC875_c19099_308 ^s	5B	68.0	519153210	0.24	0.0007
Tdurum_contig53926_455 ^s	5B	68.0	516469300	0.26	0.0006
BS00046963_51	6B	59.0	150665070	-0.19	0.0006
Excalibur_rep_c106789_271	6B	59.0	153957925	-0.20	0.0005
Kukri_c38732_225 ^s	6B	59.0	151131387	-0.18	0.0007
wsnp_Ex_rep_c102044_87296690	6B	59.0	153959656	-0.19	0.0007
wsnp_Ex_rep_c102044_87297599	6B	59.0	153957925	-0.20	0.0004

Ra_c14852_1487\$	6B	60.0	156266570	-0.18	0.0006
BS00040868_51\$	6B	63.0	228977167	-0.24	0.0001
BobWhite_c34920_228	6B	64.0	249498169	-0.21	0.0006
BS00003955_51\$	6B	64.0	260548319	-0.24	0.0001
BS00021686_51\$	6B	64.0	174566651	-0.24	0.0003
BS00045761_51\$	6B	64.0	227070547	-0.24	0.0002
BS00067871_51\$	6B	64.0	261585651	-0.24	0.0002
BS00067873_51\$	6B	64.0	261585682	-0.23	0.0003
BS00080544_51\$	6B	64.0	234560063	-0.22	0.0007
Ex_c49055_617\$	6B	64.0	260548488	-0.22	0.0005
Excalibur_c20503_382\$	6B	64.0	257568556	-0.24	0.0001
Excalibur_c47738_334	6B	64.0	262473204	-0.22	0.0004
Excalibur_c5136_2314\$	6B	64.0	231677537	-0.22	0.0003
Excalibur_c53834_416\$	6B	64.0	257993802	-0.25	0.0000
Excalibur_c79066_165\$	6B	64.0	232168465	-0.24	0.0002
GENE-2606_197\$	6B	64.0	257567774	-0.25	0.0001
Kukri_c25377_106\$	6B	64.0	174567929	-0.23	0.0005
Kukri_c38058_532	6B	64.0	257567774	-0.22	0.0009
Kukri_c52515_442	6B	64.0	259877872	-0.21	0.0005
Ra_c77985_260\$	6B	64.0	214089373	-0.22	0.0008
RAC875_c12805_908\$	6B	64.0	234559907	-0.23	0.0005
RAC875_c24962_1326	6B	64.0	229280173	-0.23	0.0003
RFL_Contig311_951	6B	64.0	231354317	-0.23	0.0003
TA005139-0719\$	6B	64.0	234559694	-0.24	0.0004
wsnp_BQ161448B_Ta_2_1\$	6B	64.0	261530574	-0.23	0.0002
wsnp_Ex_c1603_3056226	6B	64.0	221813724	-0.23	0.0004
wsnp_Ex_c23474_32717535\$	6B	64.0	276221225	-0.25	0.0001
wsnp_Ex_c27934_37093614	6B	64.0	259881611	-0.24	0.0002
wsnp_Ex_c42372_48966781	6B	64.0	229280173	-0.21	0.0007
wsnp_Ex_c46160_51746546	6B	64.0	229278919	-0.23	0.0002
wsnp_Ex_rep_c103466_88415738	6B	64.0	274209042	-0.24	0.0002
wsnp_Ex_rep_c103466_88415994	6B	64.0	274208315	-0.22	0.0006
wsnp_Ex_rep_c103497_88437811\$	6B	64.0	277165618	-0.24	0.0001
wsnp_Ex_rep_c68480_67305954	6B	64.0	259292578	-0.24	0.0001
wsnp_JD_c6448_7610859	6B	64.0	257993802	-0.24	0.0001
wsnp_Ku_c27423_37369145	6B	64.0	259883491	-0.21	0.0005
wsnp_Ra_c16850_25605248	6B	64.0	262473278	-0.23	0.0002
wsnp_Ra_c33358_42248399\$	6B	64.0	257993727	-0.22	0.0004
wsnp_Ra_rep_c111161_93528347\$	6B	64.0	277163269	-0.21	0.0005
wsnp_Ra_rep_c73725_71801179	6B	64.0	274207889	-0.21	0.0006
wsnp_Ra_rep_c73725_71801237	6B	64.0	274207831	-0.21	0.0005
wsnp_Ra_rep_c73731_71807419	6B	64.0	257996714	-0.24	0.0001
BobWhite_c32911_243\$	6B	65.0	255268901	-0.22	0.0003
Kukri_c24148_254\$	7B	136.0	705270901	-0.16	0.0007
TA005284-0990\$	7B	136.0	704271881	-0.16	0.0006
wsnp_JD_c13673_13606066\$	7B	136.0	704271648	-0.17	0.0004
2022-23					
IACX3595\$	1B	76.0	539562016	0.17	0.0009
BS00030652_51	3A	158.0	693002205	-0.25	0.0008
Excalibur_c8284_580\$	3B	57.0	736672449	-0.20	0.0009
IAAV6566	3B	84.0	736712583	-0.17	0.0003
Tdurum_contig8365_433	3B	84.0	736672399	-0.17	0.0005

Kukri_c2526_1375 [§]	4B	66.0	523447728	-0.28	0.0004
Excalibur_c25471_225	7A	64.0	34539197	0.17	0.0002
BS00094965_51 [§]	7A	150.0	669729091	-0.30	0.0002
Kukri_c30836_582	7B	73.0	699427808	0.16	0.0002

¹ = Marker position (genetic) on linkage map in cM; ² = Marker position (physical) on chromosome in bp, and [§] = Marker with candidate genes that encode a protein.

2.4. Candidate Genes

In our study, we investigated genomic regions to find candidate genes within a specific window of 200 kilobases (kb) surrounding each MTA for their association with PM resistance. This window consisted of 100 kb on each side of the MTA. We discovered a total of 94 unique CGs (89 encoding known protein domains) in 51 genomic intervals for 65 MTAs (Table S1), while no CG were spotted in defined genomic regions of the remaining 48 MTAs.

Further analysis of these 94 CGs involved screening them for the presence of genes that are already known to play a role in various pathways related to the interactions between pathogens and their host organisms. The results of this screening, as presented in Table S2 revealed that 30 CGs encoded a diverse range of protein domains relevant to plant defense and the interactions between pathogens and hosts. The identified proteins encompassed a wide array of functional domains and families. They included the following: (i) ABC transporter-like, ATP-binding domain (ii) Ankyrin repeat (iii) Aspartic peptidase A1 family (iv) Peroxidase (v) Cytochrome P450 (vi) Disease resistance protein (NB-LRR) (vii) Glutathione S-transferase (viii) P-loop containing nucleoside triphosphate hydrolase (ix) Kinase like domain superfamily (including Serine-threonine/tyrosine/cysteine-protein kinase) (x) Wall-associated receptor kinase, galacturonan-binding domain (xi) Zinc finger proteins and (xii) F-box superfamily. These CGs and their corresponding proteins are directly or indirectly involved in the response of the host organism to pathogen attacks.

3. Discussion

To uncover novel PM resistance genes/MTAs, a series of field experiments were conducted over three years: 2020-21, 2021-22, and 2022-23, utilizing the diverse WAMI panel of common wheat. The analysis of PM disease severity unveiled a wide spectrum of phenotypic variations, displaying normal distributions in two of the three environments (2020-21 and 2022-23), and a near binomial distribution in the remaining environment (2021-22) (Figure 2). This distribution pattern suggests the involvement of major and multiple quantitative trait loci (QTLs) that contribute to PM resistance. As indicated by the phenotypic analysis, GWAS analysis also identified a total of 113 MTAs associated with adult plant resistance against PM (Table 3). This can be explained by the extensive diversity present for PM resistance within the WAMI panel [47]. The presence of multiple PM resistance QTLs/MTAs in wheat germplasm sourced from diverse countries has also been reported previously [16], underlining the intricate and diverse genetic foundation of PM resistance. These findings highlight the complexity and diversity of the genetic basis underlying PM resistance and reinforce the importance of the WAMI panel in dissecting PM resistance and emphasizing the need for further investigations to unravel the specific PM resistance mechanisms involved in different wheat populations. All the important MTAs are discussed in greater detail below.

3.1. Investigating the Concordance among Significant MTAs and Previously Mapped QTL/Pm Resistance Genes

This study identified a total of 113 MTAs (19, 85 and 9 MTAs in 2020-21, 2021-22 and 2022-23, respectively) with some notable findings such as clusters of MTAs at the same position (Table 3). Upon comparing the position of markers associated with significant MTAs identified in this study with the previously reported MTAs/QTLs and *Pm* resistance genes, it was observed that several MTAs were located to the positions where *Pm* resistance genes/QTLs had been reported before while others represent novel loci. For instance, on chromosome 1A, two MTAs at positions 27.0 and 74.0

cM (detected during 2020-21), were found to share similar locations with previously identified *Pm* resistance loci such as resistance genes *Pm3* [48] and *Pm223389* [49], QTLs *Qpm.osu.1A* [50], *Qpm.mgb-1AS* [20] and *QPm.Caas-1A* [51], SNP markers SNP_BS00021714_51 [46], SNP_tplb0041a22_935, SNP_Excilibur_c15098_59116 [17] and five other MTAs identified by Liu et al. [12], and a meta-QTL *MQTL1* [29].

Similarly, on chromosome 1B, an MTA was detected at 76.0 cM during 2022-23 which shared the chromosomal region with previously identified loci *Qaprpm.cgb-1B* [52] and *QPm.osu-1B* [50], as well as five MTAs identified by Liu et al. [12].

Our analysis revealed that five SNPs detected between 132.0 to 144.0 cM on chromosome 2B during 2020-21 and 2021-22 were found in close proximity to previously detected QTLs *QPm.crag-2B* and *Qaprpm.cgb-2B*, and major resistance genes such as a recessive gene *pm42* and *PmWE99* [10,53].

On chromosome 3B, we detected twelve PM resistance associated SNPs between 57.0 and 84.0 cM during 2021-22 and 2022-23. Comparison of the chromosome intervals with the previously identified loci such as *PM_3B1* and *PM_3B2* [16], *QPm.inra-3B* [54], *QPm.osu-3B* [50], *QPm.caas-3BS* [55], *CP2* [29], *Qpm.mgb-3BL.3* [20], as well as other MTAs identified by Liu et al. [12] and Alemu et al. [46], showed that markers identified in our study possibly shared chromosomal intervals with *QPm.inra-3B* [54], *QPm.osu-3B* [50], *QPm.caas-3BS* [55] and *CP2* [29].

On chromosome 4A, an MTA was detected at 48.0 cM during 2021-22. This MTA seems to share same chromosome region as previously identified QTLs *QPm.inra-4A* [56] and *Qpm.mgb-4AL* [20] and a meta-QTL *MQTL11* [29].

The presence of two MTAs at 68.0 cM on chromosome 5B (detected during 2021-22) seems associated with previously reported loci *QPm.nuls-5B* and *QPm.umb-5BS* [27], and a major gene *Pm16* [57], highlights its potential for marker-assisted selection in breeding programs targeting PM resistance.

Among the chromosomes examined, chromosome 6B stood out with the largest number of MTAs, totaling 57, one at 0.0 cM while remaining within the 59.0 to 65.0 cM region. PM resistance MTA at 0.0 cM seems to be associated with *QPm.umb-6BS* [27]. However no previously identified loci were found in second region, suggesting the presence of novel genes or regulatory elements associated with PM resistance in this region.

On chromosome 7A, six MTAs were identified at different positions (at 57.0, 64.0, 65.0, 73.0, 74.0, and 150.0 cM) during 2020-21 and 2022-23. It seems that MTAs at 57.0, 64.0, 65.0, 73.0, and 74.0 represent a previously repetitively detected locus associated with QTLs *QPm.caas-7AS* at 26.0 to 28.0 cM [58] and meta-QTL *MQTL21* at 22.6 to 53.2 cM [29], while MTA at 150.0 seems associated with *Pm1*, the SNP marker RAC875_c37085_317 (at 152.8 cM) linked with *QPm.icg.7A* cM [17], and QTL *Qpm.mgb-7AS* at 111 cM or *Qpm.mgb-7AL* at 191.4 cM [20]. The second region, which appears to be associate with *Pm1*, present a valuable target for marker-assisted selection.

Chromosome 7B exhibited four MTAs, with one MTA detected at 73.0 cM during 2022-23 and the other three detected at 136.0 cM during 2021-22. Several major QTLs such as *Qpm.mgb-7BS1*, *Qpm.mgb-7BS.2*, *Qpm.mgb-7BL* [20], and major PM resistance genes such as *PmE* [59], *Pm47* [60] and *Pm40* [61] have been reported on in vicinity of the MTA detected at 73.0 cM on chromosome 7B. It seems markers detected at 136.0 cM represent a novel PM resistance locus since no resistance loci has been observed in this region previously.

In addition to the above-discussed MTAs, a number of other novel MTAs were detected on chromosomes 3A (at 158.0 cM, detected during 2022-23), 4B (at 66.0 cM, detected during 2022-23), and 5A (at 144.0 cM, detected during 2021-22). No QTLs/MTAs or genes were previously reported by other studies in their vicinity. These findings emphasize the importance of further investigations focused on these and other novel MTA regions such as those on chromosome 6B (between 59.0 to 65.0 cM, detected during 2020-21 and 2021-22) for a deeper understanding of the genetic mechanisms underlying PM resistance.

The co-localization of MTAs with previously identified genetic markers, MTAs, QTLs and genes provides further evidence for their involvement in the resistance mechanism against PM. Identified novel MTAs could also be valuable for diversifying the sources of genetic resistance in breeding

programs and provide valuable targets to enhance PM resistance by combining with repetitively detected loci using the associated SNP markers identified in this study. Moreover, these findings provide valuable information for breeders to prioritize genomic regions and genetic markers associated with PM resistance and contribute to a better understanding of the genetic basis of PM resistance in common wheat.

3.2. Candidate Genes for PM Resistance

To identify potential target genes for breeding, GWAS is often used as a starting point [62]. However, a challenge arises when significant markers associated with the trait of interest encompass a wide range of genes within their confidence intervals, making it difficult to pinpoint the exact causal genes [36]. Thus, in this study, we addressed this issue by focusing on MTAs found associated with genomic intervals carrying disease resistance associated candidate genes. Identified CGs (30 out of the 96 detected) broadly represent twelve plant protein families that exhibited clear connections to disease resistance and host-pathogen interactions. These proteins have been previously reported to impart disease resistance to various pathogens such as *Blumeria graminis* f. sp. *Tritici*, *Fusarium graminearum*, *Puccinia striiformis*, *Bipolaris sorokiniana*, *Puccinia triticina*, *Parastagonospora nodorum* and *Pyrenophora tritici-repentis* in plants [63–72].

It may be recalled that the 30 CGs encode proteins with distinctive domains, such as the ABC transporter-like domain, ATP-binding domain, ankyrin repeat, aspartic peptidase A1 family, peroxidase, cytochrome P450, disease resistance protein (NB-LRR), Glutathione S-transferase, NB-ARC P-loop containing nucleoside triphosphate hydrolase, kinase-like domain superfamily, wall-associated receptor kinase, galacturonan-binding domain, zinc finger proteins, and F-box superfamily domain (Table S2), which are widely recognized as essential components of disease resistance mechanisms. These results reinforce findings from previous research. For instance, Peng and Yang's [73] comprehensive analysis delved into ABC, NLR, and START genes in hexaploid wheat, revealing their co-localization with disease resistance quantitative trait loci (QTLs) associated with adult leaf rust resistance. Molecular characterization of the leaf rust-resistance gene *Lr34*, which encodes an ABC transporter with transmembrane (TM) and nucleotide binding site (NB) domains, was elucidated in studies by Krattinger et al. [74,75]. Similarly, *Lr14a*, a race-specific leaf rust resistance gene, was found to encode an ANKTM protein [76]. The YrU1 protein, conferring wheat stripe rust resistance, also featured an integrated ANK domain from ANKTM proteins, suggesting their role in resistance [76,77]. The up-regulation of A1 aspartic peptidase and G1 families during *Zymoseptoria tritici* infection [65] underscores their defensive roles. Similarly, peroxidases' positive contribution against *Puccinia striiformis* infection [78] highlights their significance in immunity. Disease sensitivity genes like *Tsn1* which encode a protein with S/TPK and NBS-LRR domains [64,67] reveal their significance in recognizing necrotrophic effectors. The critical role of the NB-ARC domain in regulating R protein activity [63] deepens our understanding of pathogen recognition. Wall-associated receptor kinases (WAKs), exemplified by *Stb6* [66] and *TaWAK6* [69], emerge as potent defenders against STB and leaf rust. During defense, identified proteins exhibit versatile functions [68,72], encompassing stress resilience, apoptosis, transcription, and interactions. The F-box family protein, known for its role in numerous biological processes, including biotic stress resistance [79], highlights the multifaceted engagement of these proteins in wheat's resistance responses.

Despite identification of candidate genes and their association with disease resistance, no candidate gene were found in genomic intervals of 48 MTAs which may potentially carry other novel structural variants responsible for regulating plant defense against *Bgt* and wheat-*Bgt* interaction; however, further investigations are required to determine the significance and potential functional relevance of these specific SNPs in *Bgt* resistance.

In conclusion, our findings unveiled the intricate genetic landscape of PM resistance within the WAMI panel. This collection showcases a valuable pool of significant PM resistance MTAs/genes embedded in elite genetic backgrounds. Finally, through a comparison with previous studies on disease resistance in wheat, we have validated the functions of key MTAs.

4. Materials and Methods

4.1. Plant Material

WAMI panel consisting of 286 genetically diverse and elite advanced wheat lines was utilized for GWAS of PM resistance. The panel was assembled and distributed through the International Wheat Improvement Network (IWIN) the by International Maize and Wheat Improvement Center (CIMMYT) and possesses a narrow range of variation for days to heading and plant height which is appropriate for gene discovery without the confounding effects of phenology and plant height [47]. The seed of WAMI panel was obtained from CIMMYT. WL711, a highly PM susceptible common wheat cultivar [80], was used as a susceptible check and disease spreader.

4.2. Experimental Design and Trait Evaluation

The field evaluations of genotypes were conducted at the research farm of Eternal University, Baru Sahib, Himachal Pradesh, India, spanning three consecutive growing seasons (2020-21, 2021-2022, and 2022-23). The Baru Sahib PM disease nursery research farm is situated in a north Indian hilly state, Himachal Pradesh, and is known for climatic conditions favorable for the natural infection, growth, and the development of PM [82].

The experiments adhered to a randomized block design and were replicated twice. Random assignment of lines to each replication was carried out using the Fisher and Yates Random Table method [81]. Fertilizers included 120 kg N, 60 kg P₂O₅, and 40 kg K₂O. Except N, fertilizers were thoroughly applied at the time of sowing. The application of nitrogen was divided into three doses: half at sowing, one-fourth at the first irrigation (21 days after sowing), and the remaining one-fourth at the second irrigation (45 days after sowing).

The genotypes under investigation were exposed to natural infections of PM. To enhance disease stress, two rows of WL711, a highly PM susceptible cultivar, were planted around the field, with an additional row seeded between the plots one month before sowing the WAMI panel in November each year. The WL711 was also sown alongside the experimental materials during main season sowing. Planting distances were maintained at 20 cm between rows and 5 cm between plants.

Disease severity was scored using a rating scale of 1 to 9 developed by Bennett and Westcott [83] on randomly chosen five plants from each plot. Once maximum expression of PM (when reached a score of 9) observed on susceptible check cultivar WL711, whole panel was rated following the established scoring criteria [83]. This approach enabled the tracking of disease progression over time and the identification of an appropriate growth stage exhibiting varying degrees of resistance or susceptibility to PM.

4.3. DNA Extraction and Genotyping

DNA extraction, genotyping of samples and data processing was performed as described previously [84]. Briefly, DNA was extracted from fresh leaves of each line using the CTAB procedure, as outlined by Saghai-maroo et al. [85]. Subsequently, genotyping was conducted at the USDA-ARS Small Grain Genotyping Center, Fargo (<http://wheat.pw.usda.gov/GenotypingLabs>), utilizing the Illumina iSelect 90K beadchip assay [43]. The SNP calling process utilized the default clustering algorithm integrated into Genome Studio v2011.1, resulting in the identification of a total of 26,814 bi-allelic SNPs [84,86]. To uphold data quality standards, SNPs characterized by a minor allele frequency (MAF) lower than 0.05 were omitted from the analysis, alongside monomorphic and low-quality SNPs. This meticulous filtration procedure led to the retention of approximately 21,132 polymorphic SNPs [84,87], which were subsequently harnessed for the purpose of conducting the GWAS in this study.

4.4. Descriptive Statistics Analyses

ANOVA and correlation coefficients analyses were performed using the Agricolae (version 1.2–4) package of the R (version 4.0.3) software [88]. The BLUP values were obtained using the 'lme4'

package [89] in R [88]. Descriptive statistics, such as mean, standard deviation, and coefficient of variation (CV), were calculated using SPSS v. 17.0 (SPSS Inc 2008).

4.5. Population Structure, Kinship Matrix and Principal Components Analyses

Population structure (population structure matrix or Q matrix) and relatedness matrix (Kinship or K matrix) were modeled using principal components analysis (PCA) utilizing the genotypic data belonging to a total of 21,132 high-quality SNPs as described earlier [90]. The optimal numbers of PCAs were determined using the Bayesian information criterion (BIC) [91]. The analysis was performed with R (version 4.0.3) software [84,88] employing the parameters suggested by VanRaden [92] and Yin et al. [93]. The first two principal components were used to create a scatter plot that visualized the distribution of genotypes into sub-groups.

4.6. Linkage Disequilibrium and Genome-Wide Association Analyses

Linkage disequilibrium (LD) and genome-wide association analyses were conducted using a dataset of 21,132 high-quality single nucleotide polymorphisms (SNPs) available from the CIMMYT, Mexico website [84]. Pairwise squared allele-frequency correlations (r^2) between SNP markers were calculated using the TASSEL (Trait Analysis by Association, Evolution, and Linkage) software with a sliding window size of 100. To assess LD between loci, r^2 values were plotted against physical distance in centimorgans (cM). The LD decay curve was fitted using a smoothing spline regression line at the genome level, following the method outlined by Hill and Weir [45] and implemented in the R environment with a script previously employed by Marroni et al. [94].

Single- and multilocus models including the general linear model (GLM) [90], mixed linear model (MLM) [95], and the fixed and random model circulating probability unification (FarmCPU) [96] method were fitted to identify marker-trait associations (MTAs). Among these, FarmCPU was selected based on the best fit and used for the final GWAS. In FarmCPU, both fixed and random effects are incorporated to improve the accuracy of association mapping. It is a two-step procedure where the fixed effect model is initially fitted to control for population structure, and then the random effect model is used to further account for relatedness among individuals. The rMVP (R based Memory-efficient, Visualization-enhanced, and Parallel-accelerated Tool For Genome-Wide Association Study) software package [93] was employed for the analysis.

A significance threshold of $P < 0.001$ ($-\log_{10}(P) > 3.0$) was applied to identify statistically significant MTAs as done previously [12,46,97,98]. The position of significant SNPs on chromosome is indicated in cM according to consensus maps of common wheat [43].

4.7. Identification of Candidate Genes

To identify potential candidate genes (CGs), we focused on the most significant MTAs. The marker sequences associated with these MTAs were aligned with the wheat genome assembly IWGSC v.1 downloaded from the Ensembl database. Specifically, we analyzed a 200 kb window around each MTA marker to extract highly significant and annotated CGs. For the gene ontology (GO) annotation of these CGs, we utilized IWGSC (<http://www.wheatgenome.org>), which offered the requisite information for annotating the GO terms associated with the identified candidate genes.

5. Conclusions

Incorporating the insights gleaned from the three-year investigation, a focused set of significant MTAs emerged, each holding the potential to drive advancements in resistance to PM disease in wheat. Notably, recurrent novel MTAs, consistently observed on chromosome 6B across 2020-21 and 2021-22 (Table 3), underscore the enduring influence of key genomic regions on PM resistance. By harnessing the knowledge embedded within these MTAs, breeders can refine their selection processes, develop tailored markers, and ultimately expedite the development of superior PM-resistant cultivars through precision-guided approaches. This study not only sheds light on the genetic architecture underpinning PM resistance but also paves the way for innovative and

accelerated wheat breeding programs that align with the demands of sustainable and resilient agricultural practices. Moreover, we identified potential candidate genes involved in disease resistance mechanisms in wheat by analyzing significant SNPs and their associated gene sequences.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary File 1.xlsx. Table S1: Details of candidate genes identified in genomic intervals of marker trait associations detected during experimental years 2020-21, 2021-22 and 2022-23.; Table S2: Details of defense and host-pathogen associated proteins encoded by candidate genes identified in genomic intervals of marker trait associations detected during this study.

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Data Availability Statement: The single nucleotide polymorphism (SNP) genotyping data of the Wheat Association Mapping Initiative (WAMI) germplasm panel of spring wheat, which is used for the present study, has been published previously by Sukumaran et al. [84], and is available publicly to download from the link: <http://hdl.handle.net/11529/10714>. All other data generated or analyzed during this study are included in this published article.

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