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Unlocking the potential of spring wheat genetic resources: Uncovering unused resistance sources against leaf rust and yellow rust resistance

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Research Article

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Abstract

The causal agents of yellow and leaf rust in wheat, *Puccinia striiformis f. sp. tritici* and *Puccinia triticina*, pose a serious threat to grain yield and quality worldwide. Growing durable resistant wheat cultivars is an effective protection measure contributing to sustainable agriculture. Many of the known resistance genes, however, have been overcome due to the high genetic diversity and adaptability of pathogen populations. Therefore, the present study aimed to identify novel loci associated with yellow rust and leaf rust in a genome-wide association study (GWAS) using 1,984 spring wheat accessions from the German Federal ex situ Genebank. Phenotypic data obtained from a detached leaf assay were combined with 90,283 genotyping-by-sequencing genome wide markers. Six significant peak marker-trait associations (MTA) were identified for yellow rust and leaf rust, respectively. These are located on chromosomes 1D, 2B, 3B, 4A, 4B, 4D, 5A, 6B, and 7D. Six candidate genes were identified in close proximity to the identified loci. These findings may be valuable for identifying and deploying genetic resources to broaden the genetic basis of resistance and safeguard durability of resistance against yellow and leaf rust.

Introduction

Wheat (*Triticum aestivum* L.), is one of the world´s most important staple crops and provides food for approximately 35% of the global population. However, its yield and quality are severely threatened by fungal diseases such as leaf rust (LR), caused by *Puccinia triticina* and yellow rust (YR), caused by *P. striiformis* (Rampitsch et al. 2019). Both diseases can lead to substantial yield losses and negatively impact grain quality.

Due to the significant impact of rust diseases, breeding programs are focused on developing wheat cultivars with enhanced long-lasting resistance. Developing and cultivating disease-resistant wheat varieties is considered an effective, economical, and environmentally sustainable way to mitigate these losses. Resistance to fungal pathogens is generally classified as either race-specific (qualitative) resistance or non-race-specific (quantitative) resistance. To date, approximately 90 resistance genes/loci (Supplementary Table S4) have been identified for LR, and 83 (Supplementary Table S5) for YR (McIntosh et al. 1995; McIntosh et al. 2005; McIntosh et al. 2009; McIntosh et al. 2013; McIntosh et al. 2015; McIntosh et al. 2017; Baranwal 2022).

Most known resistance genes are race-specific, typically governed by single major genes, and confer effective protection during the seedling stage. This form of resistance is usually durable throughout the plant's life and is often associated with a hypersensitive response (Bolton et al. 2008). While such resistance has proven valuable in breeding programs for European wheat varieties (Park et al. 2001; Pathan and Park 2006; Serfling et al. 2011), its durability is limited. Due to the high genetic diversity and evolutionary adaptability of *Puccinia* species (Dyck and Kerber 1985), new pathogen races can frequently overcome these resistance genes, rendering them ineffective over time. In contrast, non-race-specific

resistance genes, often referred to as adult plant resistance, are generally controlled by multiple genes, providing broader and more durable protection.

Rust pathogens undergo rapid evolutionary changes, driven by high mutation/recombination rates, resulting in the recurrent emergence of races that can overcome deployed resistance genes. Pyramiding multiple resistance genes is reported as an effective strategy to enhance durable resistance against rust diseases, as combination of major resistance genes can reduce disease levels compared to single genes (Mundt 2018). Therefore, it is crucial to identify and combine resistance genes or loci, including those genes or loci that have not previously been used in breeding programs. To achieve the longest possible effectiveness of resistance, it is therefore essential to strategically incorporate previously unexploited resistance loci, thereby safeguarding and extending the durability of resistance. A key resource for achieving this goal is the genetic diversity preserved in genebanks. These collections contain a wide variety of plant genetic resources, including alleles and traits that have been lost or neglected in elite breeding lines. Many of these traits confer resistance to biotic stresses, such as rust and other significant diseases (Dinglasan et al. 2022).

Accurate phenotyping and genotyping are essential for the efficient use of genetic resources in resistance breeding. Traditional phenotyping methods, however, are expensive, labor-intensive, and often destructive, which limits the significance and accuracy of the results. Automated high-throughput phenotyping platforms, such as the Macrobot system (Lück et al., 2020a), have been developed to address these limitations. These platforms enable the rapid, standardized, and non-destructive analysis of disease resistance in large plant populations (Gill et al. 2022). Combining detached leaf assays with robotics further enhances throughput and reliability (Beukert et al. 2021).

Technological progress in high-throughput genotyping complements advances in phenotyping by providing comprehensive insights into the plant genome and the genetic basis of complex agronomic traits. Molecular markers assisted techniques have significantly accelerated resistance breeding. Examples of these strategies include marker-assisted recurrent selection (MARS; Johnson 2004; Eathington et al. 2007), marker-assisted gene pyramiding (Ragagnin et al. 2009; Costa et al. 2010), marker-assisted backcrossing (MABC; Hospital et al. 1992; Frisch et al. 1999; Septiningsih et al. 2009), and genomic selection (GS; Meuwissen et al. 2001; Bernardo 2010; Poland et al. 2012). However, modern plant breeding relies on high-throughput phenotyping and genotyping and the combination of these techniques provides comprehensive characterization of ex-situ genebank resources (Volk et al. 2021), which allows identification of useful accessions in large and diverse ex situ collections which could be useful to develop cultivars with enhanced pathogen defense.

The development of high-throughput genotyping platforms including genotyping-by-sequencing (GBS) has made it possible to identify quantitative trait loci (QTL) and allelic variations underlying important traits through genome-wide association studies (GWAS) in various crops. Numerous studies in wheat have used GWAS to identify marker-trait associations (MTAs) conferring resistance to LR (El Messoadi et al. 2022; Kumar et al. 2020; Li et al. 2020; Ahmed et al. 2021; Leonova et al. 2021; El Messoadi et al.

2022; Vikas et al. 2022; Lhamo et al. 2023), and YR (Elbasyoni et al. 2017; Long et al. 2019; Yao et al. 2021; Vikram et al. 2021; Zhang et al. 2021; Qiao et al. 2024; Sharma et al. 2025). Although several studies have examined LR and YR resistance, they typically evaluated relatively small genotype sets. In contrast, our study assessed a large and diverse collection of genebank accession genotypes, enabling us to more comprehensively characterize genetic variation for disease resistance, increase the discovery of novel alleles, and enhance the power and resolution of GWAS—ultimately providing greater clarity and robustness for MTAs.

The objectives of this study were (i) to evaluate the phenotypic variability for seedling resistance to *Puccinia striiformis and Puccinia triticina* in a large spring wheat genebank collection using a semi-automated, high-throughput phenotyping platform; (ii) to perform GWAS in order to identify genomic regions associated with resistance to these pathogens, (iii) to compare the detected associations with previously reported resistance loci; and (iv) to prioritize candidate genes within the identified QTL regions based on gene annotation, GO terms, transcriptome data, and sequence similarity to published resistance loci.

Material and Method

Plant material:

The analyzed spring wheat collection comprised 1,984 genetic resources (PGRs), which were provided as seed samples by the German *ex situ* genebank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany) and propagated according to the protocols outlined by Schulthess et al. (2021) and Hinterberger et al. (2022). A subset of 1,984 genotypes with complete LR and YR phenotypic and genotypic data was considered for GWAS and further analysis.

The genotypes originate from 65 countries (http://gbis.ipk-gatersleben.de/) including 215 genotypes from Africa, 207 from America (67, 68 and 72 from central, North and South America, respectively), 1,040 from Asia, 432 from Europe, 28 from Australia and New Zealand and 62 of unknown origin. This set represents a wide diversity in years of acquisition and growth habits (see Supplementary Table S1).

High-throughput phenotyping of seedlings with assays for detached leaves

Independent greenhouse experiments were conducted to characterize the seedlings using the semi-automated, high-throughput phenotyping platform Macrobot (Lück et al., 2020a, b). Sowing, inoculation, and incubation were conducted according to the protocol described by Pfrieme et al. (2025). Up to seven leaf segments per genotype were taken as replicates after a ten-day germination and growth period (EC12). The leaf segments were inoculated with the aggressive leaf rust isolate 77WxR (Naz et al. 2008) and the aggressive yellow rust isolate WxYr27 (Rollar et al. 2021). The highly susceptible variety Borenos (registration year 1987, Strube Research, Söllingen, Germany) was used as a susceptible control line for

LR trials, Akteur (registration year 2003, Deutsche Saatveredelung AG, Lippstadt, Germany) for YR trials. Automated and multimodal image analysis of the infected segments was performed using the Macrobot platform after an 8-day incubation for LR and a 15-day incubation for YR. The degree of infection was evaluated using the BlueVision software by quantifying the percentage of the infested leaf area as described by Lueck Lück et al. (2020a, b).

Quality Assessment of Phenotypic Data and Estimation of Degree Statistics

A manual quality check was not feasible due to the large volume of data. Therefore, we implemented an automated, standardized data quality control pipeline as described by Hinterberger et al. (2022). All data processing and analysis were performed in the R statistical environment R (version 4.3.3; R Core Team, 2024) following the methodology outlined by Pfrieme et al. (2025). According to Henderson (1975), a linear mixed model (Eq. 1) was fitted, and a nominal significance level of 0.05 was used to identify outliers based on model residuals.

$y_{ijk} = \mu + g_i + e_j + t_k\ (e_j) + \epsilon_{ijk}$ (1) (1)

In this model, y represents the percentage of infested leaf area, and μ denotes the global mean. g_i is the fixed effect of the *i*th genotype, e_j is the fixed effect of the *j*th experiment and $t_k\ (e_j)$ is the fixed effect of the *k*th tray, nested in the *j*th experiment and ϵ denotes the residual.

Residuals from this model were extracted for outlier detection. Data points with residuals exceeding the nominal significance threshold were classified as outliers and excluded from subsequent analyses. To estimate relationship between YR and LR, Pearson correlation coefficients were calculated between best linear unbiased estimates (BLUEs) after inoculation with LR and YR using R function cor(). To estimate the variance components of the phenotypic traits, model (1) was employed, specifying the overall mean μ as a fixed effect, while including all remaining terms as random effects. Genotype-specific BLUEs were derived using the same model, with genotype as a fixed effect (Nyquist and Baker, 1991). All estimations of variance components and BLUEs were performed in the linear mixed model (Eq. 1) using ASReml-R software, version 4 (Butler et al. 2009).

Broad-sense heritability (H^2) was calculated using the method described by Falconer and Mackay (1996), following equation:

$H^2 = \sigma^2_g / (\sigma^2_g + (\sigma^2_e/R))$ (2)

where σ^2_g represents the genetic variance among genotypes derived from Eq. 1, σ^2_e represents the residual variance, and R represents the average number of replicates per genotype.

Genotyping:

Genotyping-By-Sequencing (GBS) was conducted as described previously (Zhang et al. 2024) using a two-enzyme (PstI and MspI) approach (Poland et al. 2012) and genomic DNA isolated from seedling tissue. In typical experiments, 288 individually barcoded samples were pooled and sequenced (Illumina NovaSeq 6000 device,122 cycles single read, one lane S1 flowcell, XP-workflow) at IPK-Gatersleben, yielding an average of 2.5 million reads per genotype. Prior to downstream analysis, adapter sequences and low-quality bases were removed from the raw sequence data prior to downstream analysis (Schulthess et al. 2021). Raw sequence processing was conducted as described previously for GBS read mapping and SNP calling (Schulthess et al. 2022) with the distinction that reads were aligned to the wheat genome assembly of Chinese Spring V2.1 (Zhu et al. 2021).

Filtering of genome wide marker data

The marker set was mapped to the Chinese Spring RefSeq V2.1 reference genome using physical positions. Markers that were not assigned to specific chromosomes (n = 2,218) were removed. The remaining markers filtered to exclude those with ≥ 30% missing value. SNP imputation was performed using the Beagle software package (version 4.1; Browning, and Browning 2007, 2007). Subsequently, markers with a minor allele frequency (MAF) of ≤ 1% and heterozygosity ≥ 10% were removed from the imputed marker set. After these filtering steps, 90,283 GBS markers remained and were used to estimate population structure and kinship.

Population structure was analyzed using informative markers by Bayesian clustering within the STRUCTURE software (version 2.3.4 software; Pritchard et al. 2000). Ten independent runs were performed for each number of clusters (K) from one to ten. Each run consisted of 50,000 burn-in steps followed by 50,000 Markov Chain Monte Carlo (MCMC) iterations. The optimal number of subpopulations was determined using the Evanno ΔK method, (based on the rate of change in the log probability of the data between successive K values), as implemented in the STRUCTURE HARVESTER (version 2.3.4, Pritchard et al. 2000). Additionally, a principal coordinate analysis (PCoA) was performed using DARwin 6 software (Perrier, X. & Jacquemoud-Collet, J. P. 2006, IRAD, Montpellier, France).

Genome wide Association Study (GWAS)

Phenotypic and genotypic data were used to perform GWAS and to identify marker trait associations (MTAs) by using four different tools: TASSEL (Trait Analysis by Association, Evolution and Linkage, Bradbury et al. 2007), GAPIT (Genome Association and Prediction Integrated Tool, Lipka et al. 2012), FARMCPU (Fixed and Random Model Circulating Probability Unification, Liu et al. 2016) and GenABEL (Aulchenko et al. 2007) in R.

In both TASSEL and GAPIT, a compressed mixed linear model (CMLM) was used that incorporated a kinship (K) matrix and a population structure (Q) matrix as a correction factor for relatedness and population structure. FARMCPU used a fixed and random model circulating probability unification algorithm in R, and GenABEL used a mixed linear model containing both the kinship matrix (K) and the

population structure matrix (Q) was used to identify significant MTAs. To illustrate the number of identified markers and also common markers between four different GWAS methods, a Venn diagram was created using R.

Initially, the Bonferroni–Holm-adjusted significance threshold of $-\log_{10}$ (p-value) ≥ 6.3 at α = 0.05 was applied to identity significant MTAs. However, this stringent cutoff yielded only one significant marker for LR. To reduce the probability of detection of false positive associations, we considered only marker trait associations which were detected by all four mapping methods for downstream analysis. Therefore, an additional, less stringent threshold (LOD ≥ 3) was considered to be significantly associated with *Puccinia triticina* and *Puccinia striiformis*. The identified significant markers were assigned to the QTL region based on their physical chromosomal position which was estimated using linkage disequilibrium (LD) decay (2.6 million base pairs). The LD decay was calculated as the squared allelic correlation (r^2) between all pairs of markers within a chromosome using the "genetics" (Warnes et al. 2013) and "LDheatmap" (Shin et al. 2006) packages in R. The obtained r^2 values were plotted against the estimated genetic distance between markers in base pairs. LD was determined by the intersection of the fitted locally weighted polynomial regression (LOESS) curve and critical r^2 values. LD was calculated for each chromosome separately and across all chromosomes. Next, the identified markers were compared with those in previous studies to confirm and interpret the results of the present study. Finally, candidate genes were identified by screening all flanking sequences of associated disease markers within ± 2.6 Mb (corresponding to the LD decay) for published functional gene annotations of Chinese Spring (IWGSC RefSeq V2.1).

Results

Response of wheat seedling to P. striiformis f. sp. tritici and P.triticina:

A total of 1,984 spring wheat genotypes were phenotyped using detached leaf assays and the Macrobot system after being inoculated with yellow rust (YR) and leaf rust (LR) under greenhouse conditions. After quality control, for technical, experimental and biological replicates BLUEs were calculated and their distributions showed a unimodal frequency pattern, which is consistent with a quantitative, polygenic inheritance of resistance (Fig. 1A + B).

Genotypes were considered as more resistant relative to the susceptible control variety if their BLUE value was below zero relative to the respective control (Borenos for LR, 5.4%; Akteur for YR, 9.9%) which was set as reference (0). A total of 1,323 and 1,287 genotypes exhibited lower infection rates for LR and YR, compared to the respective control variety. Among these, 875 genotypes exhibited lower infection rates than the controls for both rust diseases. To examine the relationship between LR and YR resistance, the Pearson correlation coefficient was calculated for the BLUE values, resulting in a significant albeit weak, positive correlation ($r = 0.14$, $p < 0.001$).

Analysis of variance components revealed a high genotypic variance and a broad-sense heritability H^2= 0.54 for LR infection, indicating strong genetic determination of resistance. In contrast, high residual variance for YR infection led to a reduced heritability (H^2 = 0.38), suggesting a higher influence of environmental factors or lower phenotyping accuracy in this case (Fig. 2).

Genotyping, filtering and population structure

Genotyping 1,984 wheat genotypes resulted in the identification of 1,021,664 GBS markers. After removing markers with more than 30% missing data and those without assigned chromosomal positions, 432,995 markers remained for further analysis. These markers were then filtered based on minor allele frequency (MAF ≤ 1%) and heterozygosity (≥ 10%), resulting in a dataset of 90,283 markers All of these markers were subsequently used for GWAS analyses without further selection. Only markers showing significant associations in the GWAS were selected for downstream analyses and interpretation. The number of markers per chromosome ranged from 965 (chromosome 4D) to 7,495 (chromosome 7A) (Fig. 3). The software Plink, was used to identify 15,771 informative markers, which were then used to estimate population structure and kinship.

To estimate the population structure, a set of 15,771 informative markers was analyzed using the STRUCTURE software. Based on the ΔK method, the optimal number of three sub-populations (K = 3) was determined (Fig. 4A). Genotypes were then assigned to clusters according to their membership coefficients; individuals with a coefficient of at least 0.5 for a particular cluster were assigned to the corresponding cluster (Fig. 4B). Of the 1,984 genotypes, 1040 originated from Asia and these genotypes were distributed across all three identified clusters, which indicates representation of Asian germplasm in the population.

The first cluster (K1) comprised mainly Asian genotypes (379 genotypes, 97.7%), 67.3 of which were from Southern Asia. India contributed the largest number of genotypes to K1 (149 genotypes), followed by Eastern Asia, with 119 genotypes from China (31.4%) (Supplementary Fig. 1). Minor proportions originated from Africa (0.5%), America (0.5%), Australia (1%) and Europe (0.3%). The second subpopulation (K2) included 494 genotypes, 407 (82.4%) of which originated from Asia. Within this group, 75.6% were from Southern Asia, predominantly from Iran (Supplementary Fig. 1). Genotypes from Africa and Europe constituted 13.6% and 3.4% of the second cluster, respectively. The distribution of South Asian genotypes across both clusters (K1 and K2) might be due to variations in climatic conditions within the region. The third cluster (K3) contained 1,057 genotypes (53.3% of the total), which were more evenly distributed across the continents: Europe (38.6%), Asia (20.5%), America (19.3%), Africa (13.5%), and Australia (2.6%) (Supplementary Fig. 1).

Genotypes with a membership coefficient of less than 0.5 were classified as admixed (n = 45), the majority of which were from Asia (37 genotypes, or 82.2%). Genotypes for which geographical origin was unknown made up 0.6% of K2 and 5.8% of K3 (Supplementary Fig. 1). The PCoA showed that the first and second principal coordinates explained 4.6% and 2.9% of the genetic variation, respectively.

Genome -Wide Association Study (GWAS)

To identify marker-trait associations for LR and YR, different GWAS models were performed. The most suitable model was selected based on QQ plots (Supplementary Fig. 2) and calculation of mean squared difference (MSD, Supplementary Table S2), with model 1 and model 2 selected as suitable model for YR and LR, respectively.

The identified peak markers were assigned to QTL regions based on their physical chromosomal position, which was estimated using the LD decay. The LD varied between 946,330.7 bp (chromosome 6B) and 8,950,806 (chromosome 2D). A LD of 2,614,573 was calculated across all 21 chromosomes. The QTL region was adjusted to ± 2.6 million base pairs from each identified significant marker based on LD decay across all chromosomes. MTAs, with LOD ≥ 3 (− log10(p value)) considered significant and identified MTAs by all four methods, were considered reliable (Table 1).

For LR, 130, 69, 45 and 66 peak markers were identified using TASSEL, GAPIT, FARMCPU and GenABEL, respectively, across all wheat chromosomes (Fig. 5A and 6A as well as supplementary Table S3). GAPIT and GenABEL showed the highest number of common LR peak markers (41) across all wheat chromosomes, except for chromosomes 1D, 2B, 2D, 3D, 5D and 6D.

As with YR, FARMCPU had relatively few common markers with the other methods used. For example, 10 common peak markers were shared between FARMCPU and GenABEL, 9 were shared between FARMCPU and GAPIT, and 31 markers were shared between GAPIT and TASSEL. All four methods identified six markers for LR (Fig. 5A) on chromosomes 3B, 4B, 4D, 5A and 7D, with two markers on chromosomes 4D (Table 1).

For YR, 100, 92, 52 and 110 peak markers were identified using TASSEL, GAPIT, FARMCPU and GenABEL, respectively (Figs. 5B and 6B, as well as Supplementary Table S3). GenABEL detected the highest number of peak markers for YR, which were distributed across all wheat chromosomes except 4B. GenABEL and TASSEL shared 36 peak markers across nearly all chromosomes, except 1A, 3A, 4B, 5A and 7A. A total of 61 common peak markers were identified between GAPIT and GenABEL on 19 chromosomes. GenABEL and FARMCPU shared 15 common peak markers on chromosomes 1A, 1D, 2A, 2B, 3B, 4A, 4D, 5B, 6B, 7A and 7B. Meanwhile, TASSEL and GAPIT shared 37 markers distributed on chromosomes 1D, 2A, 2B, 2D, 3B, 3D,4A, 4D, 5B, 5D, 6B, 6D, 7A, 7B and 7D. Ten markers were identified between TASSEL and FARMCPU on seven wheat chromosomes (1D, 2B, 2D, 4A, 5B, 6B and 6D). Thirteen markers were identified as common markers between GAPIT and FARMCPU on chromosomes 1D, 2B, 2D, 4A, 4B, 5A, 6B, 6D, 7B.

In total, six peak markers were found to be common to all four methods used (Fig. 5B). These markers were located on six wheat chromosomes (1D, 2B, 4A, 5B and 6B), with chromosomes 6B comprising three identified markers for YR (Table 1).

These results indicate that although each GWAS method identifies unique associations, a subset of peak markers is consistently identified by different methods and therefore, provides reliable candidate loci for further analysis. Furthermore, chromosomes 6B (for YR) and 4D (for LR) have multiple stable peaks, indicating these regions as potential biological importance.

Table 1
Summary of significant MTAs for YR and LR using four GWAS methods (LOD (− log10(p value), minor allele frequency (MAF ≤ 1%) and effect of FARMCPU is presented in the Table). Markers identified for the first time for YR and LR are shown in bold format.

Trait	Marker	Chrom	Pos	P.value	LOD	MAF	Effect
YR	Chr1D_8677328	1D	8677328	8.34E-08	7.08	0.3	0.93
YR	Chr2B_660133069	2B	660133069	2.51E-04	3.6	0.02	2.05
YR	**Chr4A_110976029**	**4A**	**110976029**	**1.01E-08**	**8**	**0.32**	**0.98**
YR	**Chr6B_51649618**	**6B**	**51649618**	**9.68E-04**	**3.01**	**0.18**	**0.7**
YR	**Chr6B_54841657**	**6B**	**54841657**	**7.58E-04**	**3.12**	**0.18**	**0.72**
YR	**Chr6B_243663920**	**6B**	**243663920**	**5.41E-16**	**15.27**	**0.03**	**-3.74**
LR	**Chr3B_53685308**	**3B**	**53685308**	**1.74E-07**	**6.76**	**0.03**	**1.21**
LR	**Chr4B_554311160**	**4B**	**554311160**	**4.83E-05**	**4.32**	**0.02**	**-0.99**
LR	**Chr4D_15502361**	**4D**	**15502361**	**1.06E-15**	**14.98**	**0.01**	**-1.89**
LR	**Chr4D_396886837**	**4D**	**396886837**	**3.58E-05**	**4.45**	**0.01**	**-0.82**
LR	**Chr5A_459494322**	**5A**	**459494322**	**1.25E-04**	**3.9**	**0.07**	**-0.42**
LR	**Chr7D_611401245**	**7D**	**611401245**	**8.31E-26**	**25.08**	**0.01**	**3.24**

The significant peak markers identified in this study were compared with previously reported QTLs and genomic regions associated with resistance toYR and LR (Supplementary Tables S4 and S5, respectively). In the present study, GBS markers aligned to reference genome Chinese Spring v2.1, while previous studies relied on different genotyping platforms (array-based SNP platforms) and previous version of the reference genome. Therefore, direct comparison of identical SNP markers was not possible and comparisons were performed based on overlapping chromosomal regions or QTL intervals. Of the six markers identified in the present study for YR, two MATs (“Chr1D 8677328” and “Chr2B_660133069”) were located within genomic regions on chromosomes 1D and 2B that co-localize with previously reported YR resistance QTLs (Zhu et al. 2023; Vazquez et al. 2012; Huang et al. 2021; Cheng et al. 2022; Mahmood et al. 2022; Zhou et al. 2022 and Luo et al. 2005).

For LR, six MATs were located in regions of the genome that had not previously been reported to be associated with LR resistance and were described here for the first time. To investigate the possible functional relevance of the associated markers, the flanking sequences of all LR and YR associated

markers were mapped to the reference genome sequence to identify nearby high-confidence (HC) genes. For YR, a total of 5952, 5204, 3547 and 6820 HC genes (were located in the vicinity of significant markers detected by TASSEL, GAPIT, FARMCPU, and GenABEL, respectively (Supplementary Table S6). For LR, 6867, 3697, 2670, 3690 HC genes were identified for associated markers by TASSEL, GAPIT, FARMCPU and GenABEL, respectively (Supplementary Table S7). Six candidate HC genes were found for YR and seven for LR among the markers that were commonly identified by all four methods (Table 2). Some of these genes are annotated as being related to plant defense responses and may contribute to improved resistance against biotic and abiotic stresses.

Table 2
List of identified high confidence for identified common MTA for YR and LR. The bold highlighted markers, indicating novel identified MTAs for YR and LR in the present study.

Trait	Chr[a]	Position	Marker	gene ID	Description
YR	1D	8677328	Chr1D_8677328	TraesCS1D03G0036900	UDP-glycosyltransferase
YR	2B	660133069	Chr2B_660133069	TraesCS2B03G1160100	p-loop containing nucleoside triphosphate hydrolases superfamily protein, putative
YR	**4A**	**110976029**	**Chr4A_110976029**	**TraesCS4A03G0204200**	**Cysteine-rich receptor-kinase-like protein**
YR	**6B**	**51649618**	**Chr6B_51649618**	**TraesCS6B03G0166000**	**NBS-LRR-like resistance protein**
YR	**6B**	**54841657**	**Chr6B_54841657**	**TraesCS6B03G0172900**	**Ubiquitin thioesterase**
YR	**6B**	**243663920**	**Chr6B_243663920**	**TraesCS6B03G0510000**	**Serine/threonine-protein phosphatase 2A regulatory subunit B" subunit alpha**
LR	**3B**	**53685308**	**Chr3B_53685308**	**TraesCS3B03G0165900**	**Mitochondrial glycoprotein**
LR	**4B**	**554311160**	**Chr4B_554311160**	**TraesCS4B03G0732600**	**Heat shock protein**
LR	**4D**	**15502361**	**Chr4D_15502361**	**TraesCS4D03G0054700**	**Lysine–tRNA ligase**
LR	**4D**	**396886837**	**Chr4D_396886837**	**TraesCS4D03G0569000**	**CAAX amino terminal protease family protein**
LR	**5A**	**459494322**	**Chr5A_459494322**	**TraesCS5A03G0614500**	**Basic helix-loop-helix transcription factor**
LR	**7D**	**611401245**	**Chr7D_611401245**	**TraesCS7D03G1191900**	**receptor kinase 1**

[a]: chromosome

Identification of Reliable Resistance-Associated Loci

According to the resistance test of 1,984 genotypes, a set of 113 and 50 genotypes showed lower infection (BLUE values < 0) for YR and LR, respectively, compared to control genotypes. Among these

sets, six genotypes were observed carrying favorable alleles for both LR and YR, originating from three distinct geographic regions (Europe (3), Asia (2) and America (1)). Therefore, favorable resistance alleles exist in the germplasm of different continents which point out the potential of combining resistance from diverse genetic resources to increase rust resistance. To uncover the genetic factors underlying YR and LR resistance, BLUEs were linked with allele effects from GWAS. The favorable alleles (allele with negative effect value driven from GWAS output) which reduced the disease severity may be considered as promising genetic resources to improve the durability of rust resistance in breeding programs. incidence matrix according to allele-effect indicates six genotypes have multiple favorable alleles for both rusts (Fig. 7).

Discussion

In the present study, several novel MTAs for LR and YR were identified in a diverse panel of 1984 spring wheat genotypes. Of these, seven MTAs for YR and six MTAs for LR were not previously reported and could provide novel targets for wheat breeding programs. Candidate genes associated with these MTAs are involved in plant defense pathways and represent potential mechanisms of resistance to rust diseases. Notably, some genotypes carried a combination of several unknown QTLs for Lr and Yr, which may confer potential resistance and persistence to evolving pathogen populations. These findings indicate the effectiveness of combining high-throughput phenotyping with sequencing-based genotyping (GBS) and GWAS in elucidating the genetic architecture of disease resistance in wheat. Considering that breed-specific resistance genes can be rapidly overcome by new pathogenic strains, identified MTAs in the present study might be valuable resources for developing wheat cultivars with more stable and long-term resistance. Therefore, these results highlight novel genomic regions associated with rust resistance and provide practical strategies for using diverse germplasm to enhance durable resistance in wheat breeding programs.

Distinct Genetic Bases for Leaf Rust and Yellow Rust Resistance

The present study investigated a diverse set of 1,984 spring wheat genotypes regarding their resistance to LR and YR. The unimodal distribution of infection rates observed after inoculation with YR and LR reflects quantitative variation in resistance. A similar observation was reported in segregating populations with polygenic resistance (Kolmer, 2013; Poland et al. 2009) in a diverse genetic panel, which they suggest continuous variability in resistance and do not prove the underlying genetic architecture. The higher heritability of the resistance values recorded for LR ($H^2 = 0.54$) confirms the high precision of the system used and has already been observed in other studies (Pfrieme et al. 2025). In comparison, high residual variance for YR infection led to lower heritability ($H^2=0.38$), which in this case indicates a higher influence of environmental factors or lower phenotyping accuracy. This has already been observed in previous studies (Pfrieme et al. 2025). A significant, albeit weak, positive correlation ($r = 0.14$) was found between BLUEs for LR and YR, suggesting that resistance to these two diseases is

largely governed by different genetic factors. This is in accordance with previous studies, which have shown that cross-resistance between LR and YR can occur but is generally limited in wheat germplasm. While some colocalized or pleiotropic QTL conferring resistance to multiple rusts have been reported (Ponce-Molina et al. 2018; Ye et al. 2022; Tong et al. 2024), the low correlation observed here suggests that these loci are not predominant in this panel.

Methodological Advances and Model Performance in GWAS

We performed GWAS to investigate the genetic basis of resistance genes to YR and LR. Here, a single-locus model such as compressed Mixed Linear Model (CMLM)) and multilocus models (FARMCPU and GenABEL) were applied to identify markers associated with YR and LR resistance. Multilocus models can overcome limitations of single-locus models for complex traits which are controlled by multiple loci simultaneously and reduce false-positive associations. As reported by Kaler et al. (2020), multilocus models are suitable for detecting traits which are controlled by many small effect loci. All approaches provided suitable model fits based on QQ-plots and MSD values. However, GAPIT demonstrated slightly improved performance for both traits.

Comparison of markers with previous studies

In the present study, two out of 8 identified QTL regions for YR, namely QTL1_YR and QTL5_YR are overlapping with previously reported QTL regions (Supplementary Table 4). Zhu et al. (2023) reported QTL for YR on chromosome 1D located at 580.935 which is close to the physical position of our most significant marker “Chr1D_8677328”. Likewise, Mahmood et al. (2022) identified a QTL on chromosome 5B, located 1.4Mb apart from the identified marker “Chr5B_469983889”.

Similar to YR, two out of 8 identified QTL regions in the present study were reported in previous studies for leaf rust. The marker “Chr1A_11338370” on chromosome 1A is located in a distance of 1.3Mb and 1.9Mb of two reported QTLs by Rollar et al. (2021) and Azzimonti et al. (2014), respectively (Supplementary Table 5). In 2019, Zhang et al. reported a marker at the physical position 623512618.5 which is close to the identified marker on chromosome 5A, in the present study. The genetic distance between these two markers is 214,065 bp. The commonly identified QTL region between present and previous studies are confirming our GWAS results, and also refer to stable and durable resistance loci which can be useful for marker-assisted breeding.

Functional Insights into Candidate Genes Underlying Rust Resistance

Markers that were consistently detected by all four GWAS models were prioritized for candidate gene analysis. Candidate genes were selected based on their physical proximity to significant markers within the LD-defined QTL intervals and their functional annotation related to plant defense. In total, six high-confidence candidate genes associated with yellow rust (YR) and leaf rust (LR) resistance were

identified. As this study is based on association mapping, all functional interpretations are descriptive and do not imply causality.

The first gene of interest, potentially involved in YR resistance was associated with Cysteine-rich receptor-kinase-like protein (CRKs). CRKs belong to subfamily of receptor-like kinases which play important roles in pathogen recognition and the activation of defense signaling pathways. For instance, *TaCRK10* as a sensor of *Puccinia striiformis f. sp. Tritici* and resistance to yellow rust through regulating nuclear processes (Wang et al. 2021). In addition, CRKs have been reported to positively regulate resistance to *Puccinia triticina* through regulating the hypersensitive response and defense gene expression in wheat (Gu et al. 2020, Wang et al. 2021). Previous studies reported the distribution of CRKs on 18 wheat chromosomes, except chromosome 4A, 4B, or 4D (Liu et al. 2024). In contrast, the CRK-encoding gene identified on chromosome 4A in the present study. This discrepancy might be explained by annotation or genotype-specific genomic variation.

NBS-LRR proteins in plants, by participating in complex signaling networks, induce a wide range of defense responses, including oxidative burst, changes in ion and calcium flux, activation of mitogen-activated protein kinase (MAPK) cascades, induction of genes related to disease response, and generation of hypersensitive responses (McHale et al. 2006). In the present study, NBS_LRR was identified on chromosome 6B at physical position 51,661,310 bp, which was associated with YR resistance.

Basic helix-loop-helix (bHLH) transcription factors constitute a large family of plant transcriptional regulators characterized by a conserved basic DNA binding region and a helix-loop-helix domain that enables protein dimerization and specific recognition of DNA motifs such as the E-box (CANNTG) (Song et al. 2025). These transcription factors are involved in the control of numerous processes, including growth and development, hormone signaling, metabolism, and biotic stress responses. Plant responses to biotic stresses include regulation of defense-related gene expression, interference with hormonal signaling pathways including jasmonic acid and salicylic acid, and modulation of immune responses resulting from pathogen recognition. For instance, response to LR includes changes in expression of transcription factor families such as WRKY, NAC, bZIP and potentially bHLH (Wang et al. 2019). The bHLH was identified on chromosome 5A (at physical position 459,494,243 bp) which was associated to LR.

Receptor kinase 1 belongs to the family of receptor-like kinases (RLKs), which play a key role in pathogen recognition and activation of plant immune responses. RLKs function as pattern recognition receptors (PRRs) and recognize pathogen-associated molecular patterns (PAMPs). This recognition leads to the initiation of pattern-mediated immunity (PTI) and ultimately involves the production of reactive oxygen species (ROS), activation of MAPK pathways, and transcriptional reprogramming of defense genes (Boller and Felix 2009). In wheat, leaf rust resistance gene Lr10 encodes a receptor-like kinase (LRK10) is co-segregated with rust resistance, indicating the role of receptor-dependent signaling defense against *Puccinia triticina* (Feuillet et al. 2003). Therefore, receptor kinase 1 identified in the present study

might be contributed to leaf rust resistance through early pathogen recognition and activation of defense signaling pathways.

The QTL regions identified in this study span approximately 2.6 Mb, reflecting local linkage disequilibrium patterns. This resolution allows the identification of positional candidate genes based on their physical proximity to significant peak markers, but does not permit definitive assignment of causality to individual genes. Further fine-mapping, allelic diversity analyses, and functional validation studies will be required to confirm the role of these candidate genes in yellow rust and leaf rust resistance.

Limitations and Implications for Wheat Improvement

Genetic validation is essential to confirm the involvement of candidate genes in wheat's resistance to rust diseases before performing functional validation. Limitations of this study include the potential environmental effects on the phenotyping accuracy and the complex genetic backgrounds of the wheat panel, which may have influenced the detection of associations. Therefore, future research should focus on conducting additional genetic validations of these loci, followed by their functional characterization and integration them into marker-assisted selection to improve disease resistance in wheat.

Conclusion

In this study, the effectiveness of combining high-throughput phenotyping with genotyping data and employing multiple GWAS models to elucidate the genetic architecture of resistance to YR and LR in a diverse set of spring wheat was demonstrated. Analysis of the 1984 genetic resource using four complementary GWAS methods led to the identification of stable and reliable marker-trait associations for both diseases, including several novel and previously unreported loci. The simultaneous use of multiple GWAS approaches highlights the efficacy of this analytical framework for specifically investigating complex disease resistance traits, by reducing method-dependent bias and increasing confidence in the identified MTAs. Furthermore, the identification of genotypes with the accumulation of several unknown YR and LR resistance QTLs demonstrates the potential of this set of genotypes to achieve stable resistance. These results not only expand our understanding of the genetic basis of rust resistance in wheat, but also clearly demonstrate the practical utility of the high-throughput GWAS platform in identifying novel resistance loci and elite germplasm that can be used in wheat breeding programs.

Declarations

Conflicts of Interest:

The authors declare no conflict of interest. The authors declare that the experiments comply with the current laws of Germany. The funders had no role in the design of the study; in the collection, analyses,

or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Author Contribution

All authors contributed to the study conception and design. Albrecht Serfling, Jochen Christoph Reif, and Frank Ordon conceived and designed the experiments. Anne-Kathrin Pfrieme performed the experiments. Max Haupt and Jochen Christoph Reif provide the genotypic data. Behnaz Soleimani and Anne-Kathrin Pfrieme analysed the data and wrote the first draft of the manuscript. Behnaz Soleimani, Anne-Kathrin Pfrieme, Albrecht Serfling and Andreas Stahl edited the manuscript. Axel Himmelbach and Nils Stein generated GBS data and contributed to writing. All authors read and approved the final manuscript.We would also like to note that Behnaz Soleimani and Anne-Kathrin Pfrieme contributed equally to this work and should be considered co-first authors

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Data Availability

The phenotypic datasets generated for this study are available in the Supplementary Materials. Genotyping-by-sequencing (GBS) reads are available at the European Nucleotide Archive (ENA) under study PRJEB93924.”

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Figures

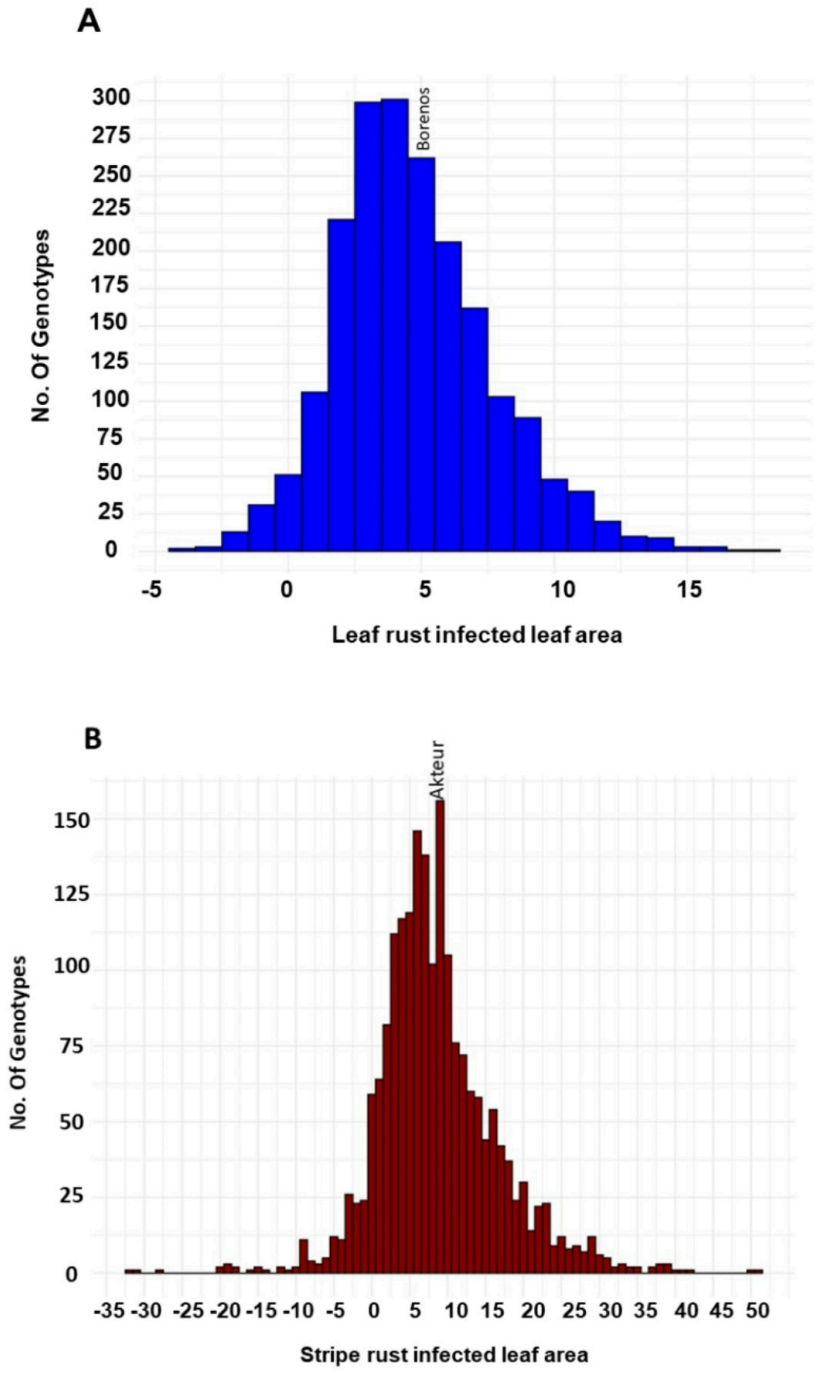


Figure 1

Distribution of the best linear unbiased estimates (BLUEs) of the percentage of infected leaf area of 1,984 spring wheat genetic resources (PGRs) after inoculation with leaf rust (LR, A) and stripe rust (YR, B).

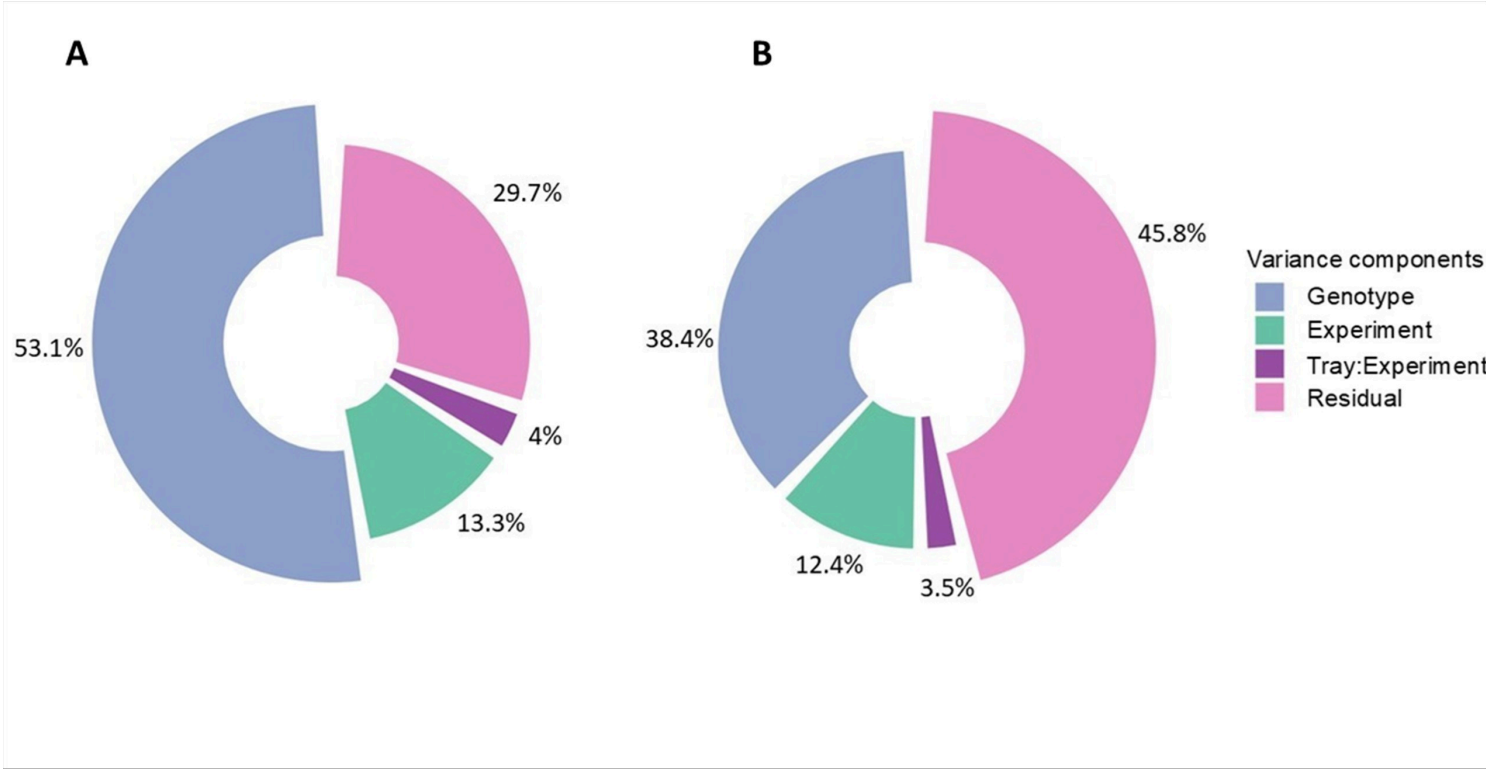


Figure 2

Variance component analysis of leaf rust (LR, A) and stripe rust (YR, B) infection. “Experiment” refers to the infection and screening batch, while “Tray” refers to all genotypes grown on a single tray in the greenhouse, nested in on infection and scanning batch. All components (genotype, experiment, tray) were significant at α = 0.01.

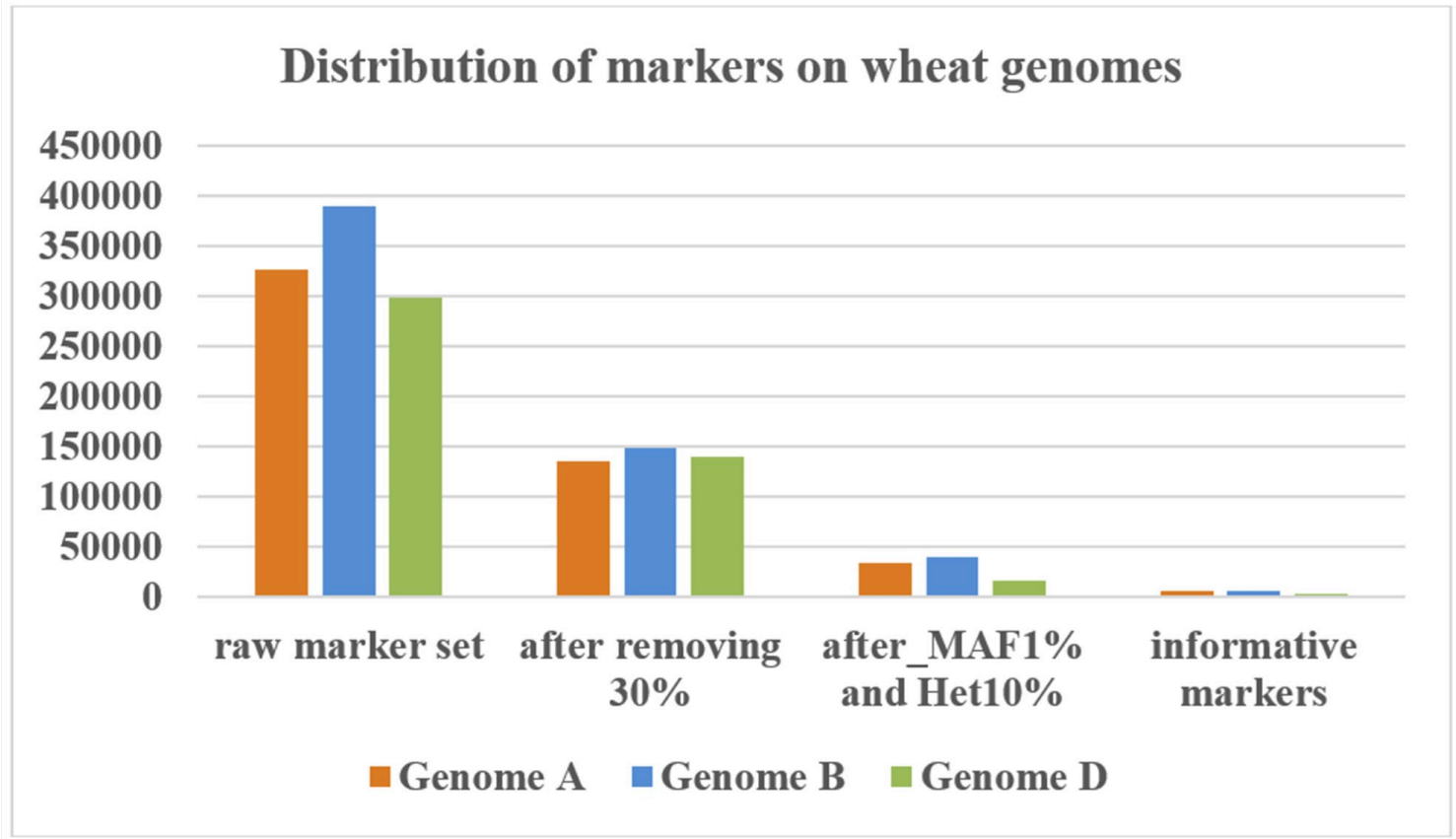


Figure 3

Distribution of markers across wheat chromosomes genomes (A, B and D) for each marker set: raw markers (without filtering), markers after excluding 30% missing value and unknown chromosome, markers after filtering 1% minor allele frequency and 10% heterozygosity, informative markers.

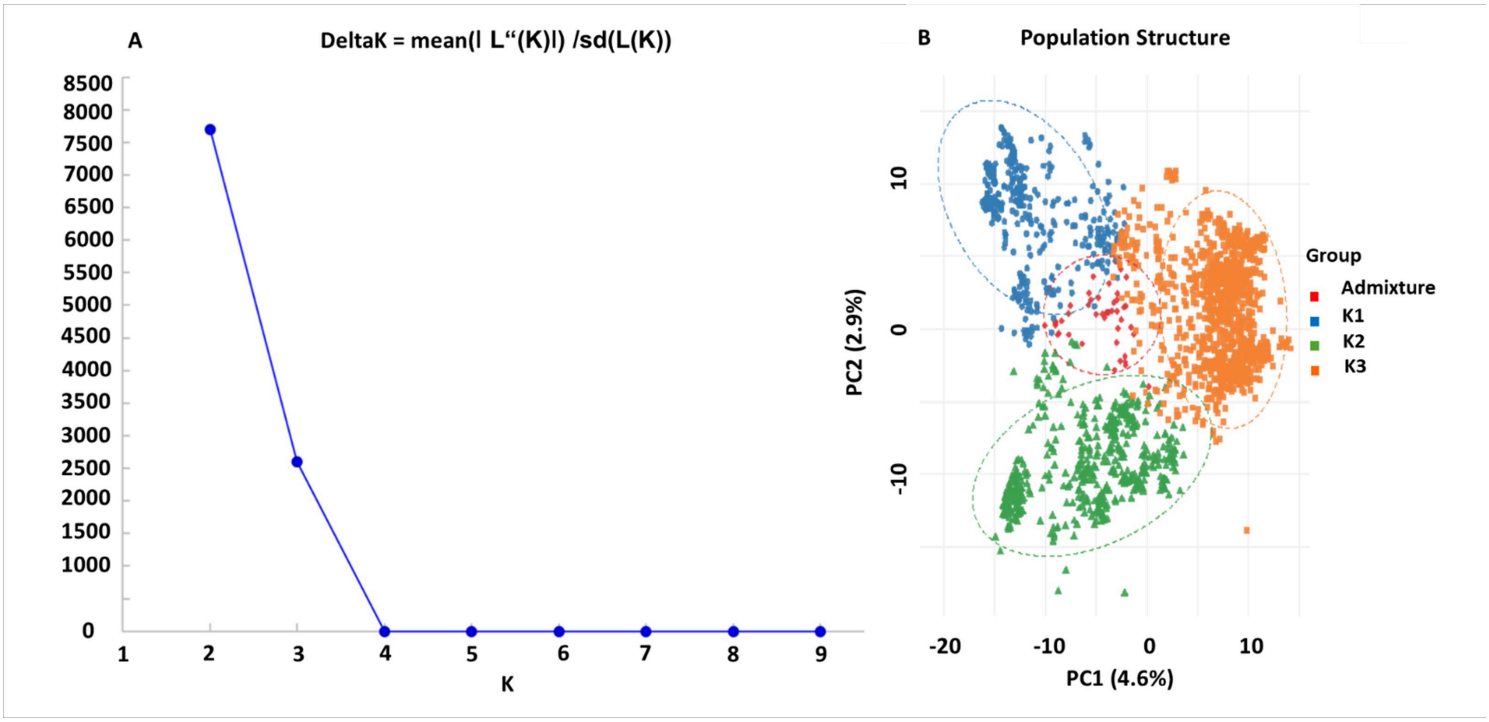


Figure 4

Population structure analysis of 1,984 spring wheat based on 90,283 GBS markers, A): Delta K plot obtained from Structure harvester. B): Principal Coordinates Analysis (PcoA) plot indicated three clusters (K1, K2 and K3) based on Structure result.

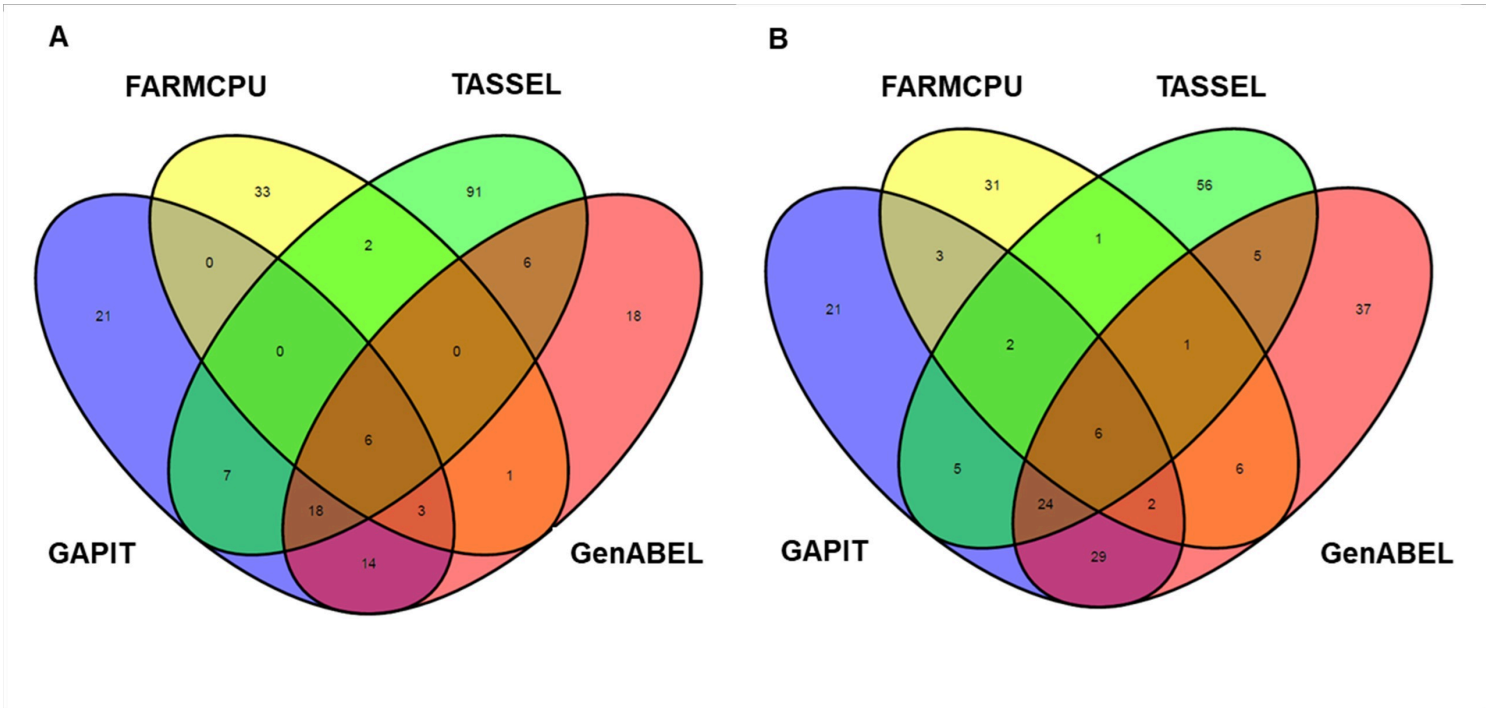


Figure 5

Venn diagrams indicating the overlapping of significant peak marker-trait associations (MTAs) for leaf rust (LR, A) and stripe rust (YR, B) using four different statistical methods for GWAS.

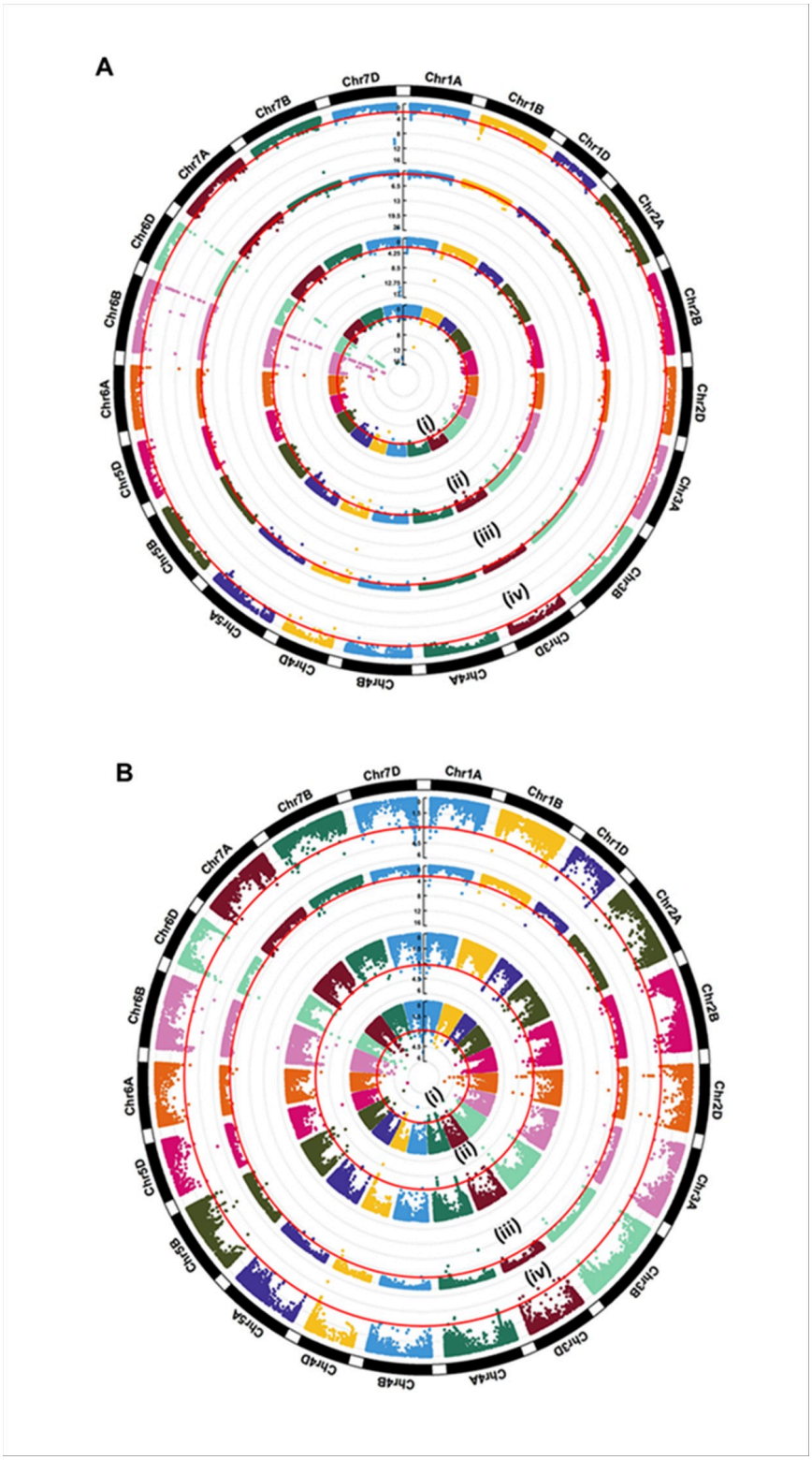


Figure 6

Circular Manhattan displaying results obtained by four different GWAS methods: (i) CMLM (in TASSEL), (ii) CMLM (in GAPIT), (iii) FarmCPU and (iv) GenABEL for leaf rust (LR, **A**), stripe rust (YR, **B**). The red line (LOD = 3, (−log10(p value)) indicates threshold for identifying significant MTAs.

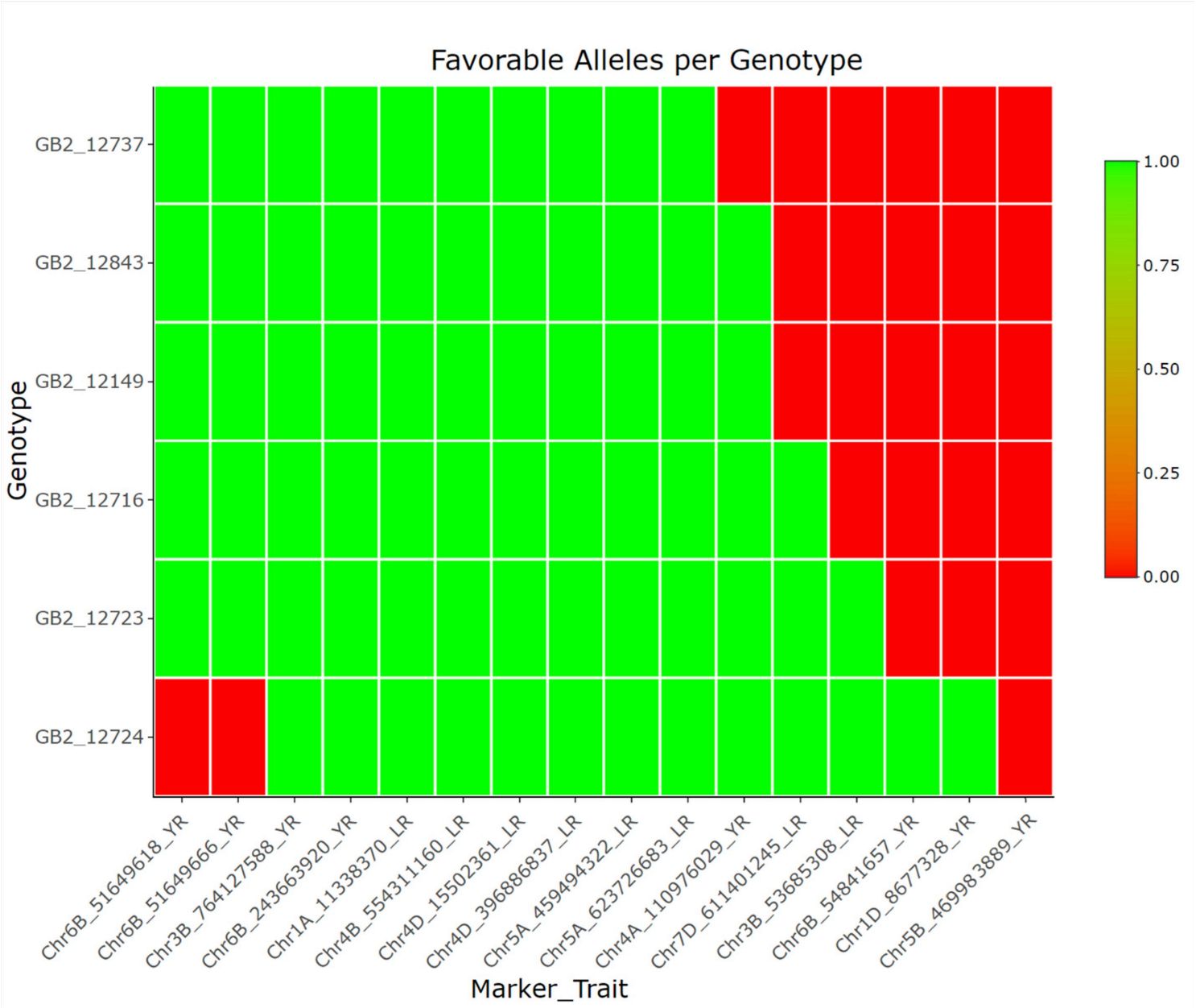


Figure 7

Incidence matrix of allele effects of identified markers for leaf rust (LR) and stripe rust (YR) in six genotypes (carrying favorable alleles for both LR and YR). Green (1): Favorable allele present (negative effect allele associated with reduced disease severity) and Red (0): Unfavorable allele present (positive effect allele associated with increased disease severity).

Supplementary Files

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