

# Dual reference genome-based association mapping enhances discovery of greenbug resistance loci in synthetic wheat

**Honglian Ye**

University of California, Davis

**Genqiao Li**

USDA-ARS Peanut and Small Grains Research Unit

**Yuzhou Xu**

Kansas State University

**Amy Bernardo**

USDA-ARS, Hard Winter Wheat Genetics Research Unit

**Santiago Bataller**

Western Regional Research Center

**Prakitchai Chotewutmontri**

Western Regional Research Center

**Han-Chang Chang**

Western Regional Research Center

**Roger Thilmony**

Western Regional Research Center

**Subhashree Subramanyam**

USDA-ARS, Crop Production & Pest Control Research Unit

**Xiaofei Zhang**

University of California, Davis

**Shuyu Liu**

Texas A&M University

**Yong Q. Gu**

Western Regional Research Center

**Xiangyang Xu**

USDA-ARS Peanut and Small Grains Research Unit

**Steven S. Xu**

`steven.xu@usda.gov`

Western Regional Research Center

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## Research Article

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# Abstract

Greenbug, *Schizaphis graminum* (Rondani), is a serious wheat pest, with periodic outbreaks that often cause substantial yield losses in many wheat-growing regions globally. Breeding and growing greenbug-resistance wheat cultivars has proven to be the most effective and efficient strategy for managing greenbug and its transmitted barley yellow dwarf virus. To identify new greenbug resistance sources, we evaluated a panel of 156 synthetic hexaploid wheat (SHW) lines for responses to greenbug biotype E and identified 98 (62.8%) resistant lines. Genotyping by sequencing (GBS) was used to generate single nucleotide polymorphism (SNP) markers from the panel. After alignment to the Chinese Spring ReSeq v2.1 and *Aegilops tauschii* Aet v4.0 reference genomes and filtering in TASSEL, 34,809 and 31,416 SNPs were retained, respectively. Population-structure analysis resolved two subpopulations. Genome-wide association study detected eight marker-trait associations on chromosomes 3D, 5D, 6D, and 7D across the two reference genomes. Notably, the SNP S7D\_599460943 on 7D explained the largest proportion of phenotype variance (31.3%), while SNPs S3D\_364199819 and S3D\_618021552 on 3D explaining 28.9% and 18.0% of phenotypic variance, respectively. Two 7D SNPs (S7D\_599460943 and S7D\_602876826) mapped within a known resistance cluster on 7DL that contains *Gb3*, *Gb4*, *Gb7*, *Gb8*, and *Gb9*; SNPs on chromosomes 3D, 5D, and 6D are likely associated with novel greenbug resistance loci. The SHW lines carrying potentially new resistance loci provide essential resources for further identification and cloning of the new *Gb* genes. The GBS datasets generated here also provide a resource for discovering additional beneficial alleles for other agronomic traits in wheat.

## Key message

Dual reference genome-based association mapping identified eight greenbug resistance loci in synthetic wheat, including several novel regions on chromosomes 3D, 5D, and 6D.

## Introduction

Wheat (*Triticum aestivum* L.,  $2n = 6x = 42$ ) is one of the key staple global crops that needs to double its production by 2050 to meet rising demands driven by population, shifting dietary preference, and increased biofuel consumption (Ray et al. 2013; Singh et al. 2016; Mao et al. 2023). Although wheat yields are increasing globally at a rate of 0.9% annually, it is still less than the needed 2.4% increase to meet the projected demands (Ray et al. 2013). Biotic stresses are among the several factors that negatively affect wheat production (Robles-Zazueta et al. 2024). Biotic stressors, particularly insect pests and diseases, cause an average global production loss of 20–37%, equating to approximately \$70 billion annually (Pimentel et al. 1997).

Insect pests are one of the main biotic factors affecting plant growth. These pests can inflict serious damage on crops, leading to reduced yield and quality. Insects such as aphids, Hessian fly, wheat stem sawfly, borers, and wheat midges can feed on various parts of the wheat plant, including the leaves, stems, roots, and seeds. This results in a range of symptoms from stunted growth and yellowing of leaves to impaired photosynthesis and nutrient absorption, ultimately affecting yield output (Farook et al. 2019). Among these insects, the greenbug, *Schizaphis graminum* (Rondani), is one of the destructive cereal aphids (Kumar et al. 2019). In the United States, greenbug causes an estimated annual wheat yield loss of more than \$100 million (Royer et al. 2015). They feed on cereal stems and leaves using stylets inserted into the phloem sieve elements, where they consume the phloem sap as their food source (Burd and Porter 2006). These cause leaf spotting, chlorosis, and necrosis, which are observed 2–3 days after infestation, and then an eventual decline in plant health that could lead to death.

Management strategies for greenbug in cereal crops typically involve breeding for host resistance, cultural practices, biological control, and chemical methods. Given the potential environmental and health ramifications of chemical control strategies, breeding for pest resistance becomes a sustainable alternative that is heavily reliant on the understanding of genetic resistance mechanisms in the crops considered. To date, nine designated greenbug resistance genes (*Gb1*–*Gb9*) have been documented in wheat (Xu et al. 2024). Greenbug resistance genes in wheat typically provide protection against only a limited number of biotypes. There are 10 most studied biotypes (A–C, E–K); however, new greenbug biotypes are

constantly emerging in agricultural fields (Harvey et al. 1997; Armstrong et al. 2016). To ensure sustainable wheat production, it is crucial to identify novel resistance sources that can protect against a broader range of biotypes.

Synthetic hexaploid wheat (SHW) ( $2n = 6x = 42$ , AABBDD), developed by combining tetraploid wheat (*T. turgidum* L. ssp.,  $2n = 4x = 28$ , AABB) and diploid D-genome progenitor *Aegilops tauschii* Coss. ( $2n = 2x = 14$ , DD), has garnered increasing attention for its broad genetic base, unique genomic composition, and potential to enhance wheat production (Li et al. 2018). The SHW collections have been found to be a rich source of resistance genes and quantitative trait loci (QTLs) against major wheat diseases (e.g., rusts, septoria nodorum blotch, tan spot, Fusarium head blight, and powdery mildew) and insect pests (e.g., Hessian fly) (Friesen et al. 2008; Yu et al. 2012; Li et al. 2018; Szabo-Hever et al. 2018; Sharma et al. 2021; Athiyannan et al. 2022; Subramanyam et al. 2023). For resistance to greenbugs, five designated genes (*Gb3*, *Gb4*, *Gb7*, *Gb8*, and *Gb9*) and several provisionally named loci (*Gba*, *Gbb*, *Gbc*, *Gbd*, *Gbx1*, *Gbx2*, *Gbz*, and *Gb49921*) have been identified in various SHW, *Ae. tauschii*, and their derived wheat lines (McIntosh et al. 2024; Li et al. 2025). The *Gb3* gene, originally identified in the SHW line Largo (Joppa et al. 1980; Joppa and Williams 1982; Hollenhorst and Joppa 1983), has been deployed in multiple wheat cultivars, including 'TAM 110' (Lazar et al. 1997), 'TAM 112' (Rudd et al. 2014), and 'TAM 204' (Rudd et al. 2019). These varieties have been widely grown in the southern Great Plains in the U.S., where they have effectively reduced damage caused by recurrent greenbug outbreaks (Royer et al. 2015).

To identify genes and QTLs conferring resistance to major wheat diseases and insect pests, we recently assembled a diverse collection of SHW lines. In this study, we aimed to identify resistant SHW lines and potential genes or QTLs underlying greenbug resistance, thereby facilitating the discovery of novel *Gb* genes. Toward this objective, we evaluated a panel of SHW lines for greenbug resistance and conducted a high-density genotyping-by-sequencing (GBS)-based genome-wide association study (GWAS) to identify genetic markers associated with resistance.

## Materials and Methods

### Plant materials

A total of 189 accessions were utilized for greenbug resistance evaluation, consisting of 156 SHW lines and 33 available tetraploid wheat parental lines (Table S1). The SHW lines included 112 lines from CIMMYT (International Maize and Wheat Improvement Center, Mexico) and 44 lines from our laboratory at USDA-ARS (Albany, CA, USA). The 112 CIMMYT SHW lines and their corresponding durum wheat parents were obtained from the Wheat Genetic and Genomic Resources Center at Kansas State University (Manhattan, KS, USA).

### Phenotypic evaluations of Greenbug

The SHW lines were evaluated for responses to greenbug biotype E, which were collected, characterized, and maintained by the USDA-ARS. A randomized complete block design with two replicates was applied and ten seeds from each accession were planted in two consecutive cells of a 73-cell flat (Growing Systems, Inc., Milwaukee, WI) filled with Sunshine Redi-earth growing mix (SunGro Horticulture Canada Ltd.). Wheat 'Jagalene' was planted in two cells in each flat as a susceptible control, while the resistant control which was planted in one cell in each flat, with PI 697274 as the resistant control for greenbug biotype E. The flats were grown in a greenhouse at  $22 \pm 2^\circ\text{C}$  with supplemental light at a 14:10 (light: dark) photoperiod until seedling emergence.

Seedlings were infested with greenbug one day after seedling emergence by laying greenbug-infested barley seedling between rows. Each plant was scored for resistance to Gb about 2 weeks after infestation when the susceptible control seedlings died. Plants that grew normally without damage symptoms were scored as resistant ( $R = 2$ ), whereas those that completely turned yellow, withered or died were rated as susceptible ( $S = -2$ ). Plants that were still alive with over and less than 50% chlorosis were rated as being moderately susceptible ( $MS = -1$  and moderately resistant ( $MR = 1$ ), respectively).

## Genotyping

Leaf samples from the SHW lines were harvested at the two-leaf stage into 96-well plates, frozen with liquid nitrogen, and homogenized using Retsch Mixer Mill MM 400 (Retsch, Düsseldorf, Germany). Genomic DNA was extracted following by previously described (Tai and Tanksley 1990). The concentration of DNA was quantified using by a Nanodrop<sup>TM</sup> 8000 spectrophotometer (Thermo Scientific, MA, USA) and stored at -80 °C for Genotyping-by-Sequencing (GBS) analysis.

GBS library preparation and sequencing followed the protocol previously described by Taranto et al. (2016). SNP calling was performed using the TASSEL-GBS pipeline (Glaubitz et al. 2014) and aligned to Wheat Chinese Spring IWGSC RefSeq v2.1 (Zhu et al. 2021) and the *Ae. tauschii* Aet v4.0 genome (Luo et al. 2017), respectively.

## Population structure and Phylogenetic tree

Population structure analysis was conducted using Structure v2.3.4 (Pritchard et al. 2010), employing the Markov Chain Monte Carlo (MCMC) method in Bayesian statistics. The length of the burn-in period was set to 1000, and the number of MCMC repetitions after the burn-in was set to 10,000. For each simulated value of K, ranging from 1 to 15, a total of five iterations were performed. The optimal K value for the SHW population structure was determined using Structure Selector (<https://lmme.qdio.ac.cn/StructureSelector/>) (Li and Liu 2018), based on the logarithm of the data probability [LnP(D)] and the rate of change in [LnP(D)] between consecutive K values (delta K,  $\Delta K$ ). The phylogenetic tree was constructed using 34,809 and 31,416 imputed SNPs from 156 SHW lines, employing the UPGMA (unweighted pair group method with arithmetic mean) hierarchical clustering method in Tassel 5.0 (Bradbury et al. 2007). The visualization of the tree was facilitated using Interactive Tree Of Life (iTOL v5) (Letunic and Bork 2021).

## Genome-wide association studies and gene annotations

The initial dataset was filtered based on a minor allele frequency (MAF) > 0.01 on Tassel 5.0 (Bradbury et al. 2007). Subsequently, genotypes with  $\geq 10\%$  missing data were removed, and SNPs with a minimum allele frequency of  $\geq 0.05$  and a maximum heterozygous proportion of 0.2 were retained. The filtered SNPs were imputed using the LD KNNi (*k*-nearest neighbor genotype imputation) algorithm and subjected to another filtering round. The resulting dataset was utilized for GWAS using R packages GAPIT (Huang et al. 2019). The available models for GWAS analysis in this software suite include the Mixed Linear Model (MLM), Multi-Locus Mixed Model (MLMM), Fixed and Random Model Circulating Probability Unification (FarmCPU), and Bayesian Information and Linkage Disequilibrium Iteratively Nested Keyway (BLINK).

The optimal models for greenbug resistance in SHW lines from two reference genome datasets were assessed based on the fitting results of the Q-Q plots. For MTAs, the significant thresholds were established at  $p \leq 0.000001$  [equivalent to  $-\log_{10}(P) \geq 6$ ]. An R package was used to construct Manhattan plots (Huang et al. 2019). The percentage of phenotypic variation explained (% PVE,  $R^2$ ) by the individual SNPs was determined using the BLINK model.

High-confidence gene annotations for markers associated to greenbug resistance were selected based on the shortest proximity to the physical positions of the significant SNPs on the Wheat Chinese Spring IWGSC RefSeq v2.1 (Zhu et al. 2021) and the *Ae. tauschii* Aet v4.0 genome (Luo et al. 2017). To discover new resistance genes in the SHW population, we compared the physical positions of our significant SNPs to the markers associated with the known *Gb* genes (Curtis et al. 1960; Dubcovsky et al. 1998; Boyko et al. 2004; Weng et al. 2005; Lu et al. 2010; Azhaguvel et al. 2012; Xu et al. 2020), which were previously mapped to the Chinese Spring IWGSC RefSeq v1.0 (IWGSC 2018).

## Results

### Greenbug resistance in SHW lines

A total of 156 SHW lines, along 33 tetraploid wheat parental lines, were successfully evaluated for resistance to greenbug biotype E (Table S1). Among the SHW lines, 98 (62.8%) exhibited resistance, including 95 resistant (R) and 3 moderately resistant (MR) to greenbug (Fig. 1, Table 1). In contrast, 58 SHW lines were classified as susceptible (S), comprising 7 moderately susceptible (MS) and 51 susceptible lines (Fig. 1, Table S1). All tetraploid wheat parental lines were susceptible, indicating that the observed resistance in SHW lines is contributed by *Ae. tauschii* parents.

Table 1  
Synthetic hexploid wheat (SHW) panel with resistant (R) and moderately resistant (MR) lines to greenbug biotype E.

| Line <sup>a</sup> | Pedigree <sup>b</sup>                                                 | Response <sup>c</sup> |
|-------------------|-----------------------------------------------------------------------|-----------------------|
| TA4152-28         | 68.111 / RGB-U // WARD /3/ <i>Ae. tauschii</i> (316)                  | R                     |
| TA4152-29         | 68.111 / RGB-U // WARD /3/ <i>Ae. tauschii</i> (326)                  | R                     |
| TA4152-37         | 68.111 / RGB-U // WARD /3/ <i>Ae. tauschii</i> (629)                  | R                     |
| TA4152-49         | 68.111 / RGB-U // WARD /3/ FGO /4/ RABI /5/ <i>Ae. tauschii</i> (890) | R                     |
| TA4152-30         | 68112 / WARD // <i>Ae. tauschii</i> (369)                             | R                     |
| TA4152-31         | 68112 / WARD // <i>Ae. tauschii</i> (369)                             | R                     |
| TA4152-40         | 68.111 / RGB-U // WARD RESEL /3/ STIL /4/ <i>Ae. tauschii</i> (781)   | R                     |
| TA4152-41         | 68.111 / RGB-U // WARD RESEL /3/ STIL /4/ <i>Ae. tauschii</i> (783)   | R                     |
| TA4153-30         | 68.111 / RGB-U // WARD RESEL /3/ STIL /4/ <i>Ae. tauschii</i> (385)   | R                     |
| TA4153-32         | 68.111 / RGB-U // WARD RESEL /3/ STIL /4/ <i>Ae. tauschii</i> (431)   | R                     |
| TA4152-1          | Altar 84 / <i>Ae. tauschii</i> (188)                                  | R                     |
| TA4152-3          | Altar 84 / <i>Ae. tauschii</i> [TA 1651] (192)                        | R                     |
| TA4152-4          | Altar 84 / <i>Ae. tauschii</i> (193)                                  | R                     |
| TA4152-7          | Altar 84 / <i>Ae. tauschii</i> (205)                                  | R                     |
| TA4152-9          | Altar 84 / <i>Ae. tauschii</i> (211)                                  | R                     |
| TA4152-16         | Altar 84 / <i>Ae. tauschii</i> (219)                                  | R                     |
| TA4152-20         | Altar 84 / <i>Ae. tauschii</i> (221)                                  | R                     |
| TA4152-90         | Altar 84 / <i>Ae. tauschii</i> (502)                                  | R                     |
| TA4152-75         | Arlin 1 / <i>Ae. tauschii</i> (283)                                   | R                     |
| TA4153-4          | Arlin 1 / <i>Ae. tauschii</i> (218)                                   | R                     |
| TA4153-28         | Arlin 1 / <i>Ae. tauschii</i> (335)                                   | R                     |
| TA4152-85         | Cerceta / <i>Ae. tauschii</i> (174)                                   | R                     |
| TA4152-92         | Cerceta / <i>Ae. tauschii</i> (1024)                                  | R                     |
| TA4152-94         | Cerceta / <i>Ae. tauschii</i> (1027)                                  | R                     |
| TA4153-12         | Cerceta / <i>Ae. tauschii</i> (1025)                                  | R                     |

<sup>a</sup>TA numbers refer to the accessions maintained at the Wheat Genetics Resource Center (WGRC) at Kansas State University (Manhattan, KS, USA). Within this collection, TA4152 and TA4153 represent two sets (Elite 1 and Elite 2, respectively) of elite SHW lines originally developed at the International Maize and Wheat Improvement Center (CIMMYT), Mexico. TA4154 denotes the durum wheat parents of CIMMYT SHW lines. SW numbers identify the SHW lines developed at USDA-ARS, Fargo, ND, USA.

<sup>b</sup>In the pedigrees of the CIMMYT SHW lines, numbers in parentheses denote the *Ae. tauschii* accession identifiers used by CIMMYT. For SHW lines developed by the USDA-ARS, Clae and PI prefixes indicate *Ae. tauschii* accessions from the USDA-ARS National Small Grains Collection (Aberdeen, ID, USA), while the RL prefix refers to accessions held by Agriculture and Agri-Food Canada (Winnipeg, MB, Canada).

<sup>c</sup>Reaction type: R, resistant; MR, moderately resistant.

| Line <sup>a</sup> | Pedigree <sup>b</sup>                                         | Response <sup>c</sup> |
|-------------------|---------------------------------------------------------------|-----------------------|
| TA4153-14         | Cerceta / <i>Ae. tauschii</i> (386)                           | R                     |
| TA4153-15         | Cerceta / <i>Ae. tauschii</i> (392)                           | R                     |
| TA4153-16         | Cerceta / <i>Ae. tauschii</i> (533)                           | R                     |
| TA4153-20         | Cerceta / <i>Ae. tauschii</i> (1046)                          | R                     |
| TA4153-23         | Cerceta / <i>Ae. tauschii</i> (368)                           | R                     |
| TA4153-31         | Cerceta / <i>Ae. tauschii</i> (417)                           | MR                    |
| TA4152-8          | CPI / GEDIZ /3/GOO //JO /CRA /4/ <i>Ae. tauschii</i> (208)    | R                     |
| TA4152-88         | CPI / GEDIZ /3/ GOO // JO / CRA /4/ <i>Ae. tauschii</i> (409) | R                     |
| TA4152-39         | Croc 1 / <i>Ae. tauschii</i> (725)                            | R                     |
| TA4152-91         | Croc 1 / <i>Ae. tauschii</i> (517)                            | R                     |
| TA4152-10         | D67.2 / P66.270 // <i>Ae. tauschii</i> (211)                  | R                     |
| TA4152-13         | D67.2 / P66.270 // <i>Ae. tauschii</i> (217)                  | R                     |
| TA4152-15         | D67.2 / P66.270 // <i>Ae. tauschii</i> (218)                  | R                     |
| TA4152-18         | D67.2 / P66.270 // <i>Ae. tauschii</i> (220)                  | R                     |
| TA4152-21         | D67.2 / P66.270 // <i>Ae. tauschii</i> (221)                  | R                     |
| TA4152-22         | D67.2 / P66.270 // <i>Ae. tauschii</i> (222)                  | R                     |
| TA4152-56         | D67.2 / P66.270 // <i>Ae. tauschii</i> (257)                  | R                     |
| TA4152-68         | D67.2 / P66.270 // <i>Ae. tauschii</i> (633)                  | R                     |
| TA4152-69         | D67.2 / P66.270 // <i>Ae. tauschii</i> (659)                  | R                     |
| TA4153-5          | TK SN1081 / <i>Ae. tauschii</i> (222)                         | R                     |
| TA4152-63         | Yar / <i>Ae. tauschii</i> (518)                               | R                     |
| TA4152-53         | YAV 2 / TEZ // <i>Ae. tauschii</i> (249)                      | R                     |
| TA4152-74         | YAV 2 / TEZ // <i>Ae. tauschii</i> (895)                      | R                     |
| TA4152-19         | Dverd 2 / <i>Ae. tauschii</i> (221)                           | R                     |
| TA4152-55         | Gan / <i>Ae. tauschii</i> (180)                               | R                     |
| TA4152-73         | Gan / <i>Ae. tauschii</i> (897)                               | R                     |

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<sup>b</sup>In the pedigrees of the CIMMYT SHW lines, numbers in parentheses denote the *Ae. tauschii* accession identifiers used by CIMMYT. For SHW lines developed by the USDA-ARS, Clae and PI prefixes indicate *Ae. tauschii* accessions from the USDA-ARS National Small Grains Collection (Aberdeen, ID, USA), while the RL prefix refers to accessions held by Agriculture and Agri-Food Canada (Winnipeg, MB, Canada).

<sup>c</sup>Reaction type: R, resistant; MR, moderately resistant.

| Line <sup>a</sup> | Pedigree <sup>b</sup>                                             | Response <sup>c</sup> |
|-------------------|-------------------------------------------------------------------|-----------------------|
| TA4153-27         | Gan / <i>Ae. tauschii</i> (206)                                   | R                     |
| TA4153-29         | Gan / <i>Ae. tauschii</i> (335)                                   | R                     |
| TA4152-57         | LCK59.61 / <i>Ae. tauschii</i> (313)                              | R                     |
| TA4152-77         | Rascon 37 / <i>Ae. tauschii</i> (312)                             | R                     |
| TA4152-84         | Green 3 / <i>Ae. tauschii</i> (458)                               | R                     |
| TA4152-62         | Scaup / <i>Ae. tauschii</i> (518)                                 | R                     |
| TA4152-87         | Scaup / <i>Ae. tauschii</i> (409)                                 | R                     |
| TA4152-78         | SCOT / MEXI 1 // <i>Ae. tauschii</i> (314)                        | R                     |
| TA4153-11         | SKARV 2 / <i>Ae. tauschii</i> (304)                               | R                     |
| TA4152-72         | SNIPe / YAV79 / DACK / TEAL /3/ <i>Ae. tauschii</i> (877)         | MR                    |
| TA4152-48         | Sora / <i>Ae. tauschii</i> (884)                                  | R                     |
| TA4153-1          | Sora / <i>Ae. tauschii</i> (192)                                  | R                     |
| TA4152-59         | Sterna-DW/ <i>Ae. tauschii</i> (358)                              | R                     |
| TA4152-14         | Yarmuk / <i>Ae. tauschii</i> (217)                                | R                     |
| TA4152-2          | Decoy 1 / <i>Ae. tauschii</i> (188)                               | R                     |
| TA4152-32         | Decoy 1/ <i>Ae. tauschii</i> (447)                                | R                     |
| TA4152-36         | Decoy 1/ <i>Ae. tauschii</i> (515)                                | R                     |
| TA4152-79         | Decoy 1 / <i>Ae. tauschii</i> (333)                               | R                     |
| TA4152-95         | Decoy 1 / <i>Ae. tauschii</i> (1030)                              | R                     |
| TA4153-13         | Decoy 1/ <i>Ae. tauschii</i> (1027)                               | R                     |
| TA4153-33         | Decoy 1/ <i>Ae. tauschii</i> (534)                                | R                     |
| TA4152-33         | YAV 3 / SCO // JO69 / CRA /3/ YAV79 /4/ <i>Ae. tauschii</i> (498) | MR                    |
| SW1               | T. durum Langdon/ <i>Ae. tauschii</i> Clae1                       | R                     |
| SW2               | T. durum Langdon/ <i>Ae. tauschii</i> Clae5                       | R                     |
| SW7               | T. durum Langdon/ <i>Ae. tauschii</i> Clae22                      | R                     |
| SW8               | T. durum Langdon/ <i>Ae. tauschii</i> Clae25                      | R                     |

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<sup>c</sup>Reaction type: R, resistant; MR, moderately resistant.

| Line <sup>a</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Pedigree <sup>b</sup>                           | Response <sup>c</sup> |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------|-----------------------|
| SW10                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> H80-101-4 | R                     |
| SW14                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> PI220641  | R                     |
| SW15                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> PI317392  | R                     |
| SW16                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5003    | R                     |
| SW19                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5259    | R                     |
| SW20                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5261    | R                     |
| SW21                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5263    | R                     |
| SW26                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5392    | R                     |
| SW32                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5532    | R                     |
| SW35                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5552    | R                     |
| SW37                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5557    | R                     |
| SW41                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5570    | R                     |
| SW44                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> PI476874  | R                     |
| SW53                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> PI268210  | R                     |
| SW55                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5257    | R                     |
| SW57                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> RL5270    | R                     |
| SW59                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> Ciae19    | R                     |
| SW61                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> TA 1645   | R                     |
| SW65                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Langdon/ <i>Ae. tauschii</i> Ciae 27   | R                     |
| SW70                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum Rusty/ <i>Ae. tauschii</i> Ciae 17     | R                     |
| SW78                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | T. durum 47 – 1/ <i>Ae. tauschii</i> Ciae 24    | R                     |
| <sup>a</sup> TA numbers refer to the accessions maintained at the Wheat Genetics Resource Center (WGRC) at Kansas State University (Manhattan, KS, USA). Within this collection, TA4152 and TA4153 represent two sets (Elite 1 and Elite 2, respectively) of elite SHW lines originally developed at the International Maize and Wheat Improvement Center (CIMMYT), Mexico. TA4154 denotes the durum wheat parents of CIMMYT SHW lines. SW numbers identify the SHW lines developed at USDA-ARS, Fargo, ND, USA. |                                                 |                       |
| <sup>b</sup> In the pedigrees of the CIMMYT SHW lines, numbers in parentheses denote the <i>Ae. tauschii</i> accession identifiers used by CIMMYT. For SHW lines developed by the USDA-ARS, Ciae and PI prefixes indicate <i>Ae. tauschii</i> accessions from the USDA-ARS National Small Grains Collection (Aberdeen, ID, USA), while the RL prefix refers to accessions held by Agriculture and Agri-Food Canada (Winnipeg, MB, Canada).                                                                       |                                                 |                       |
| <sup>c</sup> Reaction type: R, resistant; MR, moderately resistant.                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                 |                       |

## SNP distribution and population structure

The SNPs generated from 156 SHW lines for GWAS were aligned to two reference genomes, the Wheat Chinese Spring IWGSC RefSeq v2.1 and the *Ae. tauschii* Aet v4.0 genomes. SNP calling with a MAF threshold > 0.01 identified 698,118 and 392,556 polymorphic SNPs in the Chinese Spring and the *Ae. tauschii* genomes, respectively. After quality control filtering and LD KNNI imputation, 34,809 (Table S2) and 31,416 (Table S3) high-quality markers were retained for GWAS analysis in

the respective genomes (Fig. 2). A higher number of polymorphic SNPs were generated using the Chinese Spring reference genome than the *Ae. tauschii*. In the Chinese Spring genome, the highest number of SNPs was observed on chromosome 7D, followed by 5D (Figure S1a and Fig. 2a). In contrast, when aligned to the *Ae. tauschii* genome, SNP density was highest on chromosome 2D, followed by 7D (Figure S1b and Fig. 2b). Notably, SNPs more uniformly distributed across chromosomes in the *Ae. tauschii* genome. Both reference genomes yielded approximately 400 SNPs with unknown chromosomal positions.

Population structure analysis indicated that  $\Delta K$ , calculated using Structure Selector, showed a clear peak at  $K = 2$  (Fig. 3a,b). Consistent results were obtained for both reference genomes based on Bayesian STRUCTURE analysis, indicating a shared underlying genetic structure. Accordingly, the population was divided into two subpopulations (Fig. 3c,d), with 129 and 27 genotypes assigned to populations 1 and 2, respectively (Table S4). Population 1 comprised 83% of the panel, whereas population 2 predominantly consisted of durum Langdon-derived SHW lines. The phylogenetic tree constructed using the 34,809 and 31,416 imputed SNPs further supported this population structure (Fig. 4). It also showed clustering of resistant and susceptible SHW lines, reflecting their genetic relatedness.

## Marker-trait associations in different reference genomes

Using GBS dataset mapped to two distinct reference genomes, four statistic models were applied for GWAS analysis to identify the marker-trait associations (MTAs) for resistance to greenbug (Table S3 and S4). In total, 13 and 18 MTAs were detected based on the Chinese Spring and *Ae. tauschii* reference genomes, respectively (Table S5 and S6). Based on the Q-Q plots (Figure S2 and S3), the BLINK models demonstrated superior performance among the four models evaluated across both reference genomes.

Two MTAs ( $P \leq 0.000001$ ) were identified in the Chinese Spring dataset (Fig. 5a). These markers are located across chromosomes 6D (1), and 7D (1), explaining phenotype variances 20.3% and 31.3%, respectively. Notably, the SNP S7D\_599460943 on chromosome 7D explained the highest proportion of phenotypic variance (31.3%), highlighting its significant allelic substitution effect based on the Chinese Spring reference genome. For *Ae. tauschii* reference genome, the analysis revealed 6 significant SNPs ( $P \leq 0.000001$ ) located on chromosomes 3D (2), 5D (1), 6D (1), and 7D (2) (Fig. 5c). The phenotype variance explained by these markers ranged from 1.33% to 28.8%, with the marker S3D\_364199819 on chromosome 3D being the most significant contributor. Subsequently, the marker S3D\_618021552, located on chromosome 3D, was identified as the second most significant determinant.

Furthermore, the Welch's t-test ( $P \leq 0.05$ ) was used to validate the associated SNPs, confirming the significant phenotypic difference between the alternative alleles. These results are consistent to those obtained from the BLINK model. In total, eight SNPs associated with resistance to greenbug were identified across the two reference genomes (Table 2). None of these SNPs were shared between the two genomes. Marker on chromosome 7D explained the highest proportion of phenotypic variance ( $R^2$ , %) in the Chinese Spring genome and was located near genes encoding Serine-threonine/tyrosine-protein kinase. In *Ae. tauschii* genome, markers S3D\_364199819 and S3D\_618021552 exhibited the largest effects and were mapped close to genes encoding uncharacterized subfamily of plant invertase/pectin methylesterase inhibitors and disease resistance protein in plants, respectively.

Table 2

The most significant SNPs associated with greenbug resistance in a panel of 156 Synthetic hexaploid wheat (SHW) lines.

| No. <sup>a</sup>                                                                                                                                                                                                                | SNP           | Chr | Position (bp) | -log <sub>10</sub> (P) | MAF <sup>b</sup> | Effect | PVE <sup>b</sup> (R <sup>2</sup> , %) | Known Gb genes <sup>c</sup> | Candidate gene annotation                                                     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|-----|---------------|------------------------|------------------|--------|---------------------------------------|-----------------------------|-------------------------------------------------------------------------------|
| 1                                                                                                                                                                                                                               | S6D_58461544  | 6D  | 58,461,544    | 10.3                   | 0.4              | 0.6    | 20.3                                  |                             | Eukaryotic translation initiation factor 3 subunit F                          |
| 2                                                                                                                                                                                                                               | S7D_599460943 | 7D  | 599,460,943   | 26.6                   | 0.4              | -1.3   | 31.3                                  | Gb3, 4, 7, 8, 9             | Serine-threonine/tyrosine-protein kinase                                      |
| 3                                                                                                                                                                                                                               | S3D_364199819 | 3D  | 364,199,819   | 6.8                    | -0.7             | 0.1    | 28.9                                  |                             | Uncharacterized subfamily of plant invertase/pectin methylesterase inhibitors |
| 4                                                                                                                                                                                                                               | S3D_618021552 | 3D  | 618,021,552   | 8.0                    | 0.8              | 0.1    | 18.0                                  |                             | Disease resistance protein, plants                                            |
| 5                                                                                                                                                                                                                               | S5D_542251865 | 5D  | 542,251,865   | 15.2                   | -1.5             | 0.1    | 13.1                                  |                             | Dual specificity protein phosphatase domain profile                           |
| 6                                                                                                                                                                                                                               | S6D_476379816 | 6D  | 476,379,816   | 7.1                    | -0.3             | 0.4    | 1.3                                   |                             | Ankyrin repeat domain-containing protein 13                                   |
| 7                                                                                                                                                                                                                               | S7D_602476826 | 7D  | 602,476,826   | 12.4                   | -0.6             | 0.2    | 3.7                                   |                             | B3 domain-containing protein REM16-like                                       |
| 8                                                                                                                                                                                                                               | S7D_602850309 | 7D  | 602,850,309   | 24.7                   | -1.2             | 0.3    | 15.6                                  | Gb3, 4, 7, 8, 9, Gb49921    | Chloramphenicol acetyltransferase-like domain superfamily                     |
| <sup>a</sup> No.1 and 2 indicate the two significant SNPs identified from Wheat Chinese Spring IWGSC RefSeq v2.1 genome; No. 3 through No. 8 indicate the significant SNPs identified from <i>Ae. tauschii</i> Aet v4.0 genome. |               |     |               |                        |                  |        |                                       |                             |                                                                               |
| <sup>b</sup> MAF: Minor allele frequency, PVE: Phenotype_Variance_Explained                                                                                                                                                     |               |     |               |                        |                  |        |                                       |                             |                                                                               |
| <sup>c</sup> Known Gb genes in the region previously identified by Weng et al. (2005), Lu et al. (2010), Xu et al. (2024), and Li et al. (2025).                                                                                |               |     |               |                        |                  |        |                                       |                             |                                                                               |

## Discussion

Synthetic hexaploid wheat is artificially created from a cross between a tetraploid species carrying A and B genomes and the D-genome progenitor *Ae. tauschii*. It can be readily hybridized with common wheat germplasm for transferring desired traits from tetraploid wheat and *Ae. tauschii*. Therefore, the SHW collections have served an invaluable genetic resource for improving modern wheat germplasms. In this study, we identified 98 SHW lines with resistance to greenbug biotype E, constituting 62.8% of the SHW lines. The evaluation data showed that the tetraploid wheat parents of these SHW are susceptible; and the eight MTAs are all located on D-genome chromosomes (3D, 5D, 6D, and 7D). Therefore, the greenbug resistance genes in these SHW lines are contributed by *Ae. tauschii* parents, which is consistent with several previous studies (Lage et al. 2003; Subramanyam et al. 2023). This result also confirmed that *Ae. tauschii* is a good source of resistance to greenbugs (Xu et al. 2023b).

Of the nine formally designated greenbug resistance genes (*Gb1–Gb9*), *Gb1* maps to chromosome arm 1AS (Xu et al. 2023a), while *Gb2* and *Gb6* originated from rye via T1AL·1RS translocations (Hollenhorst and Joppa 1983; Lu et al. 2010). *Gb5* was introgressed to 7AL from an *Ae. speltooides* 7SL segment through a T7AS·7AL-7SL-7AL interstitial translocation (Dubcovsky et al. 1998). The remaining five genes (*Gb3*, *Gb4*, *Gb7*, *Gb8*, and *Gb9*) all derived from *Ae. tauschii* map to the terminal deletion bin 7DL3-0.82-1.0 (Weng et al. 2005; Xu et al. 2020, 2024). Furthermore, all temporarily named *Gb* genes identified in *Ae. tauschii* or its derivatives, including *Gba*, *Gbb*, *Gbc*, *Gbd*, *Gbx1*, *Gbx2*, *Gbz*, and *Gb49921*, map to the distal region of 7DL (Zhu et al. 2005; Li et al. 2025). In the present study, SNP S7D\_599460943 corresponds precisely to the *Gb9* co-segregating KASP marker *Stars-KASP869* (Xu et al. 2024), providing direct evidence that this SNP tags the *Gb9* locus. The second SNP, S7D\_602876826, is located distal to the *Gb9* interval within the region harboring *Gb3* and *Gb4*. Consequently, these 7D SNPs are most likely associated with the *Gb* genes previously identified in *Ae. tauschii*.

The significant SNPs on chromosomes 3D, 5D, and 6D fall outside the genomic regions of all currently designated *Gb* genes and are likely novel resistance loci. On chromosome 3D, two SNPs from the *Ae. tauschii* genome analysis explain 28.9% and 18.0% of phenotypic variance, respectively. The proximal candidate gene at S3D\_364199819 belongs to an uncharacterized subfamily of plant invertase/pectin methylesterase inhibitors, while the gene near S3D\_618021552 encodes a plant disease resistance protein. On chromosome 5D, the candidate gene at S5D\_542251865 contains a dual-specificity protein phosphatase domain. On chromosome 6D, S6D\_58461544 maps proximal to a eukaryotic translation initiation factor 3 subunit F (eIF3f) gene, and S6D\_476379816 is adjacent to an ankyrin repeat domain-containing protein 13 (Table 2).

The exclusive detection of the 3D and 5D loci in the *Ae. tauschii* reference genome dataset and their absence in the Chinese Spring genome analysis, highlights reference genome selection choice shapes the landscape of discoverable resistance loci in the SHW collections. Population structure analysis consistently identified  $K = 2$  across both datasets, confirming that the choice of reference genome did not alter the underlying genetic groupings of the SHW panel. Therefore, the absence of overlapping significant SNPs between the two genomes reflects genuine differences in genomic coverage rather than a methodological artifact. Chinese Spring represents only a narrow slice of D-genome diversity; consequently, resistance loci derived from divergent *Ae. tauschii* accessions are systematically underrepresented or entirely absent in Chinese Spring-based variant calling. This is illustrated by the failure of Chinese Spring, ‘Jagger’, and a custom durum wheat-*Ae. tauschii* assembly to capture the *Gb3* locus from Largo (Parker et al. 2026). In contrast, the *Ae. tauschii* Aet v4.0 reference recovered SNP variation in D-genome regions where Chinese Spring-based alignment fails, allowing the chromosome 3D associations to emerge exclusively from this dataset. This result aligns with the finding that each additional reference genome provides a nearly linear increase in GWAS-detectable loci (Dutta et al. 2023). Therefore, for the SHW lines derived from diverse *Ae. tauschii* backgrounds, reference genome selection is not merely a technical detail, but a direct determinant of which resistance loci are discoverable.

The resistant SHW lines identified in this study, which harbor potentially novel *Gb* loci, are available as parental lines for resistance introgression into adapted wheat germplasm. Furthermore, the associated SNPs provide a foundation for molecular marker development (e.g., KASP assays). Given the resolution limits inherent to a GWAS population of this size and the coordinate discrepancies that complicate direct comparisons across reference assemblies, these candidate *Gb* loci must be validated through linkage mapping or gene cloning. Our findings provide a strategic guide for selecting specific resistant SHW lines to serve as parents in the development of these mapping populations. Additionally, the GBS datasets generated for the 156 SHW lines against two reference genomes constitute a reusable genomic resource for future genetic analyses of resistance to other biotic stresses currently under evaluation.

## Declarations

### Conflict of interest

The authors declare no conflict of interest.

### Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

#### **Ethics approval**

Not applicable.

#### **Consent to participate**

Not applicable.

#### **Consent for publication**

Not applicable.

## **Author Contribution**

SX and XY initiated the study. XY and GL conducted phenotyping. HY and HC prepared DNA samples; AB and YX handled GBS genotyping. HY conducted all bioinformatic analyses. PC, XZ, RT, SL, SS, SB, and YG reviewed and edited the manuscript. The manuscript was drafted by HY, and SX, and revised by all.

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## **Data Availability**

All relevant data are included within the manuscript and its supplementary information files.

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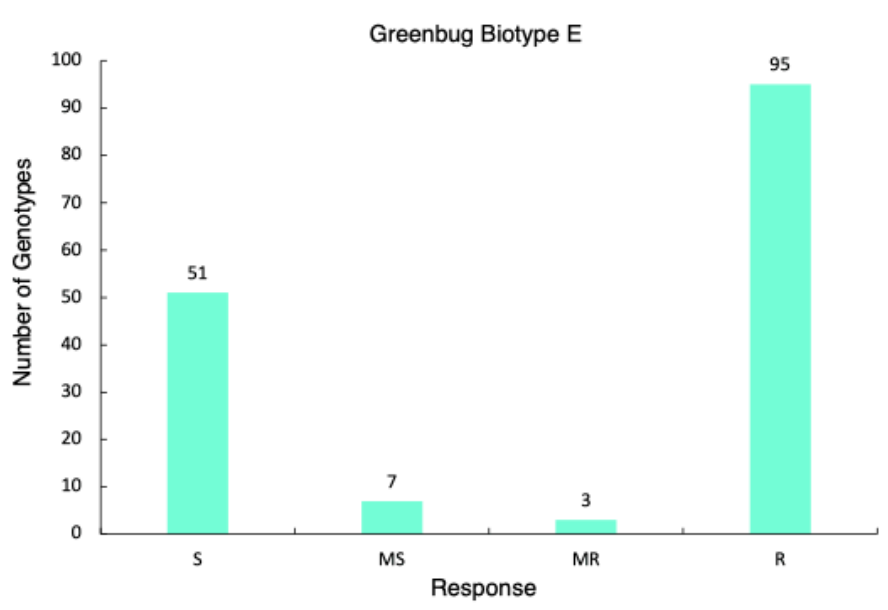
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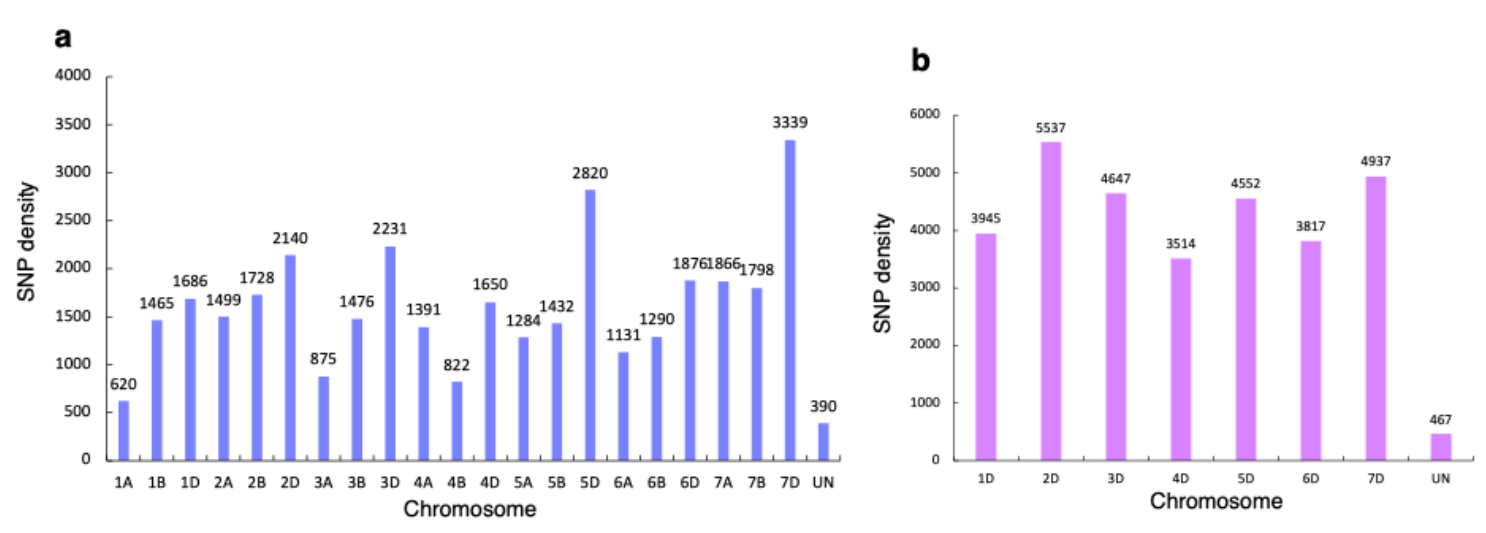
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## Figures



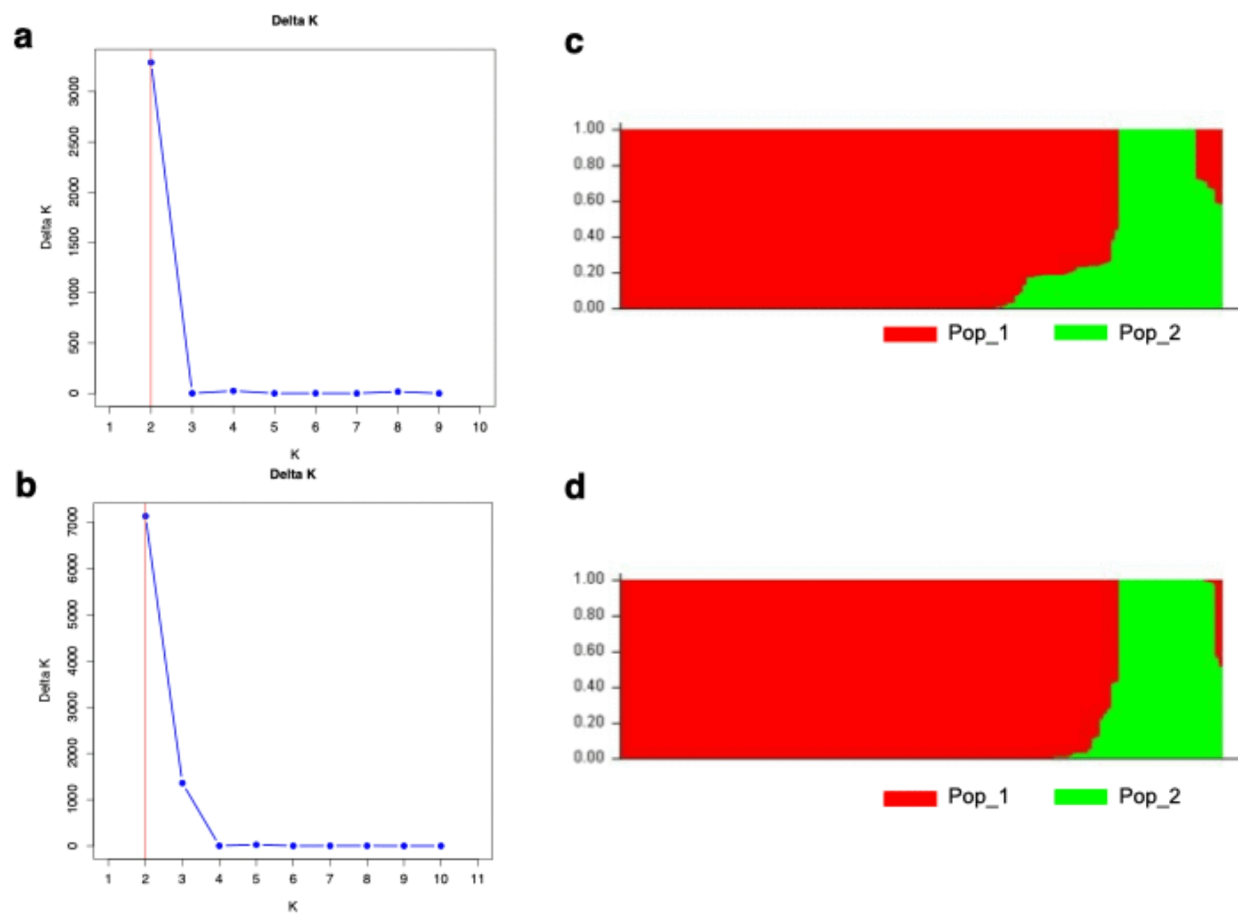
**Figure 1**

Frequency distribution of response to greenbug at seedling stage in 156 synthetic hexaploid wheat (SHW) lines. R = resistant (2), MR = moderately resistant (1), MS = moderately susceptible (-1), S = susceptible (-2).



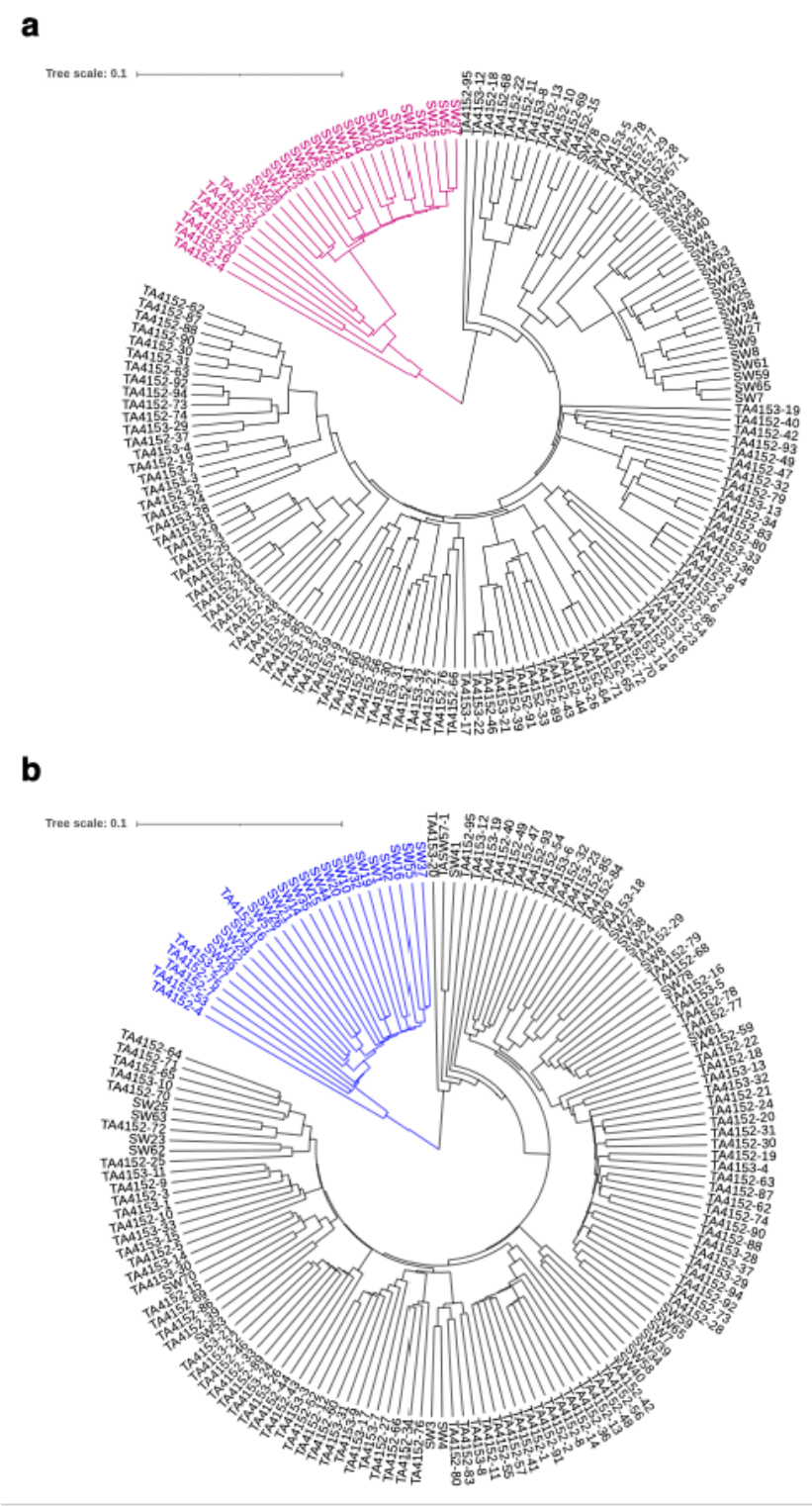
**Figure 2**

Filtered SNP distribution by chromosome in (a) Chinese Spring IWGSC RefSeq v2.1 and (b) *Ae. tauschii* Aet v4.0 genomes.



**Figure 3**

Population structure of synthetic hexaploid wheat (SHW) panel inferred using Bayesian-based program STRUCTURE. **(a, c)** Estimation of the optimal number of subpopulations using K-values. Two subpopulations (K = 2) were identified based on the Chinese Spring RefSeq v2.1 and *Ae. tauschii* Aet v4.0 genomes, respectively. **(b, d)** Population structure plots of SHW accessions at K = 2 based on inferred ancestry (Q matrix). The sub-populations were named Pop\_1 and Pop\_2.



**Figure 4**

Phylogenetic trees of 156 synthetic hexaploid wheat (SHW) lines constructed using **(a)** 34,809 imputed SNPs based on the Chinese Spring RefSeq v2.1 genome and **(b)** 31,416 imputed SNPs based on the *Ae. tauschii* Aet v4.0 genome (Table S2).

Subpopulation Pop\_1 is indicated in black, while Pop\_2 is denoted in magenta for the Chinese Spring analysis and blue for the *Ae. tauschii* analysis, respectively.

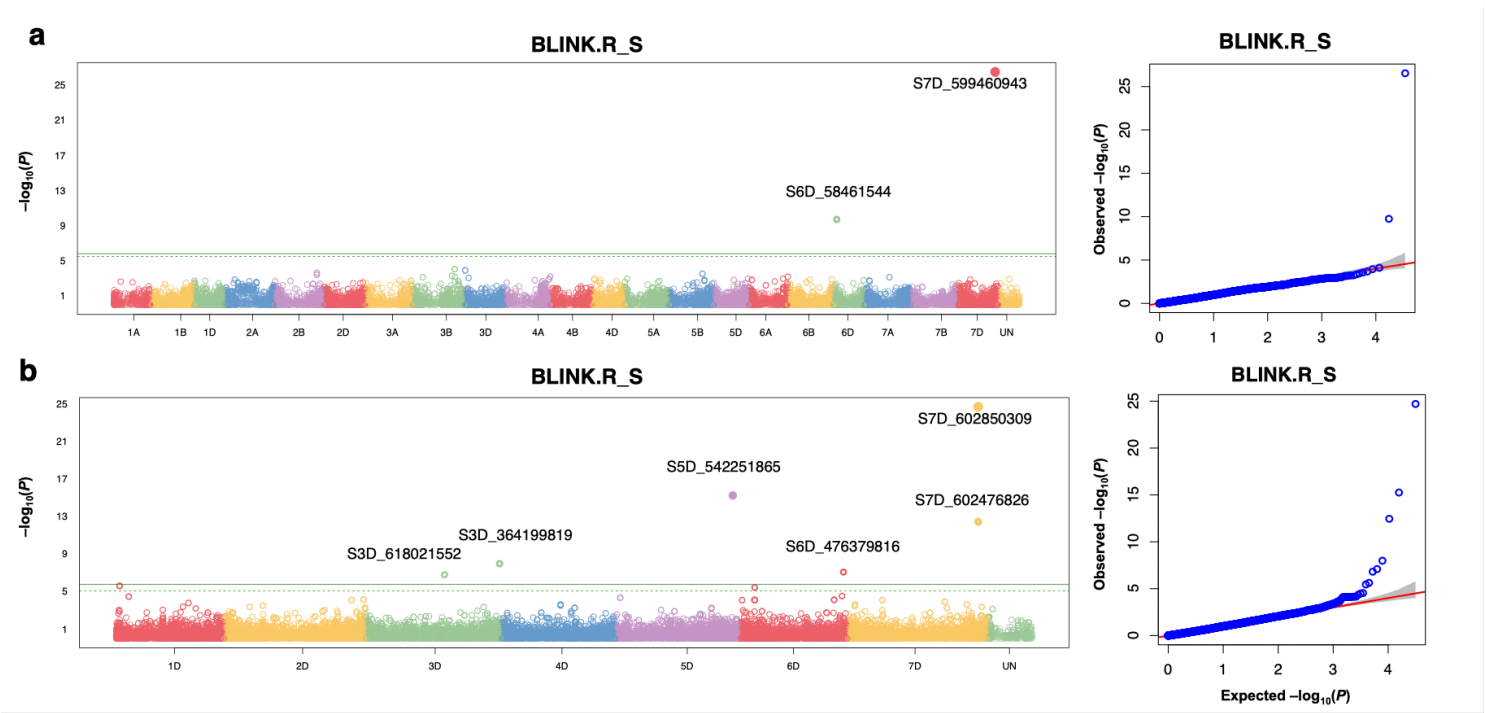


Figure 5

Manhattan plots showing SNPs significantly associated with greenbug resistance in synthetic hexaploid wheat (SHW) lines based on (a) the Chinese Spring IWGSC RefSeq v2.1 genome and (b) the *Ae. tauschii* Aet v4.0 genome. The y-axis represents observed  $-\log_{10}(P)$  values, with the significance threshold set at  $-\log_{10}(P) \geq 6$ . Quantile-quantile (Q-Q) plots for the BLINK model are shown to the right of each Manhattan plot, depicting the relationship between observed and expected  $-\log_{10}(P)$  values.

## Supplementary Files

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