Genetic Dissection of the Powdery Mildew Resistance in Wheat Breeding Line LS5082 Using BSR-Seq

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Abstract

Wheat powdery mildew is a destructive disease seriously threatening yield and quality. Comprehensive dissection of new resistance-related loci/genes is necessary to control this disease. LS5082 is a Chinese wheat breeding line with resistance to powdery mildew. Genetic analysis indicated that a single dominant gene, tentatively designated *PmLS5082*, conferred seedling resistance to different *Bgt* isolates. Bulked segregant RNA-seq (BSR-seq) was carried out to map the R gene *PmLS5082* and profile differentially expressed genes associated with *PmLS5082*. *PmLS5082* was mapped to chromosome arm 2BL and flanked by the markers WGGBH612-5 and YTU19-005 with genetic distances of 0.3 and 0.4 cM, respectively. The physical position was subsequently locked into the interval of 710.3-711.0 Mb. *PmLS5082* differs from the catalogued *Pm* genes on chromosome arm 2BL in its resistant spectrum, physical position and origin, suggesting it is most likely a new *Pm* gene. Through COG and KEGG analysis, differentially expressed genes associated with *PmLS5082* were profiled, and six genes in *PmLS5082* interval were confirmed to be associated with *PmLS5082* via RT-qPCR, using an additional set of wheat samples and time-course analysis post-inoculation with *Bgt.* In order to transfer *PmLS082* to different genetic backgrounds using marker-assisted selection (MAS), closely linked markers, including Kompetitive Allele-Specific PCR (KASP) markers, were evaluated and nine markers were confirmed to be widely applicable for MAS in different genetic backgrounds.

Introduction

Common wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD) is one of the most widely cultivated cereal crops worldwide (Li et al. 2019). Yield and quality of wheat production globally is impacted by the devastating disease, powdery mildew. The causal organism is the fungal pathogen *Blumeria graminis* f. sp. *tritici* (*Bgt*) that has complex and variable virulence structures in natural populations (Wicker et al. 2013; Tang et al. 2016; Liu et al. 2018). Infection by the *Bgt* pathogen can reduce chlorophyll content, affect photosynthesis, and typically decrease wheat yield by 10-15% and up to 62% in severe cases (Singh et al. 2016).

To control this disease, fungicides have been and currently are used, but long-term use of fungicides has led to resistance due to pathogenic variation within *Bgt* (Manoharachary et al. 2014). Alongside drug resistance, cost and environmental pollution factors caused by the use of fungicides cannot be ignored (Saharan et al. 2019). In comparison to fungicides, host resistance is considered to be the most effective and environmentally friendly means to prevent powdery mildew epidemics (Ma et al. 2015; Zhang et al. 2021). The development of resistant cultivars relies on the facts that there are abundant resistance gene resources. So far, more than 100 formally and 30 temporarily designated powdery mildew resistance (*Pm*) genes/alleles have been identified at 63 loci within common wheat and its diverse relatives (McIntosh et al. 2020; He et al. 2021).

There are two types of resistance patterns to powdery mildew: qualitative resistance and quantitative resistance (McIntosh et al. 2020). Qualitative resistance is common and accounts for a significant

proportion of the reported *Pm* genes, and these genes clearly follow Mendel's law of segregation. In contrast, several *Pm* genes, such as *Pm38* (Spielmeyer et al. 2008), *Pm39* (Lillemo et al. 2008), *Pm46* (Herrera-Foessel et al. 2014) and *Pm54* (Hao et al. 2015), are quantitatively inherited, and their inheritance follows a normal distribution. Comparatively speaking, qualitative resistance has often provided high resistance to powdery mildew but has been shown to be defeated after extended periods in production, whereas quantitative resistance is only moderately effective but rarely overcome (Spielmeyer et al. 2005). Together, the two forms of resistance have provided the genetic basis of powdery mildew resistance in wheat. Due to ease of selection during breeding, the focus has been on the *Pm* genes providing qualitative resistance but, as mentioned above, many *Pm* genes with qualitative resistance have lost their resistance to the powdery mildew plant-pathogen interaction (Ma et al. 2015; He et al. 2021). Consequently, there is an urgent need to mine and utilize more effective resistance sources to increase the genetic diversity of *Pm* genes.

To date, a number of *Pm* genes have been cloned, such as *Pm1*, *Pm2*, *Pm3*, *Pm4*, *Pm5*, *Pm8*, *Pm21*, *Pm24*, *Pm41* and *Pm60* (Yahiaoui et al. 2004; Hurni et al. 2013; He et al. 2018; Xing et al. 2018; Zou et al. 2018; Hewitt et al. 2020; Li et al. 2020a; Lu et al. 2020; Xie et al. 2020; Sánchez-Martín et al. 2021), and research on *Pm* genes has mainly focused on the isolation of R genes and their use in breeding. However, these resistance genes have multiple structural types. Additionally, disease resistance is a complicated process, since not only the R genes but also a large number of associated genes participate jointly in the resistance process.

The underlying molecular mechanism of disease resistance needs to be clarified to support the rational use of the *Pm* genes. Up to now, the mechanism for powdery mildew infection has been reported in grapevine (Fung et al. 2008), barley (Eckey et al. 2004) and *Arabidopsis thaliana* (Fauteux et al. 2006). However, wheat powdery mildew is more complex and different from those mentioned above. Relatively little is known regarding the molecular mechanism of powdery mildew resistance in wheat. Only individual key genes, including *NAC* (NAM ATAF1/2 CUC2) and *MYB* (V-myb avian myeloblastosis viral oncogene homolog) transcription factors, have been analyzed in depth and shown to play a key role in the resistance process (Zhou et al. 2018; Zheng et al. 2020).

For the identification and dissection of wheat resistance genes, bulked segregant RNA-seq (BSR-seq) combined with RNA sequencing (RNA-seq) and bulked segregant analysis (BSA), provides an highly efficient and low-cost method to rapidly map an R gene and profile the pattern of differentially expressed genes associated with that R gene (Wang et al. 2017; Hao et al. 2019). BSR-seq can also alleviate the adverse effects of complex genomes, especially the allohexaploid wheat genome, and help to obtain useful results for varying characters (Ramirez-Gonzalez et al. 2015; Wang et al. 2018b). Therefore, BSR-seq is the approach we have taken to identify and map R genes and their associated genes in wheat breeding line LS5082.

LS5082 is a Chinese wheat breeding line with elite agronomic traits. In the last few years, LS5082 was shown to exhibit high resistance to powdery mildew over its entire life cycle, indicating that it is an

attractive source of resistance gene(s) for controlling wheat powdery mildew. To better understand and use the powdery mildew resistance in LS5082, we report on the identification of the R gene(s) conferring powdery mildew resistance, analysis of resistance-related genes, and the screening of markers suitable for MAS.

Materials And Methods

Plant materials

The wheat breeding line LS5082 was bred and provided by Prof. Sishen Li, Shandong Agricultural University, China, and maintained in our lab as a resistant germplasm to powdery mildew. The wheat cultivars Shannong 29, Shimai 22 and Huixianhong, which are susceptible to all the *Bgt* isolates tested, were used as susceptible parents and crossed with LS5082 to obtain F_1 hybrids, F_2 populations and $F_{2:3}$ families for genetic analysis of the powdery mildew resistance in LS5082. Wheat cultivar Huixianhong was used as the susceptible control for phenotypic assessment. Five resistant donors, Coker 747 (with *Pm6*) (Bennett et al. 1984), Liangxing 99 (with *Pm52*) (Wu et al. 2019), CH7086 (with *Pm51*) (Zhan et al. 2014), WE35 (with *Pm64*) (Zhang et al. 2019), Qingxinmai (with *PmQ*) (Li et al. 2020b) were used to compare their phenotypic responses to different *Bgt* isolates with those of LS5082 through multi-race response experiments. Twenty-six susceptible cultivars collected from different ecological regions of China were used to evaluate the applicability of closely linked markers for MAS.

Reactions to different *Bgt* isolates and genetic analysis

To evaluate the resistant spectrum of the *Pm* gene in LS5082 and compare its reaction pattern with the catalogued *Pm* genes on the same chromosome arm, LS5082 was tested against 11 *Bgt* isolates collected from different wheat production regions of China and with different virulence patterns using genotypes Coker 747 (with *Pm6*), Liangxing 99 (with *Pm52*), CH7086 (with *Pm51*), WE35 (with *Pm64*), Qingxinmai (with *PmQ*) as controls. For each *Bgt* isolate, five seeds of each of the genotypes above were sown in 72-cell rectangular trays with each tray 3 × 3cm. The susceptible control Huixianhong was sown randomly in each tray. Each tray was then put in an independent space with high humidity to avoid cross-infection. The growing conditions were set at a daily cycle of 14 h light at 24^{o}C and 10 h of darkness at 18^{o}C. When the seedlings had grown to the one-leaf stage, they were inoculated with fresh conidiospores, previously multiplied on Huixianhong seedlings. For the first 24h after inoculation, the seedlings were put in a dark place with high humidity at 18^{o}C. From the second day, the growing conditions were set at a daily cycle of 14 h light at 22^{o}C and 10h of darkness at 18^{o}C. When the spores were fully developed on the first leaves of the susceptible control, at 10-14 days post inoculation, infection types (ITs) were surveyed using the 0-4 scale described by An et al. (2013), in which ITs 0, 0;, 1 and 2 are regarded as resistant, and ITs 3 and 4 as susceptible. For all the experiments above, three independent repeats were carried out.

To investigate the inheritance of the powdery mildew resistance in LS5082, the *Bgt* isolate E09 was firstly selected to inoculate one-leaf seedlings of LS5082, Shannong 29, Shimai 22 and Huixianhong, and their crossed F_1 plants, F_2 population and $F_{2:3}$ families for genetic analysis. For the resistant and susceptible parents and their F_1 hybrids, 10 seeds were selected for sowing; for each F_2 population, no fewer than 200 seeds were sown; and for each $F_{2:3}$ family, 20 seeds were sown. As the segregation ratio against isolate E09 was consistent with that of monogenetic inheritance, other isolates avirulent on LS5082 were all used to inoculate randomly selected 10 homozygous resistant, 10 segregating and 10 homozygous susceptible $F_{2:3}$ families for genetic analysis using the same procedures as above to clarify if the same gene conferred resistance to all the *Bgt* isolates, i.e., to confirm that no other genes were included in LS5082 conferring resistance to powdery mildew. When the phenotypic data was obtained, goodness-of-fit analysis was carried out using a chi-squared (χ^2) test to assess deviations of the observed phenotypic data from theoretically expected segregation ratios (SPSS 16.0 software, SPSS Inc, Chicago, USA) at $P \leq 0.05$.

Preparation of samples for BSR-Seq, library construction and RNA sequencing

After scoring the ITs of $F_{2:3}$ families of LS5082 × Shannong 29 using *Bgt* isolate E09, 50 resistant plants from 50 homozygous resistant $F_{2:3}$ families, and 50 susceptible plants from 50 homozygous susceptible $F_{2:3}$ families were selected for isolation of their total RNA using the Spectrum Plant Total RNA kit (Sigma-Aldrich) following the manufacturer's protocol. Equal amounts of RNA from each of the 50 resistant plants were mixed, and similarly for the 50 susceptible plants to construct resistant and susceptible RNA bulks, respectively. The RNA integrity of the resistant and susceptible bulks was assessed using the Agilent 2100 Bio analyzer (Agilent Technologies, Santa Clara, CA, USA). An RNA Integrity Number (RIN) ≥ 7 was considered to meet the sequencing standard. The cDNA libraries were constructed using TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol, and the quality of the cDNA libraries was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The eligible cDNA libraries were sequenced on the Illumina HiSeq sequencing platform (Illumina HiSeq4000), sequenced according to the standard protocol by Beijing Biomarker Technologies Company Limited (Beijing, China). The sequencing indicator was set as 20 Gb clean data for each bulk.

SNP calling and BSR association mapping

After the cDNA libraries were sequenced, the raw data were filtered. Firstly, the reads with adapter, ribosome RNA sequences, reads with N proportion ≥ 10 and poor-quality reads with more than 50% bases with a Q value ≤ 10 were eliminated, and high-quality clean data was obtained; secondly, the STAR software (https://github.com/alexdobin/STAR) was used to assemble the clean data using the reference genome of Chinese Spring (v1.1); thirdly, SNP calling was carried out following the reference flowchart aimed at RNA-Seq by the software GATK (v3.1-1), and the SNP index values in the two bulks was calculated using the MutMap method (Abe et al., 2012) with SNPs in the susceptible bulk as a reference; fourthly, the ΔSNP index between resistant and susceptible bulks for each SNP was calculated (Takagi et

al. 2013) using the following formula: ΔSNP_index = (SNP_index of resistance bulk) − (SNP_index of susceptible bulk); fifthly. Euclidean distance (ED) algorithm (Trapnell et al. 2010) was carried out to lock the associated interval based on the ΔSNP_index using the associated threshold value of 0.21. The interval with an associated threshold value > 0.21 was considered to be the candidate interval.

Development of new markers and molecular mapping of the *Pm* gene

After confirming the candidate interval, the markers linked to catalogued *Pm* genes in the candidate region were firstly used to screen polymorphic markers to preliminarily map the *Pm* gene in LS5082. To saturate the marker density, the SSR, Indel and other different sequences between resistant and susceptible bulks obtained from the BSR-Seq were used to develop new markers available for gel electrophoresis detection in BMK Cloud (supported by Biomarker Technologies Corporation). PCR amplification and separation and visualization of the PCR products followed Ma et al. (2015).

In addition to the markers available for gel electrophoresis detection, Kompetitive Allele-Specific PCR (KASP) markers were also developed for mapping the *Pm* gene(s) in LS5082. Firstly, distinctive SNPs in the candidate interval was screened; secondly, sequences of 100 bp upstream and downstream of the distinctive SNP were acquired for KASP marker development using both the Polymarker website (http://www.polymarker.info/) and Premier 5 software; thirdly, the amplification sequences of the designed primers were aligned once again in the *Triticeae* Multi-omics Center (http://202.194.139.32/) to ensure specificity of the sequences. The KASP primers were used in a Bio-Rad CFX real-time PCR system (Bio-Rad Laboratories, Inc, California, United States) with a final volume of 10 μL containing 3.0 μL of genomic DNA (125 ng), 5.6 μL of 2 × KASP Master Mix (provided by LGC), and 0.17 μL of primer mix (balanced mix of three pairs of primers for each marker). The amplification procedure was set as follows: 94°C for 15 min, followed by 10 touchdown cycles of 94°C for 20 s, 64 to 58°C (decreasing 0.6°C per cycle), and 38 cycles of regular amplification (94°C for 20s, 58°C for 60s), and the final fluorescence was detected at 20°C using software Bio-Rad CFX Manager 3.1.

All the developed markers above were tested for polymorphisms between the resistant and susceptible parents and their derived resistant and susceptible bulks. The resulting markers were then genotyped on the $F_{2:3}$ population of LS5082 × Shannong 29. After obtaining the genotyping data, a χ^2 test was carried out to assess deviations of the observed phenotypic data of the $F_{2:3}$ families from theoretically expected segregation ratios for goodness-of-fit analysis. The linkage map of the powdery mildew resistance gene(s) in LS5082 was constructed based on Lincoln et al. (1993) and Kosambi (1943) using the MAPMAKER 3.0 and the Kosambi function.

Differential expression analysis

After mapping clean reads (obtained from BSR-Seq) to the reference genome, FPKM (Fragments per kilo bases of exon per million fragments mapped) was carried out to calculate the expression level of the reads (Garber et al., 2011). Software EBSeq was used to detect differentially expressed genes (DEGs) using Fold Change ≥ 2 and FDR (False discovery rate) <0.01 as standard. Statistical significance of

DEGs was determined using a combination of multiple tests and false discovery rate (FDR) (Reiner et al., 2003). Statistics and clustering analysis of DEGs between resistant and susceptible bulks were carried out to present the expression patterns in a genome-wide scale.

Functional annotation of the DEGs was performed using IWGSC (the International Wheat Genome Sequencing Consortium) database (v1.1). COG and KEGG pathway enrichment analyses were performed using an R package for DEGs (Yu et al., 2012). For COG analysis, the Unigene sequences were aligned to the COG database to predict possible functions, and to determine the gene function distribution characteristics (http://www.ncbi.nlm.nih.gov/COG). For KEGG pathway analysis, the KEGG database was used to blast against the metabolic pathway (https://www.kegg.jp/kegg/pathway.html).

RT-qPCR

After analysis of the DEGs using COG and KEGG, the DEGs relating to disease resistance and/or stress tolerance were selected to profile their expression patterns using RT-qPCR. To detect transcript levels, primers for specific DEGs were designed based on the coding sequences of the selected genes (Table S1). Firstly, the seedlings of LS5082 and Shimai 22 were inoculated with the *Bgt* isolate E09 at the one-leaf stage, and the first leaves of LS5082 and Shimai 22 seedlings were sampled 3, 6, 12, 24, 36, 48 and 72 h after inoculation, with three parallel experiments; secondly, total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich) following the manufacturer's recommendations, and then quantified by measuring absorbance at the wavelengths of 260 and 280 nm using a Nano Drop 1000 spectrophotometer (Thermo Scientific); thirdly, Promega DNase I-treated RNA was used for cDNA synthesis using Invitrogen SuperScript-II reverse transcriptase following the manufacturer's guidelines. Finally, the cDNA was analyzed using RT-qPCR, following the procedure described by He et al. (2016; 2018) using SYBR green master mix (Applied Biosystems) with a Rotor-Gene-Q (Qiagen). Amplification was followed by melt curve analysis. Relative quantification was carried out using the $2^{-\Delta\Delta Ct}$ method (He et al. 2018). Oligonucleotides amplifying *ACTIN* were used for normalization.

Evaluation of the closely linked markers for MAS

To evaluate markers for MAS, the closely linked markers were genotyped for LS5082 and 27 susceptible wheat cultivars from different regions of China. Polymorphisms between LS5082 and 27 susceptible wheat cultivars were analyzed, and the markers that stably amplified polymorphic band(s) between LS5082 and the susceptible cultivars were regarded as effective for MAS in these genetic backgrounds. To transfer the *Pm* gene to applicable backgrounds, these cultivars were crossed with LS5082 to construct F_2 and F_3 segregation populations for MAS.

Results

Evaluation of powdery mildew resistance in LS5082

When tested against 12 *Bgt* isolates at the seedling stage, LS5082 was resistant to 10 of 12 isolates with an IT 0, accounting for a proportion of 83.3 %. LS5082 also showed an immune reaction pattern towards the highly virulent isolates E05, E20 and E21 (Figure 1 & Table 1). This suggests that LS5082 is a valuable donor for resistance breeding.

Inheritance of powdery mildew resistance in LS5082

After being inoculated with the *Bgt* isolate E09, LS5082 showed no visible symptoms or hypersensitivity on the first leaf, and hence we regarded it as immune with IT 0, while the susceptible parents Shannong 29, Shimai 22 and Huixianhong showed abundant sporulation with more than 80% of the first leaf covered with aerial hyphae, and hence were regarded as highly susceptible with IT scores of 4. The F_1 plants of LS5082 × Shannong 29, LS5082 × Shimai 22 and LS5082 × Huixianhong showed similar reaction patterns to E09 as the parent LS5082 with an IT 0, suggesting that the *Pm* gene(s) in LS5082 displayed dominant inheritance. The F_2 population of LS5082 and different susceptible parents Shannong 29, Shimai 22 and Huixianhong all fit the theoretical ratio of 3:1 for monogenic segregation (Table 2), suggesting a dominant monogenic segregation model. To further confirm this result and clarify the genotypes of the resistant F_2 plants, all the tested F_2 plants were transplanted in the field to harvest $F_{2:3}$ families. All the $F_{2:3}$ families of LS5082 and different susceptible parents fit the ratio of 1:2:1 (Table 2). Therefore, we concluded that the resistance to *Bgt* isolate E09 in LS5082 is controlled by a single dominant gene, tentatively designated as *PmLS5082.*

To further confirm if *PmLS5082* also confers resistance to other *Bgt* isolates, 10 homozygous resistant, 10 segregating, and 10 homozygous susceptible $F_{2:3}$ families of LS5082 × Shannong 29 were randomly selected and inoculated with eight additional *Bgt* isolates shown to be avirulent on LS5082. The phenotypic statistics of these selected families were all consistent with those of *Bgt* isolate E09. Therefore, we suggest that the resistance to all the tested *Bgt* isolates is controlled by the same gene, *PmLS5082*, and no other *Pm* genes are involved in LS5082 powdery mildew resistance.

Clean data, quality control, and sequence alignments

After filtering adapter, ribosome RNA sequences and low-quality reads, 37.5 and 20.2 Gb clean data were obtained from resistant and susceptible bulks, respectively. The data size exceeded setup parameter (20 Gb) and the transcript size of the wheat genome. Therefore, it was considered to cover most transcribed genes within the wheat genome. To assess the quality of sequencing, the Q30 value and GC content were calculated, and the percentages of clean reads with a Q30 were 92.68% and 92.46% for resistant and susceptible bulks, respectively, and the GC contents 56.50% and 54.68% for the resistant and susceptible bulks, respectively. After aligning clean reads of resistant and susceptible bulks to the reference genome (IWGSC v1.1), the percentages of reads mapping to the reference genome were 87.24% and 73.78% for the resistant and susceptible bulks, respectively. In summary, the sequencing quality of resistant and susceptible bulks was high and suitable for subsequent analysis.

SNP calling and confirmation of candidate interval

A total of 47,155 high quality SNPs between resistant and susceptible bulks were detected from the clean data for subsequent ΔSNP index analysis. Using the ED algorithm and 0.21 as the threshold, only one putative candidate region near the end of chromosome arm 2BL (652.4-714.4 Mb) was identified (Figure 2). There is a total of 1,028 SNPs in this candidate region, which can be used for subsequent marker development and DEGs analysis (Table S1)

Molecular mapping of *PmLS5082*

After the confirmation of the candidate region, the markers linked to *Pm63*, *Pm64* and *PmQ* were used to genotype the $F_{2:3}$ families of LS5082 and Shannong 29. The markers WGGBH612-5, WGGBH913 and Xicsq405 were linked to *PmLS5082*, and *PmLS5082* was flanked by the markers WGGBH612-5 and WGGBH913 with genetic distances of 0.3 and 7.6 cM, respectively (Figure 3). The corresponding candidate region using the IWGSC database (v1.1) was narrowed to 710.3-715.0 Mb in chromosome arm 2BL. To saturate the linkage map of the *PmLS5082* interval and further narrow down the candidate region, new gel-based markers and gel-free KASP markers were designed using the BSR-Seq derived Indels, SSR, differential sequences and SNPs between resistant and susceptible bulks in the candidate region (710.3-715.0 Mb). As a result, seven gel-based markers (*YTU19-005*, *YTU19-007*, *YTU19-009*, *YTU19-011*, *YTU19-012*, *YTU19-014* and *YTU19-016*) and two gel-free KASP markers *YTU19-KASP26* and *YTU19-KASP96* showed consistent polymorphisms between the parents and the resistant and susceptible bulks. After genotyping the $F_{2:3}$ population of LS5082 and Shannong 29, the markers were also linked with *PmLS5082* (Figure 4 & 5, Table 3). A high-density linkage map was then constructed using the newly developed markers and the reported markers *WGGBH612-5*, *WGGBH913* and *Xicsq405* (Figure 3). *PmLS5082* was flanked by *WGGBH612-5* and *YTU19-005* with genetic distances of 0.3 and 0.4 cM, respectively. The corresponding physical interval was narrowed to 710.3-711.0 Mb in the chromosome arm 2BL (Figure 3).

Comparisons of *PmLS5082* and the catalogued *Pm* genes on chromosome arm 2BL

To compare *PmLS5082* with the catalogued *Pm* genes on chromosome arm 2BL, 12 *Bgt* isolates were used to compare the reaction patterns of LS5082 with six resistance donors (Coker 747 (with *Pm6*), Am9/3 (with *Pm33*), Liangxing 99 (with *Pm52*), CH7086 (with *Pm51*), WE35 (with *Pm64*) and Qingxingmai (with *PmQ*)) (Figure 1 & Table 1). The result demonstrated that *PmLS5082* showed a significantly different response spectrum to these other catalogued *Pm* genes on chromosome arm 2BL.

Discovery and analysis of DEGs

After calculation of the expression level of the reads and functional annotation, a total of 10,646 DEGs was identified between the resistant and susceptible bulks, of which 5,280 DEGs were down-regulated and 5,366 DEGs were up-regulated using the expression index of susceptible bulk as a standard (Table S2 & Figure S1). Clusters of orthologous groups (COG) analysis was then performed on the DEGs between

resistant and susceptible bulks. The data show that the function of DEGs are most involved in two categories: signal transduction mechanisms and carbohydrate transport and metabolism. The DEGs related to defense mechanisms also account for a large proportion (Figure 6).

To further investigate the signal transduction pathways that the DEGs may be involved in, significance enrichment analysis for KEGG pathway was performed on the DEGs between resistant and susceptible bulks. A total of 126 significantly enriched ($Q \leq 0.05$) pathways involving 20 categories were found (Figure S2 & Table S3). Among them, one plant-pathogen interaction pathway was enriched, and 18 DEGs were present in this pathway (Figure S3). These genes can be used as targets for further molecular studies into the plant response to powdery mildew.

Expression patterns of the resistance-related genes in the candidate interval

To profile the expression of the resistance-related genes in LS5082, 13 DEGs related to disease-resistance and/or stress tolerance in the candidate interval (Table S4) were selected to monitor their transcriptional level at different stages after inoculation with *Bgt* isolate E09. Six of the genes showed significant differences between LS5082 and Shimai 22 in the time course analysis following *Bgt* inoculation (Figure 7). The transcriptional level of TraesCS2B02G512300.1 in LS5082 was rapidly up-regulated at 3-6 h and reached a peak at 24 h after inoculation. Only after 48 post inoculation did this gene start to up-regulate in Shimai 22 (Figure 7a) & b). The transcriptional level of TraesCS2B02G512400.1 in LS5082 was rapidly up-regulated at 24 h after inoculation, but not in Shimai 22. The transcriptional level of TraesCS2B02G524300.1 in LS5082 was rapidly up-regulated at 3-6 h and also 48 h after inoculation, but showed only low expression in Shimai 22. The transcriptional level of TraesCS2B02G520300.1 in LS5082 were first up-regulated at 24 h and again up-regulated at 48 h and 72 h after inoculation, but there are no corresponding up-regulation in Shimai 22. The transcriptional level of TraesCS2B02G480000.1 in LS5082 was rapidly up-regulated at 6 h, but only showed low expression in Shannong 29. The transcriptional level of TraesCS2B02G521800.1 in LS5082 began to up-regulate from 3-6 h and again up-regulated between 36 and 48 h after inoculation, but there no corresponding high expression in Shimai 22.

Evaluation of closely linked markers for MAS

To transfer *PmLS5082* to susceptible cultivars using MAS, nine closely linked markers available for gel electrophoresis and two KASP markers available for high throughput detection were tested for their availability for MAS (Figure 8 & 9, Table S5). The results indicated that all the markers, including the KASP markers, could detect polymorphic genotypes between LS5082 and 27 susceptible cultivars, suggesting that once *PmLS5082* is transferred into the susceptible cultivars through hybridization, these markers can be used to detect *PmLS5082* in MAS. Therefore, our work has provided different types of markers to meet the requirements of different detection platforms.

Discussion

LS5082 is an elite wheat breeding line which we have maintained in our lab as a powdery mildew resistant germplasm. A dominant R gene, *PmLS5082*, was genetically identified to confer seedling resistance in LS5082 to 10 of 12 tested *Bgt* isolates. The display of powdery mildew resistance was not only related to a new R gene, but we also showed that regulatory genes which activated and/or supported the R gene(s) were differentially expressed.

The R gene, *PmLS5082*, was mapped to chromosome arm 2BL, and locked into a 0.7 Mb physical interval (710.3-711.0 Mb). Prior to identifying *PmLS5082*, nine *Pm* genes (*Pm6*, *Pm33*, *Pm51*, *Pm52*, *Pm63*, *Pm64*, *PmQ*, *MlZec1* and *MlAB10*) have previously been reported on chromosome arm 2BL. As the gene donors are from diverse sources, including wheat cultivars, landraces, *T. persicum* Vav, *T. dicoccoides*, *T. timophevii Zhuk.*, and *Thinopyrum intermedium* introgression lines (Li et al. 2020b), this suggests that chromosome arm 2BL is most likely to be an enrichment region for resistance genes. It also implies that additional R genes and complex mechanisms might be associated with this chromosome arm.

Based on their chromosome locations and/or their origins, the nine catalogued *Pm* genes on chromosome 2BL can be distinguished from *PmLS5082* (710.3-711.0 Mb, Chinese wheat breeding line): *Pm52* (581.0-585.0 Mb) is derived from wheat cultivar Liangxing 99 (Wu et al. 2019; Zhao et al. 2013); *Pm33* (779.1-784.3 Mb) is derived from *T. persicum Vav.* (Zhu et al. 2005); *MlZec1* and *MlAB10* (both 796.7-780.0 Mb) are both derived from *T. dicoccoides* (Maxwell et al. 2010; Mohler et al. 2005); *Pm6* (698.3-699.2 Mb) is derived from *T. timopheevii* 2B/2G introgression; and *Pm64* (699.2-705.5 Mb) is derived from wild emmer (Zhang et al. 2019). Additionally, the *Pm6* interval has severe recombination suppression due to the introgression of the 2G chromosome segment (Wan et al. 2020), whereas the *PmLS5082* interval has no significant recombination suppression, and all the markers had normal recombination, indicating that *PmLS5082* is different from *Pm6*.

The physical intervals of *Pm51* (709.8-739.4 Mb), *Pm63* (710.3-723.4 Mb) and *PmQ* (710.7-715.0 Mb) overlap that of *PmLS5082*, and hence there was a need for further clarification. *Pm51* is derived from a *T. intermedium* chromosome segment that was introgressed into wheat (Zhan et al. 2014), and hence can be distinguished from *PmLS5082*. *Pm63* was detected in an Iranian wheat landrace PI 628024 (Tan et al. 2019). However, none of the *Pm63*-linked markers showed polymorphism between LS5082, Shannong 29 and their derived resistant and susceptible bulks, suggesting the genetic diversity in the *Pm63* interval is different from that of *PmLS5082*. *PmQ* is derived from the Chinese landrace, Qingxinmai, and shows a distinctive recessive inheritance (Li et al. 2020b). Further, only one *PmQ*-linked marker was also linked with *PmLS5082*. This indicated that *PmLS5082* is most likely different from *PmQ*. Furthermore, the resistance spectrum of *PmLS5082* was significantly different from all the *Pm* genes we compared it with. Therefore, we conclude that *PmLS5082* is most likely a new *Pm* gene. However, although *PmLS5082* can be distinguished from *Pm51*, *Pm63* and *PmQ* based on the evidence shown above, more and intensive analysis should be provided in the future, including allelic tests and cloning of the genes. A project is underway to fine map *PmLS5082* using larger mapping populations.

To dissect the molecular basis of the powdery mildew resistance in LS5082, we also profiled the regulatory and supported genes associated with the R gene using BSR-Seq, which is a high efficiency and low-cost means of investigating the overall expression profile of resistance-related genes (Hao et al., 2019). Following *Bgt* invasion, a large number of DEGs was activated, and three types of genes accounted for the greater proportion of these, including those involved in signal transduction mechanisms, defense mechanisms and carbohydrate transport and metabolism. This result accords with the working model of signal transduction and activation of defense mechanisms: in the case of pathogen invasion, signal transduction mechanisms are expected to be activated in order to transduce the stress signal. Subsequently, defense mechanisms are expected to be mobilized to fight the invasion. Both these processes need the support of biosynthesis and metabolism.

To further investigate the key regulatory genes involved in disease-resistance process, the expression profiles of six genes were analyzed following pathogen inoculation. The functions of these genes are related to different aspects of the resistant response to *Bgt* invasion. For example, G-type lectin S-receptor-like serine-protein and receptor-like serine-protein are powerful gene families related to adversity stress (Sun et al. 2018, Afzal et al. 2008), and they were induced with high expression in LS5082 at 24 h of inoculation which is a key node of *Bgt* invasion. The LRR receptor-like serine-protein is a typical disease resistance protein (Wang et al. 2018a), it has continuously high expression in LS5082, and most likely played the most important role in withstanding the *Bgt* invasion. The zinc finger CCCH domain-containing protein is an important transcription factor, which may activate resistant genes downstream. The peroxisomal biogenesis factor 6 is related to the functional role of the peroxisome (Sparkes et al. 2014), which is to scavenge active oxygen and hydrogen peroxide produced by the *Bgt* invasion at the early stage; the abscisic acid-inducible protein is a functional protein in the ABA signaling pathway (Anderberg et al. 1992), and one of its main functions is regulation of stomatal closure, which may mitigate *Bgt* invasion to some extent. These data provide an initial attempt at dissecting the resistance pathways and may provide leads towards the improvement of durable resistance.

After dissection of the powdery mildew resistance in a genotype, the next challenge is how to efficiently use it in breeding. In resistance breeding, selected R genes are mainly transferred into susceptible cultivars in order to improve their resistance. In the case of LS5082, due to its comparable agronomic performance with commercial wheat lines, and its broad resistance spectrum, the R gene *PmLS5082* can serve as a valuable resistance gene to broaden the genetic diversity and improve resistance in commercial wheat lines. To transfer *PmLS5082* using MAS, we screened 11 markers that can detect *PmLS5082* in the backgrounds of 27 susceptible cultivars (Table S5). It is worth mentioning that we developed two types of breeding markers (gel and non-gel based) for *PmLS5082*, which meets the needs of different laboratories with different levels of platforms for marker analysis. The gel-based markers can serve as a simple and low-cost selection tool to detect the transfer of *PmLS5082* into elite breeding materials. This especially benefits those breeding programs with a basic laboratory setup and makes it possible to use MAS to deploy *PmLS5082* into their new cultivars. The KASP markers can be assayed using a SNP genotyping system which can also clearly distinguish homozygotes from heterozygotes in

an F_2 population. The KASP markers are gel-free, analyzed by a fluorescence reader (Semagn et al. 2014), and are suitable for those breeding programs with medium-throughput marker laboratories.

Using these two types of markers, MAS of *PmLS5082* is under way. LS5082 has been crossed with five susceptible commercial cultivars, including Yannong 1212, Tainong 18, Liangxing 619, Jimai 229 and Shimai 15, to construct breeding populations. The F_2 and F_3 plants with linked marker alleles, including markers for both gel electrophoresis and high throughput detection, were selected using the relevant markers. Following selection for agronomic performance in the field, we have now selected two wheat breeding lines with superior agronomic performance and high powdery mildew resistance.

Declarations

Author contribution statement

PM, LC and GM conceived the study. LW and TZ developed the markers. LW, TZ, HG, HX, and MJ validated the markers. XC, HL, LZ and JS evaluated the resistance. PM wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest: The authors declared that they have no conflict of interest.

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Tables

Table 1 Seedling reaction patterns of LS5082 and resistance stocks with catalogued *Pm* genes on the chromosome arm 2BL (Coker 747 with *Pm6*, Liangxing 99 with *Pm52*, CH7086 with *Pm51*, WE35 with *Pm64*, Am9/3 with *Pm33*, Qingxinmai with *PmQ*) to 12 *Blumeria graminis* f. sp. *tritici* (*Bgt*) isolates collected from different regions of China. Wheat cultivar Huixianhong, which has no *Pm* genes, was used as the susceptible control.

Genotypes	A10	E05	E09	E31	E18	A3	E23-1	E20	E21	E32	E23	Y14
LS5082	0*	0	0	0	3	3	0	0	0	0	0	0
Qingxinmai	4	4	3	3	3	0	4	4	0	0	4	3
WE35	0	0	0	0	0	0	4	0	0	0	0	0
CH7086	0	0	0	0	0	0	0	0	4	0	0	0
Am9/3	0	0	0	4	3	4	0	0	2	0	0	0
Liangxing 99	0	0	0	0	0	3	0	0	0	0	0	0
Coker747	0	0	3	2	3	0	0	0	3	0	0	0
Huixianhong	4	4	4	4	4	4	4	4	4	4	4	0

*: Infection types (IT) were scored according to a 0-4 scale, of which 0, 0; 1 and 2 are considered resistant, while those with an IT score of 3 or 4 are considered susceptible.

Table 2. Segregation ratios of F_2 and $F_{2:3}$ generations of LS5082 and different susceptible cultivars following inoculation with *Blumeria graminis* f. s. *tritici* (*Bgt*) isolate E09 at the seedling stage

Cross	Plants observed			Expected ratio	χ^2	*P*
	HR	Seg	HS			
LS5082 × Shannong 29 F_2	160		57	3:1	0.18	0.66
LS5082 × Shannong 29 $F_{2:3}$	55	102	53	1:2:1	0.21	0.90
LS5082 × Shimai 22 F_2	135		47	3:1	0.07	0.80
LS5082 × Shimai 22 $F_{2:3}$	37	82	45	1:2:1	0.78	0.68
LS5082 × Huixianhong F_2	141		46	3:1	0.02	0.90
LS5082 × Huixianhong $F_{2:3}$	41	90	43	1:2:1	0.25	0.88

Note: Values of χ^2 for statistical significance at $P = 0.05$ are 3.84 (1 *df*) and 5.99 (2 *df*); HR: homozygous resistant, Seg: segregating, HS: homozygous susceptible.

Table 3 Polymorphic markers newly developed using BSR-Seq for the powdery mildew resistance gene *PmLS5082*

Marker	Location (bp)	Primer sequence
YTU19-005-F	chr2B_710900188	AAGATGAACTGCGGCTGAAT
YTU19-005-R		CAGATGGACCTCTTCTTCGG
YTU19-007-F	chr2B_712318920	ACCACAACGAACACCAACCT
YTU19-007-R		ACGGGTAACCATCGAGATCA
YTU19-009-F	chr2B_712831536	TGCACCTCGATATGTGCTTC
YTU19-009-R		ATGCACAACGTTCTTAGGGC
YTU19-011-F	chr2B_714502471	TTGGTTTTTCCAAGTCGTCC
YTU19-011-R		GCCATGGTTCCTTTTTGGTA
YTU19-012-F	chr2B_714502487	TGCCGTTCCTTTTTCAACTC
YTU19-012-R		GCCATGGTTCCTTTTTGGTA
YTU19-014-F	chr2B_714787048	AAATAAGAAAAGGGGCAGCG
YTU19-014-R		GATCTAGACGAGACGAGACGC
YTU19-016-F	chr2B_715926199	CCGTGTCCACCCTAGTCTGT
YTU19-016-R		TGTCAACACCAACCTCAGGA
KASP19-026-F	chr2B_711725488	gaaggtgaccaagttcatgctTCACTGACGGCGATGTTGC
KASP19-026-H		gaaggtcggagtcaacggattTCACTGACGGCGATGTTGT
KASP19-026-C		CCGGCCGGTGTTTAGTGC
KASP19-096-F	chr2B_714502622	gaaggtgaccaagttcatgctAGACCCTGGACGGAGAGA
KASP19-096-H		gaaggtcggagtcaacggattAGACCCTGGACGGAGAGC
KASP19-096-C		GCCGCGCTGATTATTCCT

Figures

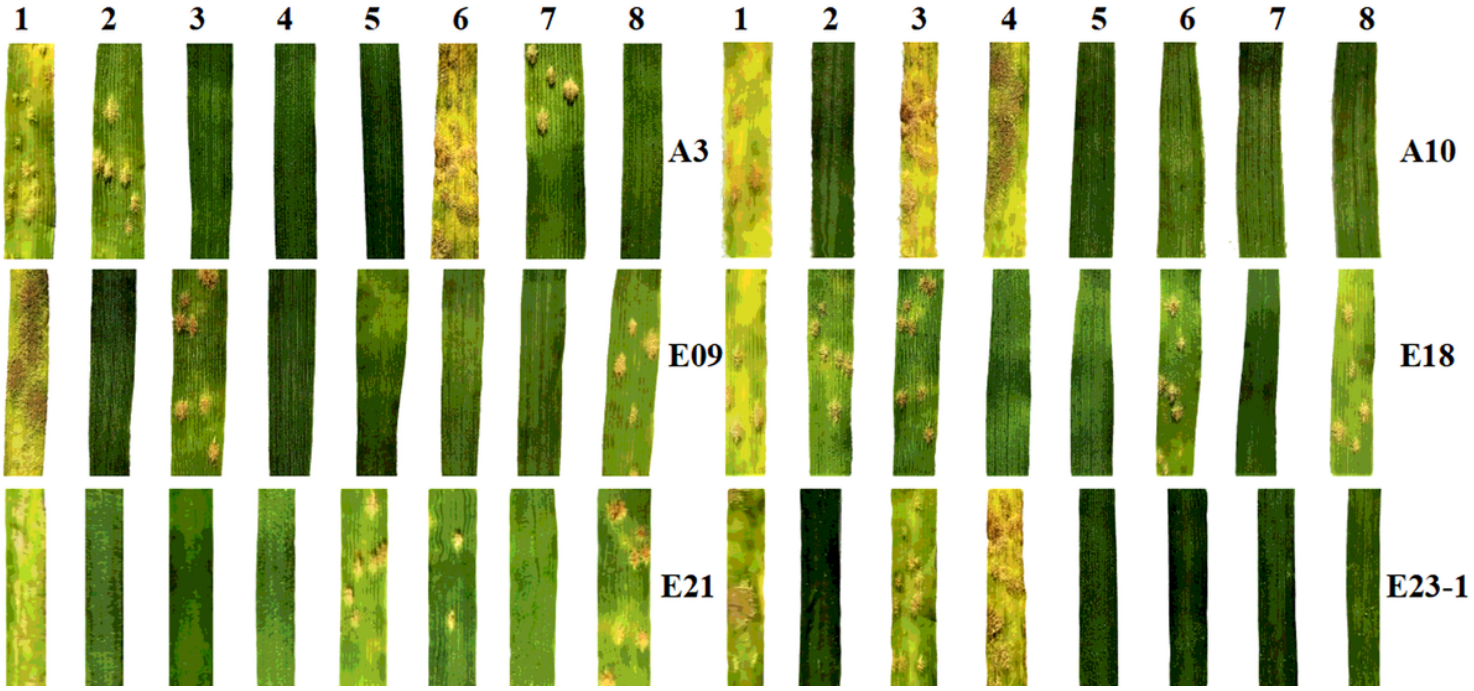

Figure 1

Reaction patterns of LS5082 and genotypes with catalogued Pm genes on chromosome arm 2BL to Bgt isolates A3, A10, E09, E18, E21 and E23-1. 1: Susceptible cultivar Huixianhong; 2: LS5082; 3: Qingxinmai (with PmQ); 3: WE35 (with Pm64); 4: CH7086 (with Pm51); 5: Am9/3 (with Pm33); 6: Liangxing 99 (with Pm52); 7: Coker 747 (Pm6).

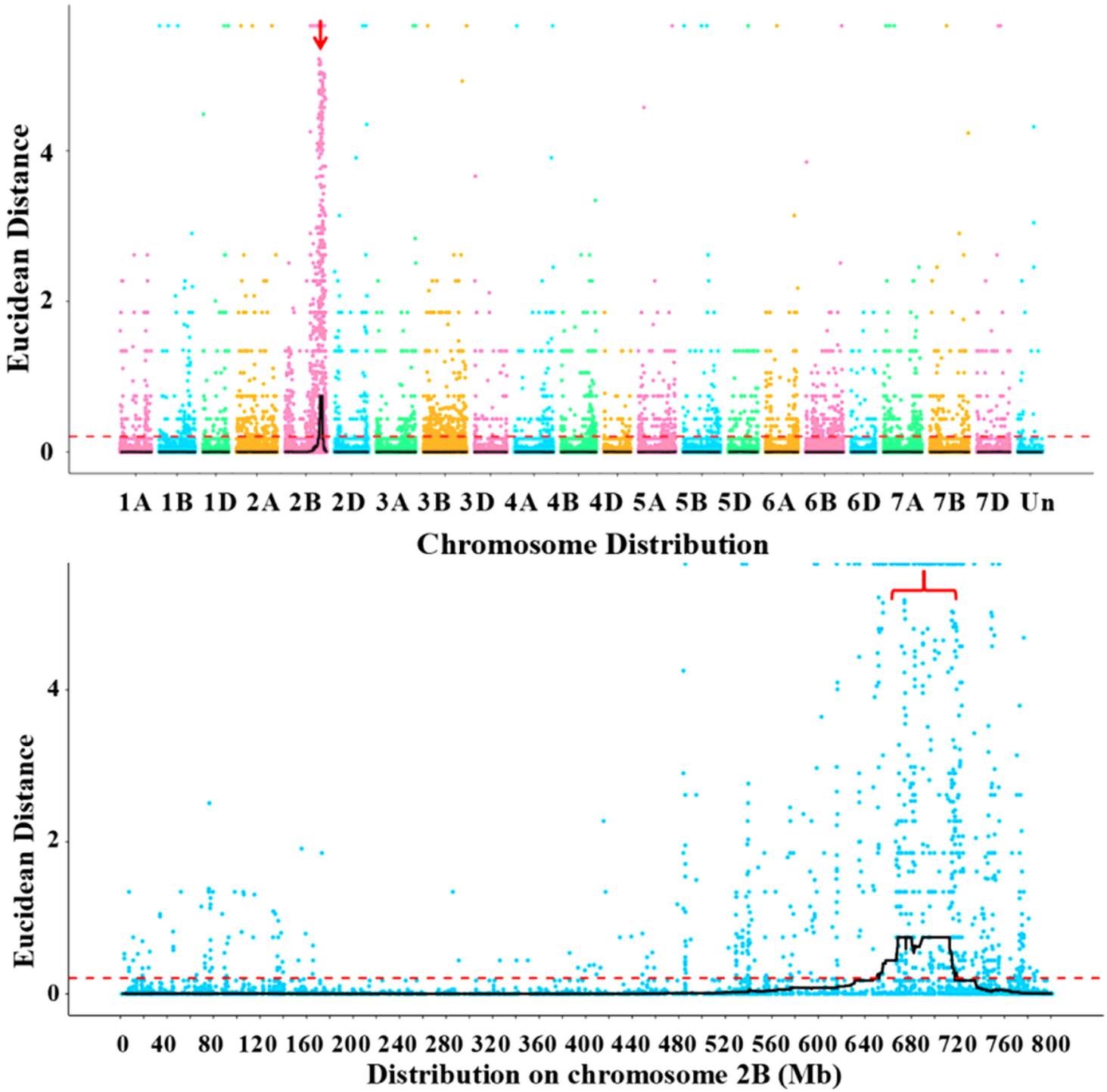

Figure 2

Distribution of the SNPs with differences between the resistant and susceptible bulks on 21 chromosomes (A) and on chromosome arm 2B (B) based on ΔSNP index value.

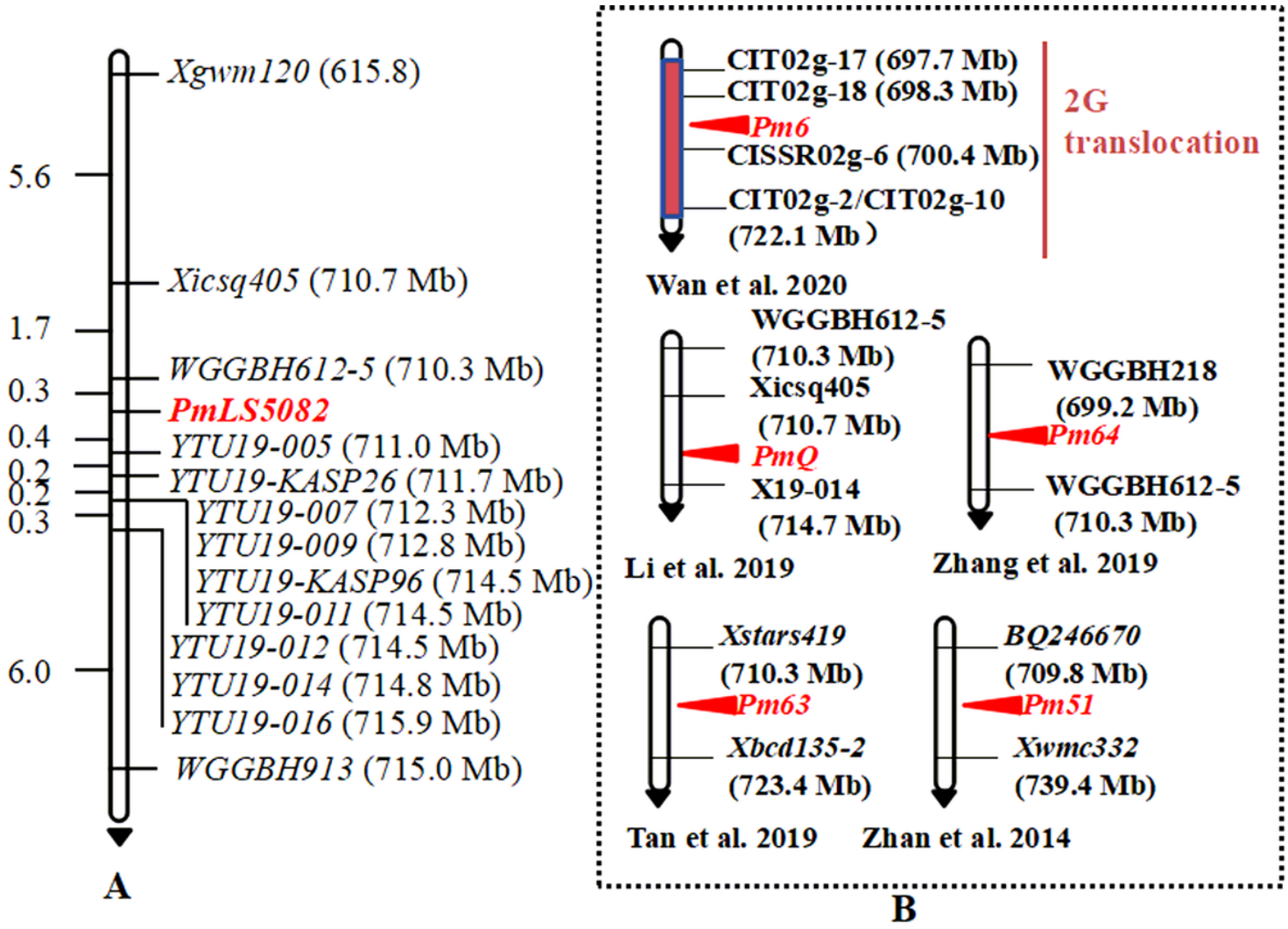

Figure 3

Linkage map of PmLS5082 using the F2:3 families of LS5082 × Shannong 29 (A) and the physical locations of catalogued Pm genes on chromosome arm 2BL (B). Genetic distances in cM are showed to the left. The black arrows point to the centromere.

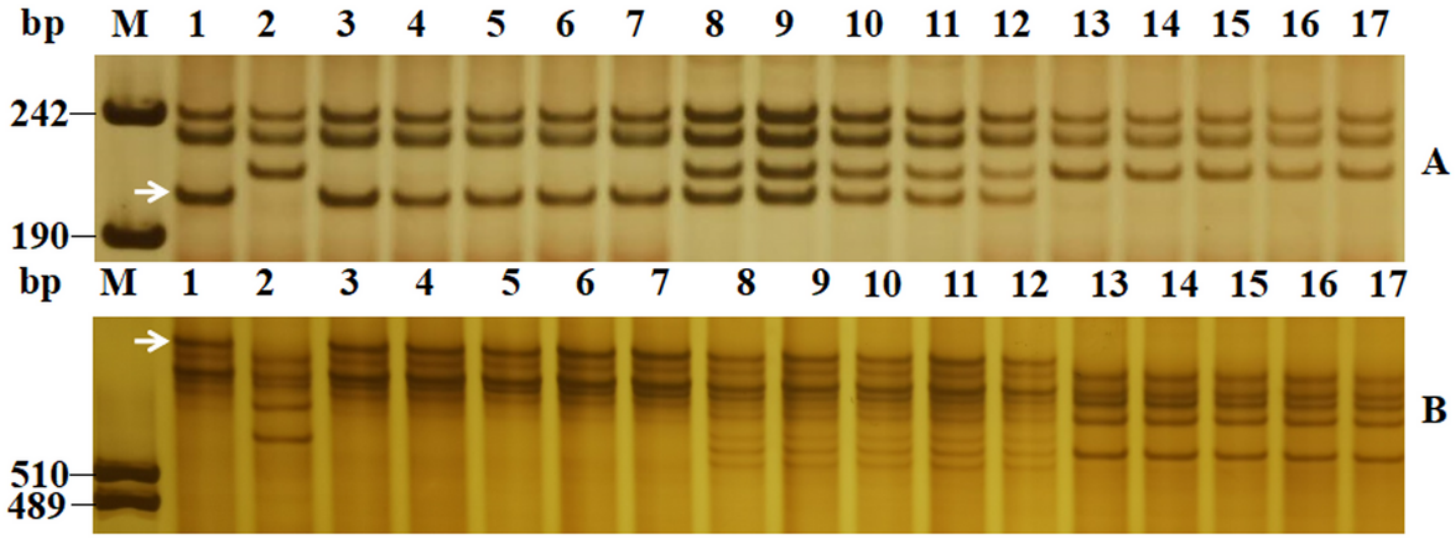

Figure 4

Amplification patterns of PmLS5082-linked markers YTU19-005 (A) and YTU19-014 (B) in genotyping LS5082, Shannong 29 and random selected F2:3 families of LS5082 × Shannong 29. Lane M, pUC18 Msp I; lanes 1-2: parents LS5082 and Shannong 29; lanes 3-7: homozygous resistant F2:3 families; lanes 8-12, homozygous susceptible F2:3 families; lanes 13-17 heterozygous F2:3 families. The white arrows indicate the polymorphic bands in LS5082.

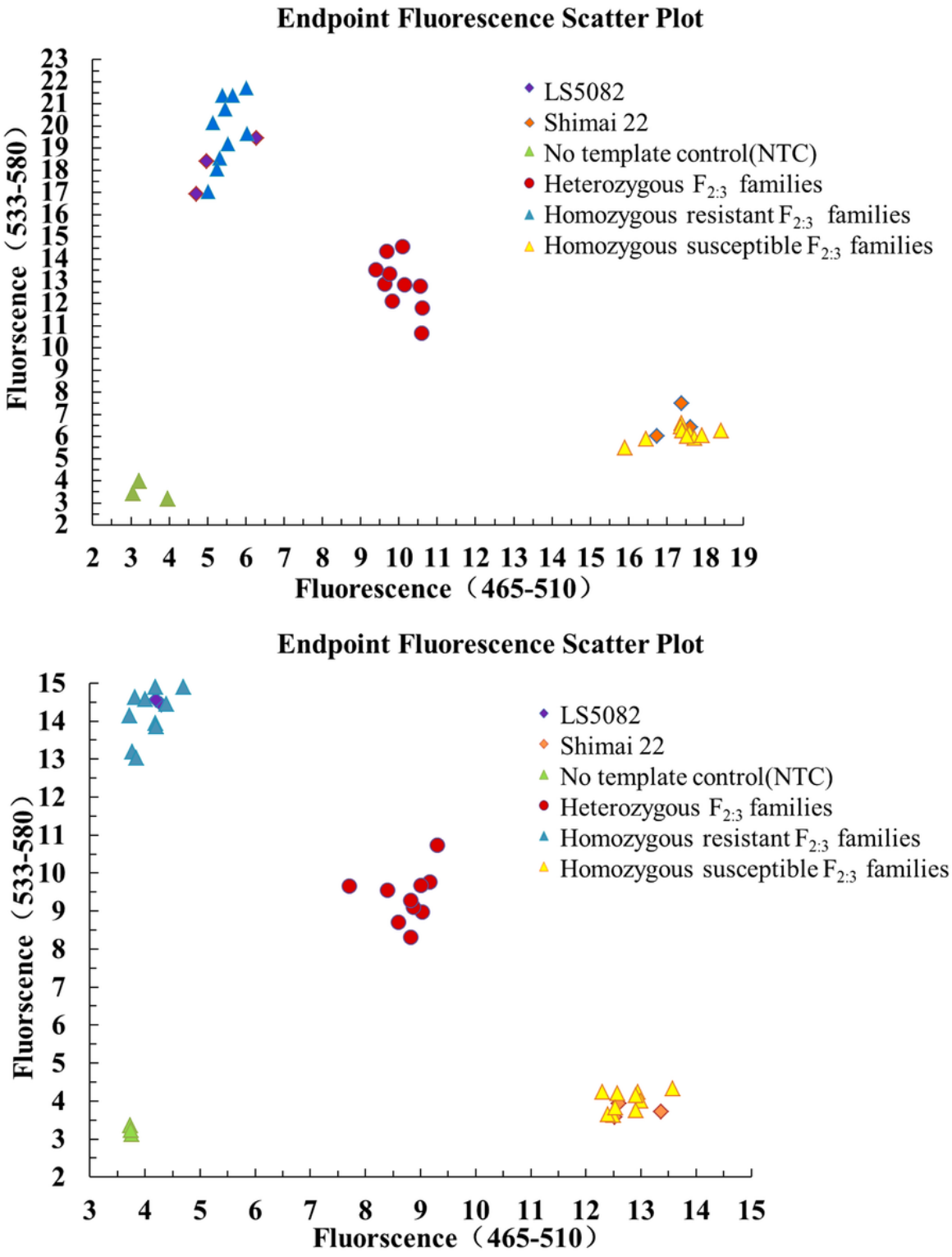

Figure 5

The genotyping results of the PmLS5082-linked KASP markers YTU19-KASP26 (A), YTU19-KASP96 (B) for random selected F2:3 families of LS5082 × Shannong 29.

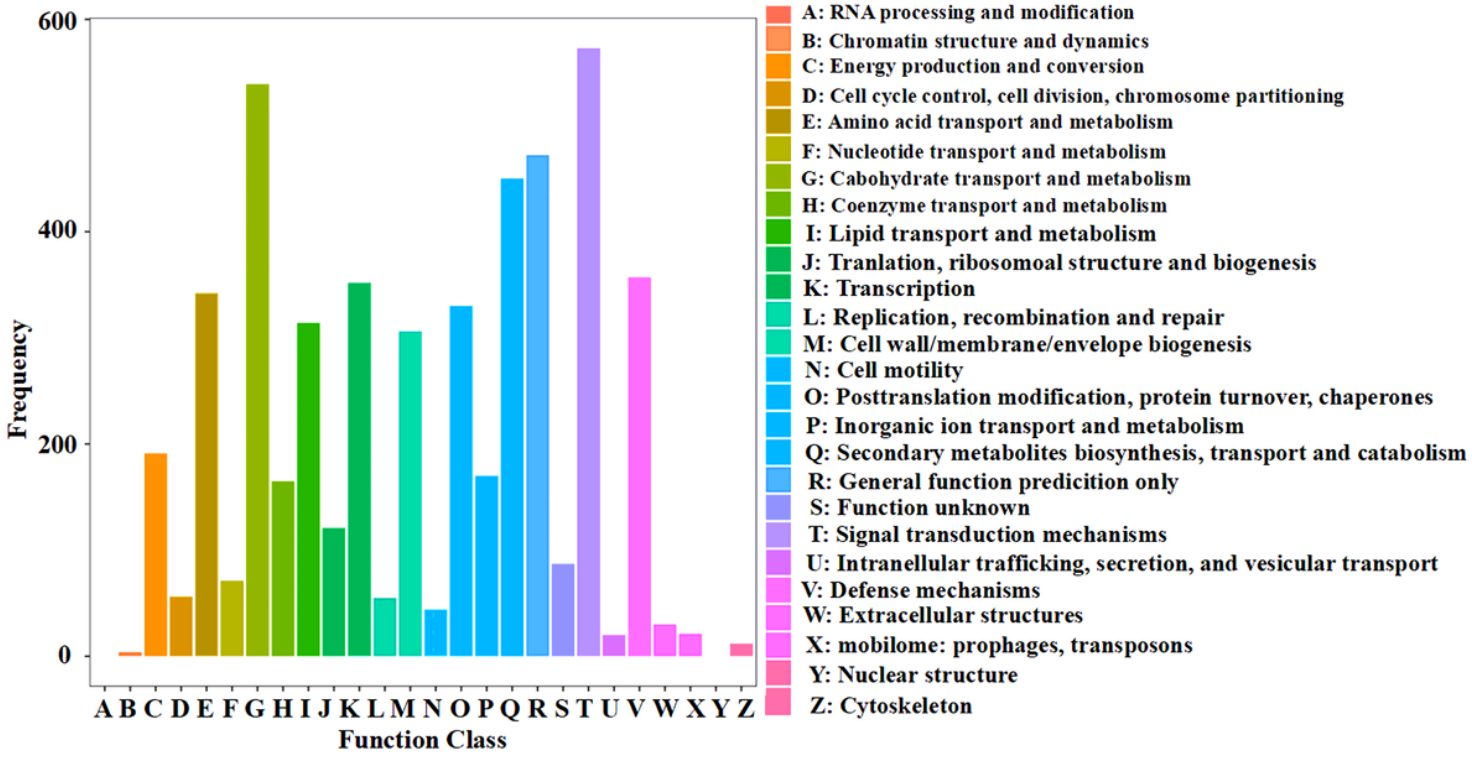

Figure 6

Clusters of orthologous groups (COG) analysis of the DEGs with consistent differences between resistant and susceptible bulks derived from the F2:3 families of LS5082 × Shannong 29.

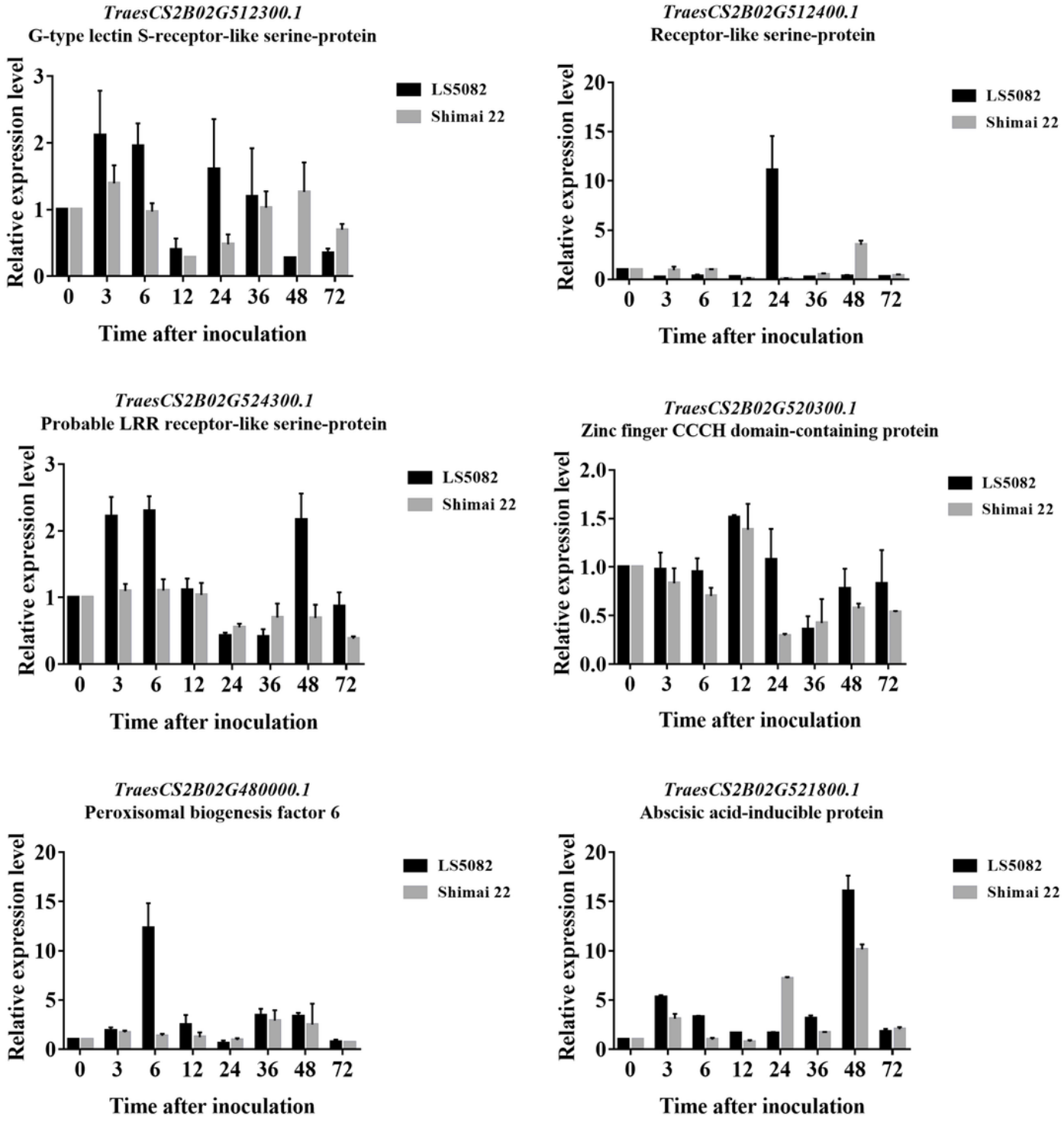

Figure 7

Expression profiles of TraesCS5D01G018000 (a), TraesCS5D01G117600 (b), TraesCS5D01G104700 (c), TraesCS5D01G105200 (d), TraesCS5D01G099200 (e), and TraesCS5D01G111400 (f) in each corresponding stage of LS5082 and Shannong 29 after Bgt infection.

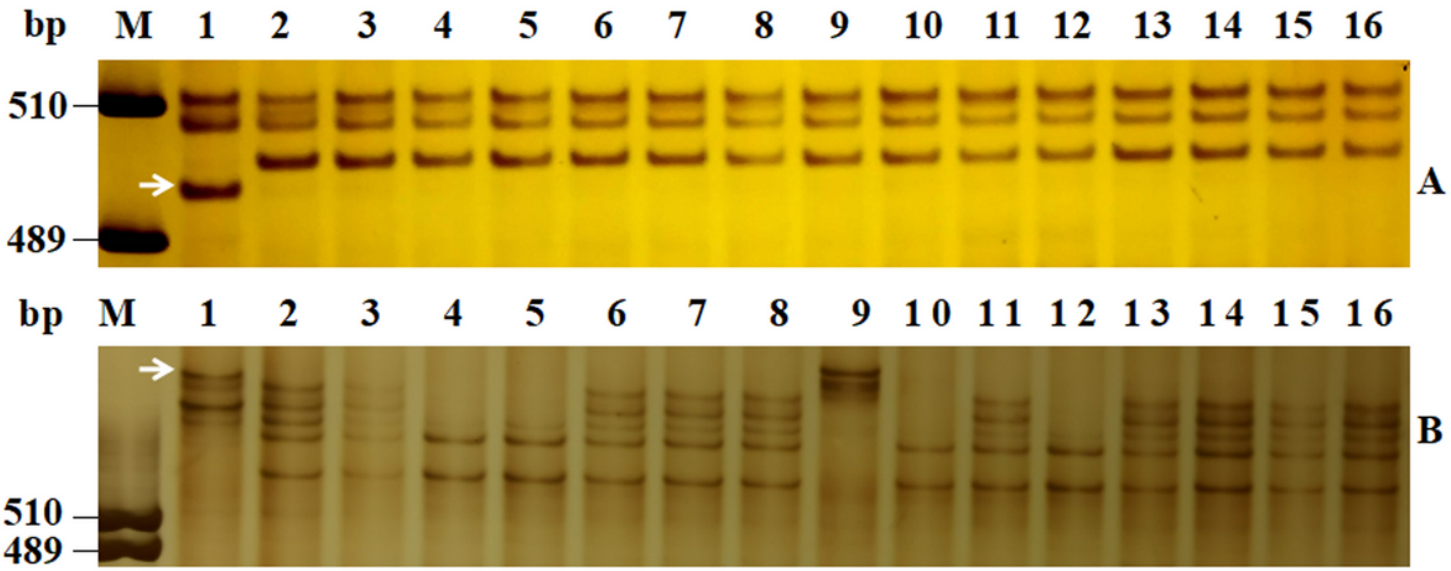

Figure 8

Amplification patterns of PmLS5082-linked markers YTU19-005 (A) and YTU19-014 (B) in LS5082, Shannong 29 and 15 wheat cultivars/breeding lines susceptible to powdery mildew. M, DNA marker pUC18 Msp I; lanes 1 and 2, LS5082 and Shannong 29; lanes 3-16: Yannong 187, Shannong 1538, Zhoumai 27, Zhongyu 1311, Yan 1212, Jimai 229, Zhongyu 9398, Womai 8, Danmai 13, Daimai 2173, Wunong 6, Zhengmai 0856、Liangxing 619、Taimai 1918. The white arrows indicate the polymorphic bands in LS5082.

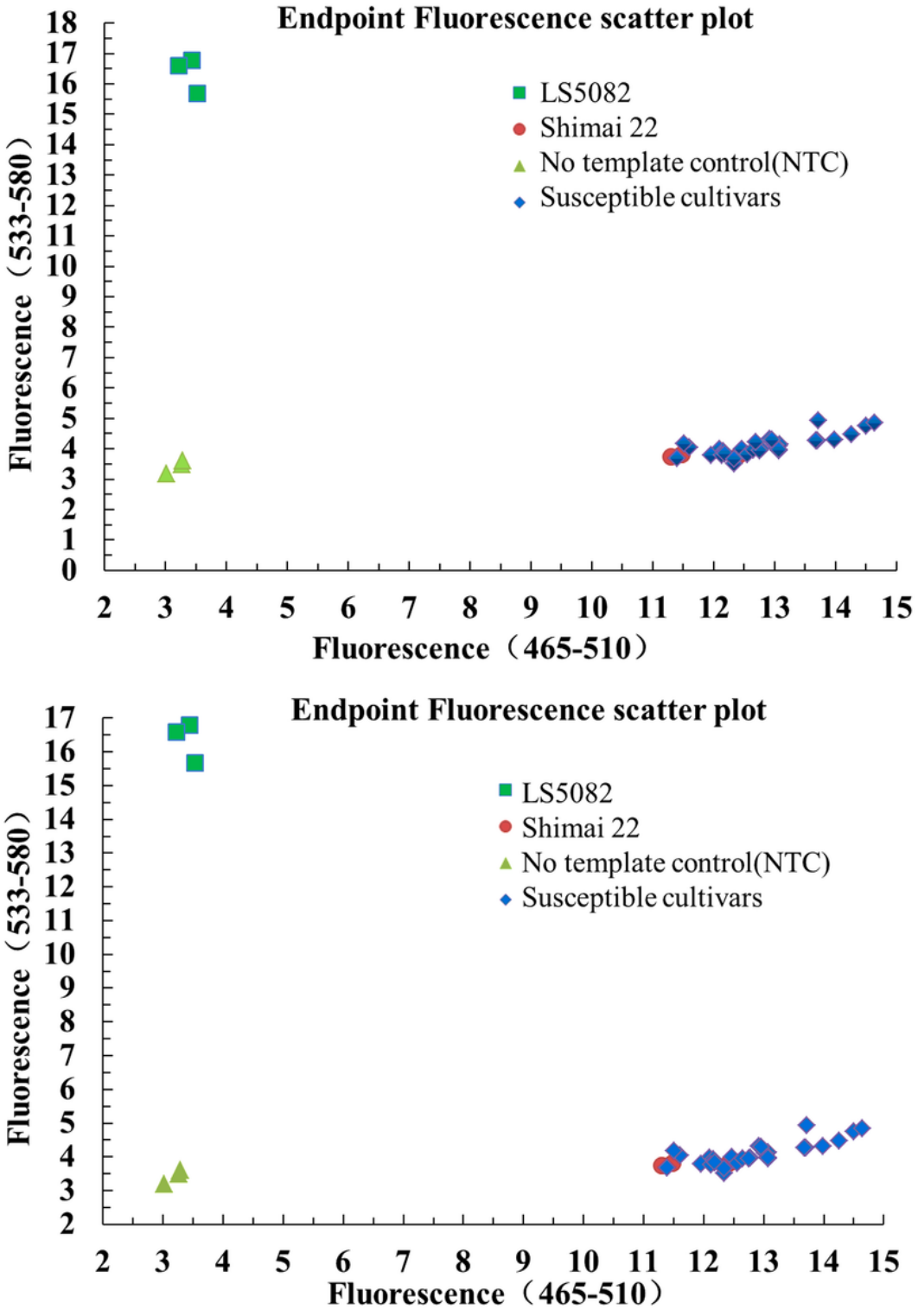

Figure 9

The genotyping results of the PmLS5082-linked KASP markers YTU19-KASP26 (A), YTU19-KASP96 (B) for LS5082, Shannong 29 and 15 wheat cultivars/breeding lines susceptible to powdery mildew. M, DNA marker pUC18 Msp I; lanes 1 and 2, LS5082 and Shannong 29; lanes 3-16: Yannong 187, Shannong 1538, Zhoumai 27, Zhongyu 1311, Yan 1212, Jimai 229, Zhongyu 9398, Womai 8, Danmai 13, Daimai 2173, Wunong 6, Zhengmai 0856、Liangxing 619、Taimai 1918.

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