Candidate powdery mildew resistance gene in wheat landrace cultivar Hongyoumai discovered using SLAF and BSR-seq

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Abstract

Background: Wheat powdery mildew, caused by Blumeria graminis f. sp. tritici (Bgt), is an important disease affecting wheat production. Planting resistant cultivars is an effective, safe, and economical method to control the disease. Map construction using next-generation sequencing facilitates gene cloning based on genetic maps and high-throughput gene expression studies. In this study, specific-locus amplified fragment sequencing (SLAF) was used to analyze Huixianhong (female parent), Hongyoumai (male parent) and two bulks (50 homozygous resistant and 50 susceptible F2:3 segregating population derived from Huixianhong × Hongyoumai to determine a candidate gene region for resistance to powdery mildew on the long arm of chromosome 7B in wheat landrace Hongyoumai. Gene expressions of candidate regions were obtained using bulked segregant RNA-seq in 10 homozygous resistant and 10 susceptible progeny inoculated by Bgt. Candidate genes were obtained using homology-based cloning in two parents.

Results: A 12.95 Mb long candidate region in chromosome 7BL was identified, and five blocks in SLAF matched the scaffold of the existing co-segregation marker Xmp1207. In the candidate region, 39 differentially expressed genes were identified using RNA-seq, including RGA4 (Wheat_Chr_Trans_newGene_16173)—a disease resistance protein whose expression was upregulated in the resistant pool at 16h post inoculation with Bgt. Quantitative reverse transcription (qRT)-PCR was used to further verify the expression patterns in Wheat_Chr_Trans_newGene_16173 that were significantly different in the two parents Hongyoumai and Huixianhong. Two RGA4 genes were cloned based on the sequence of Wheat_Chr_Trans_newGene_16173, respectively from two parent and there was one amino acid mutation: S to G in Huixianhong on 510 loci.

Conclusion: The combination of SLAF and BSR-seq methods identified a candidate region of pmHYM in the chromosome 7BL of wheat landrace cultivar Hongyoumai. Comparative analysis between the scaffold of co-segregating marker Xmp1207 and SLAF-seq showed five matching blocks. qRT-PCR showed that only the resistant gene Wheat_Chr_Trans_newGene_16173 was significantly upregulated in the resistant parent Hongyoumai after inoculation with Bgt, and gene cloning revealed a difference in one amino acid between the two parent genes, indicating it was involved in the resistance response and may be the candidate resistance gene pmHYM.

Keywords: Wheat powdery mildew, Resistance gene, Specific-locus amplified fragment sequencing, Bulked segregant analysis, Gene expression

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safest and most effective means to prevent or slow the spread of the disease. However, co-evolution and the use of single or few Pm genes can lead to rapid development of matching virulence genes in the pathogen population, resulting in breakdown of cultivar resistance [2, 3]. Therefore, it is important to discover, identify, and clone powdery mildew resistance (PMR) genes.

Mapping and homologous cloning are the major methods used to discover resistance genes; however, gene mapping is the basic of the work. Before the development of high-throughput sequencing, many types of markers, such as RFLP, RAPD, SCAR, STS, CAPs, AFLP, and SSR were used in gene marker identification. This method involved screening primers and fine mapping, which was time-consuming and tedious, thereby limiting studies on candidate genes. Single nucleotide polymorphism (SNP) refers to a single nucleotide mutation that causes DNA sequence diversity at the genome level. SNP markers are becoming major molecular markers, with high distribution density, good genetic stability, and a diallelic genotype, which is easy to detect through high-throughput automated sequencing. The increasing throughput of next-generation sequencing and de novo and reference-based SNP discovery has been applied to several species and is gradually becoming the major method for functional gene mining [4–6]. SLAF-seq, based on automated methods, has a significant advantage of high throughput screening [7]. It is an important method for excavating genomic SNP and a high-resolution strategy for large-scale de novo SNP discovery and genotyping. This technology has been made available for haplotype mapping, genetic mapping, linkage mapping, and polymorphism mapping. In previous studies, bulk segregant analysis (BSA), a classical method of marker identification in preliminary screening of primers [8–15], selects 6–10 individuals that represent an extreme phenotype. Due to the small sample sizes, markers identified via this method are usually not closely linked. SLAF uses a minimum of 50 individuals to construct a phenotypic pool, which greatly improves the accuracy of the identified candidate region. Large specific markers of any density that exist throughout the whole genome can be developed based on SLAF-seq to implement fine positioning for functional candidate regions. SLAF-seq has been used for fine mapping and screening of candidate genes in many animals and plants [16–19].

At present, Pm1 ~ Pm68 have been named as powdery mildew resistance genes, and Pm1 (Pm1a-1e), Pm2 (Pm2a-2c), Pm3 (Pm3a-3j), Pm4 (Pm4a-4d), Pm5 (Pm5a-5e), Pm8 (Pm8 and Pm17), and Pm24 (Pm24a-24b) contain alleles of these resistance genes [20]. However, there has been little progress in whole wheat sequencing because it is difficult to clone genes from polyploid organisms. Until now only a few Pm genes have been cloned, including Pm3 [21], Pm38/Yr18/Lr34/Sr57 [22], Pm8 [23], Pm46/Yr46/Lr67/Sr55 [24], Pm2 [25], Pm60 [26], Pm17 [27], Pm21 [28, 29], Pm24 [30], Pm5e [31], and Pm41 [32].

Of all the identified genes, 30 are derived from common wheat and the remainder are from wild wheat relative species. Exogenous resistance sources were usually used in resistance breeding, sometimes good resistance (R) genes exist in the far-edge materials and are difficult to incorporate. Wheat landraces in China are the products of natural and artificial selection and have better adaptability and important value in the practice of resistance breeding. Previous studies elucidated Pm gene composition in some wheat landraces in China and showed that the resistance to wheat powdery mildew in Mazama, Xiabaidongmai, Youbailan, Hongjuanlang, Aiganmangmai, Hongtoumai, Dahongtou, Hongyanglazi, Jiantouhong, and Bensanyuehuang were attributable to a single recessive gene [33–37]. These studies enrich our understanding of wheat landraces.

Wheat landrace Hongyoumai was collected and conserved by the Germplasm Resources Bank of the Wheat Research Center at Henan Academy of Agricultural Sciences. This landrace was identified to be highly resistant to wheat powdery mildew at both the seedling and adult stages [38]. Xu constructed a Chancellor*Hongyoumai F2 hybrid cross, and genetic analysis indicated that Hongyoumai carries one dominant resistance gene to powdery mildew [39]. Wang constructed F2 segregating population derived from Yumai13*Hongyoumai and reported that the resistance of Hongyoumai to the Bgt isolate GY was controlled by one dominant resistance gene, temporarily named PmHYM, which was located on chromosome 7BL near SSR markers [40]. Fu constructed F2 segregating population derived from Huixianhong*Hongyoumai, and genetic analysis indicated that the resistance of Hongyoumai to the isolate E09 was controlled by one recessive gene, named pmHYM, and performed fine mapping to identify co-segregating marker Xmp1207 of gene pmHYM using a 90k SNP chip [41]. Anti-spectral analysis of 19 wheat powdery mildew pathogen isolates showed that pmHYM was different from the Pm5 gene and its reported allele, and it was a new Pm5 allele or another gene [41]. The virulence frequencies of wheat powdery mildew isolates collected from different areas of Henan for ten years were lower than 40% according to Li [42] and our unpublished data, indicating that it can still be used in breeding for Pm resistance. Transcript sequencing was conducted using Yumai13*Hongyoumai F2.3 [43]. However, no candidate resistance genes in Hongyoumai have been identified yet.
In previous research, we have constructed the hybrid combinations of Huixianhong (female parent) × Hongyoumai (male parent) and done the identification and analysis of the resistance of the susceptible parents Huixianhong, resistant parent Hongyoumai, and F2:3 individuals derived from Huixianhong × Hongyoumai. In this study, we used the SLAF-seq approach to analyze the susceptible parent Huixianhong, resistant parent Hongyoumai, resistant pools (Rp) of 50 F2:3 homologous resistant progeny, and susceptible pools (Sp) of 50 F2 susceptible progeny derived from the Huixianhong × Hongyoumai F2:3 population. We identified differentially expressed genes (DEGs) between Rp of 10 F2:3 and Sp of 10 F2 using RNA-seq. Screening and expression patterns of target genes were analyzed in the two parents Hongyoumai and Huixianhong, using gene clone and qRT-PCR, respectively. The aim was to find the candidate region of gene pmHYM and further discover candidate PMR regions in Hongyoumai.

### Results

#### SNPs accessed using SLAF

SLAF-seq was conducted using an Illumina HiSeq 2500 at Biomarker Technologies, and 194.02 Mb reads were obtained. Two indicators of sequencing quality, Q30 value and guanine-cytosine (GC) content, were 88.09 and 43.44%, respectively (Table 1). Evaluation and monitoring of sequencing data from the control *Oryza sativa* was used to validate enzymatic digestion in this term, and 388,044,970 pair-end reads with average comparison efficiency more than 89.08% using wheat genome as a reference were obtained, along with 614,983 SLAF tags with an average depth of 52.18 in parents and 169.11 in mixed pools (Table 2 and Table 3).

A total of 292,753 SNPs were obtained from two parents, containing 851 non-synonymous-coding regions, as well as 392,556 SNPs in two bulks, containing 1283 non-synonymous-coding regions (Table 4). The distribution of all SNPs on wheat chromosomes is shown in Fig. 1.

A total of 210,495 high-quality SNPs were obtained by filtering out loci with multi-alleles, a read support degree <4, genotype consistency in mixed pools and the parent filtered site. Euclidean distance associate analysis was used to identify a region with a length of 12.95 Mb on chromosome 7BL (Fig. 2 and Table 5).

#### SNP-index analysis

SNP-index and ΔSNP-index distribution of the two bulks is shown in Fig. 3. According to separation ratio theory of progeny in the term, the association threshold was 0.667. The region above the threshold was screened and 22 regions were
identified, 40.51 Mb in length and containing 649 genes; of these, one was a non-synonymous-coding gene. The region on chromosome 7BL is shown in Table 6. From the intersection of the Euclidean distance and SNP-index, one candidate region, 12.9547 Mb in length, was identified on 7BL from 237,210,580 to 250,165,241, which contained 253 candidate genes. The average distance between the two SLAF markers was 0.0514 cM. The region located in the two SLAF markers range, corresponding to the region of co-segregating marker Xmp1207 derived from TA_TGACv1.30.dna.genome|TGACv1_scaffold_578754_7BL was in five blocks: 237371494–241,751,552; 240,463,691–243,321,483; 241,760,892–24,326,939; 241,453,352–243,128,561; 241,721,380–244,424,206.

BSR-seq analysis

Sequence assembly and analysis

Over 93 million reads were generated, with 51 million reads from T01 (R-pool) and 42 million from T02 (S-pool). A total of 23.62 Gb clean data were obtained, with a Q30 value of 86.69% and GC content of 55.96% (Table 7). The comparison efficiency of reads from two samples T01 and T02 were 70.59 and 71.76%. A total of 11,233 new genes were excavated and 7887 genes were annotated.

Differential expression analysis and candidate gene analysis

A total of 1311 DEGs were identified, including 1080 upregulated and 231 downregulated with a Fold-Change ≥ 2.0 and FDR < 0.01 criterion. Combining SLAF-BSA and RNA-seq, there were 39 known DEGs, including 11 upregulated and 28 downregulated in the candidate region (Table 8). One gene, transcript Wheat_Chr_Trans_newGene_16173, which codes one putative disease resistance protein RGA4, was identified in co-segregating candidate area 243,334,027–243,335,287, and 2.67-fold upregulated genes were expressed in the R-pool than in the S-pool.

The expression of Wheat_Chr_Trans_newGene_16173 (gene annotation: Putative disease resistance protein RGA4) in Hongyoumai were 23.4, 23.08, 23.29, 22.38 at 0, 24, 48, 72 h post inoculation (hpi), respectively, which were higher than those in Huixianhong, indicating that it may be involved in the resistance to wheat powdery mildew (Fig. 4). However, no SNPs or InDels in corresponding regions of gene Wheat_Chr_Trans_newGene_16173 were found in the SLAF results, maybe because the sequences in the corresponding region were not identified by SLAF.

Gene cloning and bioinformation analysis

Integrated genes 3780 bp in length were obtained in Huixianhong and Hongyoumai blastx analysis (http://www.ncbi.nlm.nih.gov/), and showed that ORF fragments of Hongyoumai were the same as disease resistance protein RGA4 (Accession no. XM_020324610.1) and one amino acid mutation: S to G in Huixianhong on 510 loci. SMART analysis (http://smart.embl-heide
lberg.de/) revealed that coded genes contained seven resistance structure domains: one NB-ARC (179–463), five leucine-rich repeat (LRR) (597–626; 627–649; 650–673; 1149–1172; 1173–1196), and one LRR_3 (627–940) (Fig. 5).

Discussion

SLAF is based on SNPs and can identify more abundant polymorphisms than other markers. BSA analysis uses 50 individuals of extreme phenotype. Together they improve

| Table 5  | Statistical list of associated region information based on ED associate analysis |
|----------|--------------------------------------------------------------------------------|
| Chromosome ID | Start | End  | Size(Mb) | Gene-Number |
| 7B        | 237,210,580 | 250,165,241 | 12,9547 | 253          |
the accuracy of mapping and narrow down the candidate regions. An exact resistance region 12.95 Mb in length on chromosome 7BL in wheat landrace Hongyoumai was identified in this study using these methods. Further, five blocks were identified by combining the matching scaffold of co-segregating marker \(Xmp1207\) and reducing the scope.

Transcriptome sequencing is an efficient method of screening differential expression transcripts, and is a primary method for obtaining some character-related genes combined with subsequent function analysis. In this study, BSR-seq was used to identify possible candidate genes and identified one candidate resistance transcript \(\text{Wheat}_\text{Chr}_\text{Trans_newGene}_16173\), which was differentially expressed in the two parent types, in the co-segregating region. BlastaX results showed that the homologous gene of \(\text{Wheat}_\text{Chr}_\text{Trans_newGene}_16173\) belonged to the RGA4-like resistance gene and integrated ORFs were cloned in Hongyoumai and Huixianhong based on reported disease resistance protein RGA4 [\text{Aegilops tauschii} \text{subsp. tauschii} (Accession no. XM_020324610.1)].

Resistance genes with LRR domains are widely accepted as playing major roles in plant resistance to pathogens. The LRR domains are thought to interact directly or indirectly with their avirulent effectors to specifically recognize the pathogen. A change in one acid amino in LRR domains may delete or weaken the resistance reaction [44]. Nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins are a current hot topic in plant disease resistance gene research. Genes with NBS-LRR domains are an important part of known resistance genes. NBS conserved domains in resistance genes may have specific relationships with pathogen elicitors and downstream proteins of resistance signal transduction. The NBS domain in NBS-LRR proteins is proposed to function as a molecular switch that adjusts the conformation changes of plant resistance proteins through binding ADP or ATP to regulate resistance signal conduction downstream. There are many NBS-LRR resistance genes and many cloned genes of this kind, including \(\text{Pm3} \text{[21]}, \text{Pm}21 \text{[28, 29]}, \text{Pm}60 \text{[26]}, \text{Pm}5e \text{[31]}, \text{Pm}41 \text{[32]}, \text{Yr}10 \text{[44]}, \text{Lr}1 \text{[45]}, \text{Lr}10 \text{[46]}, \text{Lr}21 \text{[47]}, \text{Sr}33 \text{[48]}, \text{Sr}35 \text{[49]}, \text{Cre1}, \text{and Cre}3 \text{[50]}, \text{Lr}24 \text{[51]} \text{and Lr}35 \text{[52]}\) are also confirmed NBS genes.

In this study, one wheat powdery mildew resistance gene, a RGA4-like gene, which has one NB-ARC, five LRR domains, and one LRR_3 was identified and cloned and belonged to the typical NBS-LRR-type gene. Also, one amino acid mutation was observed in the susceptible parent Huixianhong and was differentially expressed in two parents. This suggests that a RGA4-like gene from Hongyoumai may be the candidate \(\text{Pm}\) gene or one part plays an important role in resistance to wheat powdery mildew. The amino acid mutation locus was outside the conserved domains, the aa change indicated likely presence of another gene or variation in other regulatory elements, so further study is necessary to discover the mechanism of action of RGA4-like gene in Hongyoumai.

**Conclusions**

A candidate region for \(\text{pmHYM}\), 12.95 Mb in length, was located on chromosome 7BL in wheat landrace cultivar Hongyoumai using SLAF-BSA. There were five matching blocks between the scaffold of co-segregating marker \(Xmp1207\) and SLAF-seq. Using RNA-seq, 11 upregulated and 28 downregulated transcripts were observed in which a disease resistance protein RGA4 (\(\text{Wheat}_\text{Chr}_\text{Trans_newGene}_16173\)) was upregulated and expressed in the resistant pool at 16 hpi. Further, qRT-PCR results showed \(\text{Wheat}_\text{Chr}_\text{Trans_newGene}_16173\) was also obviously higher expression in Hongyoumai (resistant parent) than in Huixianhong (susceptible parent), indicating it maybe play a role in the resistance response.

**Methods**

**Plant materials and treatments**

All plant materials, including cultivar Huixianhong (female parent) and wheat landrace Hongyoumai (male parent) \(F_2\text{33}\) segregating population were derived from Huixianhong \(\times\) Hongyoumai. Wheat cultivar Jinfeng 1 code was selected and maintained in the wheat disease research laboratory at the Institute of Plant Protection, Henan Academy of Agricultural Sciences, China. The Bgt E09 race was provided by Prof. Yilin Zhou (Institute of Plant Protection, Chinese Academy of Agricultural Sciences).

Huixianhong, Hongyoumai 50 homozygous resistant and 50 susceptible progeny were planted in nutrition pots and placed in a climate chamber at 16–18°C, with a light/dark photoperiod of 16/8 h, seedlings at the two-leaf and 1 stem stage were collected and mailed to Biomarker Technologies for DNA extraction and SLAF analysis.
Fig. 3 (See legend on previous page.)
Two bulk DNA of the F_{2:3} segregating population were developed using Huixianhong as susceptible and Hongyoumai as resistance donors. BSA was conducted on 50 resistant and 50 susceptible progeny.

Ten susceptible progeny and ten F_{2:3} resistant progeny were planted in nutrition pots and maintained in a growth chamber at 16–18°C, with a light/dark photoperiod of 16/8h. Inoculation with the Bgt E09 race (maintained on susceptible wheat cultivar Jinfeng 1 code) was performed by shaking conidia onto wheat leaves when plants were at the three-leaf stage. At 16h post inoculation (hpi), leaves were individually collected, wrapped in tin foil, frozen in liquid nitrogen, and immediately stored at −80°C. Then, samples were sent in dry ice to Biomaker Technologies for RNA-sequencing.

Hongyoumai and Huixianhong were planted and inoculated with the Bgt E09 race. The inoculated and control wheat leaves were collected at 0, 24, 48, and 72 hpi, and were immediately frozen in liquid nitrogen and stored at −80°C prior to extraction of total RNA.

### SLAF library construction and sequencing

Total genomic DNA was extracted from wheat leaves according to the cetyltrimethylammonium bromide (CTAB) method. DNA quality and concentration were estimated using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) and electrophoresis in 0.8% agarose gels with a lambda DNA marker. Wheat genome sequences were constructed as a reference to calculate electronic enzyme digestion using restriction enzymes RsaI. The SLAF library was constructed as described by Sun et al [7], with steps as follows: Genomic DNA of each sample was digested using RsaI and a single nucleotide (A) overhang was added to the digested fragments. Dual-index sequencing adapters were ligated to the A-tailed fragments using T_{4} ligase. Then, the fragments were amplified using PCR and the products were separated using 2.0% agar electrophoresis. Fragments of 364–394bp in length were excised, purified, and sequenced to obtain target fragments. Pair-end sequencing was conducted using an Illumina HiSeq 2500 system (Illumina, Inc., San Diego, CA, USA) according to the manufacturer’s recommendations (Beijing Biomarker Company).

### SLAF-seq data analysis

SLAF-seq identification and genotyping were performed as described by Sun [7] and Zhang [53]. To assure the quality of sequence analysis, two read lengths of 100bp were used for data evaluation and analysis. The first 100bp and last 100bp in one sequence were both evaluated with a quality score Q30 (indicating a 0.1% chance of error, and 99.9% confidence). Reads <Q30 were filtered out and the ratio of the number of high-quality reads with quality scores >Q30 (indicating a 0.10% chance of error and 99.90% confidence) were added to the total number of raw reads and the GC content was calculated. After the barcodes and the terminal 5bp positions were trimmed, the high-quality reads were mapped onto the reference genome sequence using SOAP software [54]. Sequences mapping to the same position with over 95% identity were grouped into one SLAF locus [53]. The sequence error rate was estimated using the *Oryza sativa* genome sequence data as the control. SLAF-tags were identified in parents and mixed pools, and the SLAF-tags that were polymorphic in the female (Hongyoumai) and male (Huixianhong) parents were considered as SLAF markers for subsequent analysis.

### SNP accession and SNP-index analysis

SNP is the major form of polymorphic SLAF. SNP detection was conducted using GATK software and SAM tools [55]. SNPs with common variant loci identified by the two methods were considered valid. SnpEff [55, 56] was used to note SNPs with common variant loci identified by the two methods. Variations in the location and gene information of various loci on the reference genome were identified according to the location and gene information of various loci on the reference genome. High-quality SNPs were obtained as follows. First, SNP loci with many genotypes and reads with a down-port degree <4bp were filtered out. Second, SNP loci that were coincident between Rp and Sp and genes of Rp did not come from the resistant parent were also filtered out. Third, credible SNPs were obtained. Association analysis was conducted using two methods – Euclidean distance and SNP-index analysis. Genes in the candidate region were annotated using BLAST and the databases NR, Swiss-Prot, GO, KEGG, and COG [57–60].

The ΔSNP-index was calculated using significant differences between bulk genotype frequency. ΔSNP-index values approaching 1 indicated more SNP markers linked to the target trait.
Table 8  Differential expressed genes in candidate region

| Genes #ID                  | log₂FC  | regulated | nr_annotation                                      |
|---------------------------|---------|-----------|----------------------------------------------------|
| Wheat_Chr_Trans_newGene_16167 | 6.745021314 | up        | PREDICTED: polyadenylate-binding protein-interacting protein 8-like [Brachypodium distachyon] |
| Wheat_Chr_Trans_newGene_16173 | 4.657390482 | up        | Putative disease resistance protein RGA4 [Aegilops tauschii] |
| Wheat_Chr_Trans_newGene_16408 | 3.857153144 | up        | PREDICTED: putative F-box protein At4g17565-like [Setaria italica] |
| gene:Traes_7BL_E41665FE0    | 2.431156696 | up        | Putative coatamer subunit beta-3 [Aegilops tauschii] |
| gene:Traes_7BL_97AC3748A    | 1.914289176 | up        | predicted protein [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_418261D09    | 1.844998764 | up        | hypothetical protein ZEAMMB73_490100 [Zea mays] |
| gene:Traes_7BL_4437E5002    | 1.799818185 | up        | E3 ubiquitin-protein ligase KEG [Triticum urartu] |
| gene:Traes_7BL_A54A7230     | 1.594932596 | up        | hypothetical protein F77S_06825 [Aegilops tauschii] |
| gene:Traes_7BL_C09CECD07    | 1.31525402  | up        | Transcriptional repressor NF-X1 [Aegilops tauschii] |
| gene:Traes_7BL_E85DF3A5E    | 1.159372618 | up        | predicted protein [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_FD4254327    | 1.102683172 | up        | vacuolar proton-inorganic pyrophosphatase [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_Trans_newGene_16401 | -5.91836062 | down       | –                                                  |
| gene:Traes_7BL_Trans_newGene_16402 | -5.88805499 | down       | –                                                  |
| gene:Traes_7BL_Trans_newGene_16146 | -5.73120485 | down       | T-complex protein 1 subunit beta [Triticum urartu] |
| gene:Traes_7BL_95D0D023E    | -4.83760605 | down       | speckle-type POZ protein [Zea mays] |
| gene:Traes_7BL_8B348AE18    | -4.746575637 | down       | predicted protein [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_E8DE756D    | -4.737738698 | down       | Putative flavin-containing monooxygenase 1 [Aegilops tauschii] |
| gene:Traes_7BL_Trans_newGene_16411 | -4.609251169 | down       | predicted protein [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_Trans_newGene_16397 | -4.566074614 | down       | T-complex protein 1 subunit beta [Triticum urartu] |
| gene:Traes_7BL_Trans_newGene_16399 | -4.43389143 | down       | Speckle-type POZ protein-like protein B [Aegilops tauschii] |
| gene:Traes_7BL_466F7018     | -4.106259873 | down       | predicted protein [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_AF173EC17    | -4.09072315 | down       | hypothetical protein TRIUR3_06034 [Triticum urartu] |
| gene:Traes_7BL_F45C15FA2    | -4.04801723 | down       | RecName: Full = HMG1/2-like protein [Triticum aestivum] |
| gene:Traes_7BL_Trans_newGene_16409 | -3.873600139 | down       | hypothetical protein F77S_24416 [Aegilops tauschii] |
| gene:Traes_7BL_OFBOAC06    | -3.344018347 | down       | hypothetical protein F77S_07814 [Aegilops tauschii] |
| gene:Traes_7BL_AEE3C83EC    | -3.090935295 | down       | predicted protein [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_DE9710474    | -3.012246392 | down       | predicted protein [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_BAF3A0AE2    | -2.97032921 | down       | Protease 2 [Aegilops tauschii] |
| gene:Traes_7BL_B97B8AFAD    | -2.867453779 | down       | PREDICTED: callose synthase 3-like [Brachypodium distachyon] |
| gene:Traes_7BL_Trans_newGene_16174 | -2.649790705 | down       | –                                                  |
| gene:Traes_7BL_FA7046752    | -2.59224631 | down       | hypothetical protein F77S_24157 [Aegilops tauschii] |
| gene:Traes_7BL_Trans_newGene_16410 | -2.566523722 | down       | Putative disease resistance protein RGA3 [Aegilops tauschii] |
| gene:Traes_7BL_70E47404E5    | -2.432573595 | down       | ent-copalyl diphosphate synthase [Triticum aestivum] |
| gene:Traes_7BL_8A1D0A241    | -2.291330273 | down       | Ent-copalyl diphosphate synthase 1, chloroplastic [Triticum urartu] |
| gene:Traes_7BL_7A3B8A199    | -1.975271473 | down       | RecName: Full = Catalase isozyme 1 [Hordeum vulgare] |
| gene:Traes_7BL_FB21289AE    | -1.906701464 | down       | oligopeptidase B [Triticum aestivum] |
| gene:Traes_7BL_59EFF59F1    | -1.937327117 | down       | predicted protein [Hordeum vulgare subsp. vulgare] |
| gene:Traes_7BL_Trans_newGene_16407 | -1.92007674 | down       | –                                                  |
| gene:Traes_7BL_ACE459781    | -1.712545481 | down       | predicted protein [Hordeum vulgare subsp. vulgare] |

\[
\text{SNP\_index (R)} = \frac{\text{MRp}}{(\text{PRp} + \text{MRp})}
\]
\[
\text{SNP\_index (Sp)} = \frac{\text{MSp}}{(\text{PSp} + \text{MSp})}
\]
\[
\Delta(\text{SNP\_index}) = \text{SNP\_index (R)} - \text{SNP\_index (Sp)}
\]

where MRp indicates the depth of Rp in the male parent, PRp indicates the depth of Rp in the female parent, PSp indicates the depth of Sp in the male parent, and PSp indicates the depth of Sp in the female parent. False positive loci were filtered out by fitting the ΔSNP-index value of SNP markers from the same chromosome.

BSR-seq analysis

RNA-seq was conducted using Illumina HiSeq sequencing with two pooled samples of resistance F2:3 and susceptible...
offspring. Each pooled sample contained equal amounts of RNA collected from 10 individuals. Ten resistant plants and 10 susceptible plants were used individually for RNA extracts following the instruction manual of the Trizol Reagent (Life technologies, California, USA). RNA integrity and concentration were checked using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Equal amounts of RNA of qualified samples constituted T01 (R-pool) and T02 (S-pool) for library construction as follows. First, mRNA was isolated using NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB, E7490) and the enriched mRNA was fragmented into approximately 200 nt RNA inserts, which were used to synthesize the first-strand cDNA and the second cDNA. The double-stranded cDNA was end-repaired, de-A-tailed and adaptor ligated. The suitable fragments were isolated using Agencourt AMPure XP beads (Beckman Coulter, Inc.), and enriched using PCR to obtain the cDNA library. Qubit 2.0 and Agilent 2100 were used to detect the concentration and insert size of the cDNA library, respectively, and qPCR was used to detection the exact quantity for effective concentration of the library. Finally, the qualified cDNA libraries were sequenced using an Illumina HiSeq 2500 sequencing platform.

Reads containing adapter sequences, unknown nucleotides >5%, and low-quality reads of the raw sequencing data, were removed to obtain high-quality clean data (Q30>85%). Then, clean reads were mapped to the wheat genome (ftp://ftp.ensemblgenomes.org/pub/plants/release-30/fasta/triticum_aestivum/dna/) using TopHat2. Mapping data, alternative splicing analysis, gene-structure optimization analysis, and the discovery of new genes were performed and gene expression levels were calculated using the reads per kilobase per million mapped reads (RPKM) method. Clean Reads from two samples were used for comparative analysis with the reference genome using TopHat2 Transcripts were filtered for a fold-change threshold of ≥2.0 and ≤0.5. The False Discovery Rate (FDR) <0.01 was applied to identify significantly differentially expressed genes.

qRT-PCR verification
Total RNA was isolated from wheat leaf extracts using Trizol reagent (TAKARA Biomedical Technology) according to the manufacturer’s instructions. The first strand of cDNA was synthesized from 2μg of the total RNA using RT-PCR (TAKARA). The cDNA samples were diluted ×10 and used as the templates for
qRT-PCR, which was performed on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). According to the sequence of the candidate gene Wheat_chr_Trans_newGene_16173 (Putative disease resistance protein RGA4), forwards primer GGGGTCTCTCCATCTTC and reverse primer TGGTGCCACGCGTT were synthesized to analyze the transcript levels in Hongyoumai and Huixianhong. Wheat 26S rRNA (GenBank accession no. Z11889.1) was used as a control normalization parameter, with primers as follows: 26S-F: GAAGAAGGTCCAAGGGTTC; 26S-R: TCTCCCTTTAACACACAGGG. The reaction system was as follows: 2 × qPCR Mix 10 μL, 2 μL cDNA, and 1 μL/10 μmol L⁻¹ of each primer, with H₂O to a total volume of 20 μL. The temperature cycle program was 95 °C for 10 min, 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 34 s for 40 cycles. All reactions were performed in triplicate, including three non-template controls. Dissociation curves were generated for each reaction to ensure specific amplification. Transcript concentration was calculated using 2 △△Ct [61].

Gene cloning

Primers were synthesized based on the ORF of the sequence of putative disease resistance protein RGA4 [Aegilops tauschii subsp. tauschii] (Accession no. XM_020324610.1) to amplify full length gene sequences of Hongyoumai and Huixianhong. Forwards primer: 5840-F: ATGGCGGCAGCGT; reverse primer: 5840-R: CTAATCTCTACGATGGGACATTCC. Reaction volume: 2 × fast pfu master mix 25 μL, 1 μL 5840-F, 1 μL 5840-R, 3 μL cDNA, with H₂O up to 50 μL reaction volume. The PCR amplification cycle was 94 °C for 3 min, 94 °C for 30 s, 58 °C for 1 min, 72 °C for 1 kb/30 s for 33 cycles, followed by 72 °C for 10 min. Target gene fragments were isolated using 1.0% agarose gel electrophoresis recovery, purification, and sequencing.

Abbreviations

Bgt: Blumeria graminis f. sp. tritici; NGS: Next-Generation Sequencing; SLAF: Specific-Locus Amplified Fragment Sequencing; BSA: Bulked segregant analysis; hpi: hours post inoculation; DEGs: Differentially expressed genes; qRT-PCR: Quantitative reverse transcription-Polymerase Chain Reaction; aa: amino acid; PMR: powdery mildew resistance; SNP: Single nucleotide polymorphism; NBS-LRR: Nucleotide binding site-leucine-rich repeats; GC: guanine-cytosine; ED: Euclidean Distance; RPKM: the reads per kilobase per million mapped reads; FDR: False Discovery Rate.

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Ethical statement for experimental research and field studies on plants

Experimental research and field studies on plants complies with relevant institutional, national, and international guidelines and legislation.
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