

Article

GWAS-Guided Development of KASP Markers Associated with Soybean Seedling Resistance to *Fusarium solani*

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Abstract

Fusarium root rot, primarily caused by *Fusarium solani*, is a damaging soil-borne disease that restricts soybean growth and reduces yield. In the present study, a panel of 330 soybean germplasm accessions was inoculated with *F. solani*. Disease responses were evaluated using the disease severity index (DSI). The soybean accessions displayed substantial variation in their susceptibility to *F. solani*. Based on DSI, 39 accessions were classified as highly resistant, 94 as resistant, 125 as susceptible, and 72 as highly susceptible, accounting for 11.82%, 28.48%, 37.88%, and 21.82% of the panel, respectively. The genome-wide association study (GWAS) was performed using a genotyping dataset of 627,436 high-quality single nucleotide polymorphisms (SNPs) and two models, Fixed and random model Circulating Probability Unification (FarmCPU) and mixed linear model (MLM). Both GWAS models detected putative SNP associations across seven chromosomes. Based on SNP allelic-effect analysis and gene function annotation, eight genes were prioritized and subsequently evaluated by quantitative reverse transcription PCR (qRT-PCR) for their responses to *F. solani* infection. Two Kompetitive allele-specific PCR (KASP) markers, KASP-S13_37431242 and KASP-S13_37529208, were developed from resistance-associated SNPs on chromosome 13 and evaluated across the diverse soybean association panel used in this study. Accessions carrying the favorable genotypes of these markers were enriched for resistant germplasm, with positive predictive values of 65.52% and 66.67%, respectively, indicating their potential value for preliminary favorable-allele tracking and germplasm prioritization. Collectively, these results improve our understanding of the genomic components underlying soybean responses to *F. solani*. The identified loci, candidate genes, and KASP assays provide a basis for further functional validation and the development of multi-locus strategies for improving soybean resistance to *Fusarium* root rot.

Keywords: soybean; *Fusarium solani*; genome-wide association study (GWAS); KASP



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1. Introduction

Soybean [*Glycine max* (L.) Merr.] is a major food and oilseed crop worldwide. Its seeds are rich in high-quality protein and edible oil. However, its production is seriously affected by a range of soil-borne diseases. *Fusarium* root rot is among the most destructive of these diseases and can markedly impair plant growth and yield. It is prevalent in many soybean-growing areas worldwide. A range of soil-borne *Fusarium* species have been

reported to cause soybean root rot. The most commonly reported causal agents include *F. oxysporum*, *F. graminearum*, *F. solani*, *F. equiseti*, and *F. proliferatum* [1]. *F. solani* is a major soil-borne pathogen involved in soybean root rot [2]. It may also cause the characteristic foliar symptoms observed in soybean sudden death syndrome (SDS) [3]. Following infection, *F. solani* colonizes root tissues and may interfere with water and nutrient transport, resulting in root lesions, vascular discoloration, wilting, stunted growth, and yield loss [4]. At advanced stages, chlorosis, stunted growth, wilting, and premature leaf loss may occur, resulting in marked yield reductions. Despite the economic importance of this disease, strong resistance to *F. solani* is uncommon in available soybean germplasm. Only a limited number of resistance-related genes have been identified, which complicates the breeding of resistant cultivars.

Given the limited availability of characterized resistance genes, genome-wide association studies (GWAS) provide an effective approach for dissecting the genetic basis of soybean resistance to *Fusarium* root rot. This approach has uncovered numerous single nucleotide polymorphisms (SNPs) and candidate genes related to resistance against *Fusarium* spp. Several loci linked specifically to *F. solani* resistance have also been reported in common bean [5], pea [6], and sweet potato [7]. In soybean, GWAS has successfully identified resistance-associated loci for multiple biotic and abiotic stress traits, including abiotic stress tolerance [8,9], soybean sudden death syndrome [10], and soybean mosaic virus resistance [11].

This approach has also been applied to identify genomic regions associated with soybean resistance to root rot caused by different *Fusarium* species. In one study, a panel of 350 soybean accessions was examined, most of which originated from northeastern China. Eight SNPs were linked to resistance against *F. oxysporum*, and the strongest association signals were detected on chromosome 6 [12]. Another GWAS evaluated 268 USDA soybean accessions for resistance to *F. proliferatum* and identified a major QTL on chromosome 11, together with linked SNPs and three candidate genes, *Glyma.11g209000*, *Glyma.11g144500*, and *Glyma.07g087500* [13]. In addition, a GWAS of 248 USDA soybean accessions identified six SNP loci associated with resistance to *F. graminearum*, as well as eight resistant accessions that may serve as parental materials for resistance breeding [14]. A separate GWAS mapped genomic regions contributing to soybean responses to *F. proliferatum*, *F. sporotrichioides*, *F. subglutinans*, and *F. graminearum*. However, no common QTLs were detected among these pathogens, indicating that resistance to different *Fusarium* species may involve distinct genetic mechanisms [15].

Together, these observations suggest that resistance to different *Fusarium* species in soybean may involve distinct genetic components. Therefore, SNP allelic-effect analysis, together with candidate gene annotation, can help refine GWAS signals and identify favorable alleles associated with resistance. Related studies of other soil-borne pathogens have further broadened our understanding of the genetic factors governing resistance to root diseases in soybean. The development of molecular markers has facilitated genetic mapping, germplasm evaluation, marker-assisted selection, and gene pyramiding in crop breeding. Among these marker systems, SNP markers have become particularly useful because of their abundance, stability, and suitability for high-throughput genotyping. Kompetitive allele-specific PCR (KASP), as a fluorescence-based SNP genotyping platform, provides a simple, accurate, flexible, and cost-effective approach for converting trait-associated SNPs or InDels into breeder-friendly markers and has been widely applied in crop improvement programs [16]. For example, a recent study developed a comprehensive TaqMan[®] genotyping panel for soybean cyst nematode (SCN) resistance through the identification of resistance-associated genes and allelic variants [17]. These studies indicate that molecular markers can effectively accelerate the utilization of resistance-associated

loci and favorable alleles in breeding programs. Despite substantial progress in molecular marker development for soybean diseases such as soybean cyst nematode and soybean mosaic virus, markers specifically associated with soybean resistance to *F. solani* remain relatively limited.

2. Materials and Methods

2.1. Plant Materials and *Fusarium solani* Isolate

A panel of 330 soybean germplasm accessions representing diverse geographical origins was evaluated in this study. Most accessions originated from China, while the remaining accessions were introduced from Canada, the United States, Japan, Russia, Ukraine, and Italy. The soybean germplasm panel used in this study was supplied by the Soybean Research Institute of Northeast Agricultural University (NEAU), China. The panel was independently assembled from geographically diverse germplasm resources and was not derived from a single biparental population or a narrowly related breeding population.

The greenhouse trial was conducted at the Soybean Research Institute of NEAU from September to December 2025. The *Fusarium solani* isolate Fs01 used for inoculation was provided by the Soybean Research Institute of NEAU. Its identification as *F. solani* was supported by ITS sequence analysis. The ITS sequence obtained in this study showed 99.30% identity and 100% query coverage with the type-material ITS sequence of *F. solani* CBS 140079 (GenBank accession no. NR_163531.1). Before inoculum production, the fungal culture was maintained on potato dextrose agar (PDA) at 4 °C in darkness.

2.2. Inoculum Preparation and Inoculation Procedure

The pathogenicity of the *F. solani* isolate was confirmed on soybean seedlings before inoculum preparation. Inoculated seedlings showed typical root rot symptoms, while the non-inoculated controls remained healthy. To generate the inoculum, the *F. solani* isolate was propagated on PDA plates for 7 days at 25 °C under dark conditions. Sorghum grains [*Sorghum bicolor* (L.) Moench] were immersed in sterile water overnight. After soaking, the grains were divided among 250 mL Erlenmeyer flasks at 100 g per flask and sterilized by autoclaving. Mycelial plugs were excised from the actively growing margins of 7-day-old *F. solani* cultures and transferred to sterilized sorghum grains. The flasks were then incubated in darkness for 14 days until the grains were fully colonized. To ensure uniform fungal growth, each flask was shaken for approximately 5 min once daily. The colonized grains were subsequently ground and mixed with sterile vermiculite at a 1:50 volume ratio. This mixture was used as the inoculum.

To determine the viable inoculum level and check the uniformity of the final substrate, serial dilution plating was performed with minor modifications to the method described by Davis et al. [18]. For each trial, five 1.0 g samples were taken from different positions of the well-mixed inoculated substrate before sowing. Each sample was suspended in 10 mL of sterile water containing 0.05% Tween 20 and shaken for 30 min. The suspension was then filtered through eight layers of sterile gauze to remove coarse particles. The filtrate was serially diluted 10-fold to 10^{-4} , and 100 μ L aliquots of suitable dilutions were spread onto PDA plates. After incubation at 25 °C in darkness for 48 h, plates with countable, well-separated colonies were selected for colony counting. Viable propagule density was calculated and expressed as CFU g⁻¹ of substrate. The final inoculated substrate contained approximately 3.0×10^6 CFU g⁻¹, with a mean within-batch coefficient of variation of 7.81%, indicating acceptable inoculum uniformity.

Soybean seeds with no visible damage were sown in 250 mL pots containing the inoculated medium. For each accession, 30 seeds were sown per trial. After removing failed seedlings, at least 24 plants per accession were available for disease evaluation in each trial.

The pots were arranged in a randomized complete block design and maintained under greenhouse conditions. The photoperiod was set to 12 h light/12 h dark. Day and night temperatures were maintained at 28 and 18 °C, respectively, and relative humidity was kept at approximately 85%. No fertilizer was applied during plant growth. Environmental conditions were monitored regularly to minimize variation among experiments. Three independent trials were performed. The mean disease severity index across the three trials was used for subsequent analyses.

2.3. Evaluation of Resistance to *Fusarium solani* in 330 Soybean Germplasm Accessions

A panel of 330 soybean germplasm accessions was evaluated for resistance to *F. solani* using a modified protocol based on the method described by Chang et al. [19]. Disease symptoms were assessed two weeks after inoculation. Each plant was assigned a disease score of 0, 1, 3, 5, or 7 according to symptom severity. A score of 0 indicated the absence of visible symptoms and normal development. Plants were scored as 1 when mild yellowing was restricted to the primary root, while the lateral roots and overall growth remained unaffected. A score of 3 denoted browning of the primary root, necrotic lesions on the lower portion of the primary root and lateral roots, and a slight reduction in shoot growth. Plants received a score of 5 when the primary root was completely brown, clear lesions developed on the lateral roots, and shoot development was strongly suppressed. A score of 7 was assigned to plants with dark-brown, fractured primary roots and blackened lateral roots, as shown in Supplementary Figure S1. These plants were either dead or severely stunted. Disease scoring was performed blindly using coded accession labels according to the predefined disease rating scale. The disease severity index (DSI) was calculated according to Wang et al. [20] using Equation (1):

$$DSI = \frac{\sum (\text{Rating value} \times \text{Number of plants with this rating})}{\text{Total number of plants surveyed} \times 7} \times 100\% \quad (1)$$

Soybean accessions were assigned to five response categories according to their DSI: immune (I, DSI = 0), highly resistant (HR, $0 < DSI \leq 20$), resistant (R, $20 < DSI \leq 40$), susceptible (S, $40 < DSI \leq 60$), and highly susceptible (HS, $DSI > 60$). For marker screening, accessions belonging to the I, HR, and R groups were treated as resistant. Broad-sense heritability (H^2) was estimated from accession-level DSI values across replicated trials using Equation (2), following Ma et al. [21]. This estimate was used as an overall measure of phenotypic consistency across replicated trials and of the heritable component of disease resistance. In Equation (2), σ_G^2 and σ_P^2 represent the genetic and phenotypic variance, respectively. Additionally, MSG, MSE, and rep correspond to the mean square of genotype, mean square error, and the number of replications, respectively.

$$H^2 = \frac{\sigma_G^2}{\sigma_P^2}, \sigma_G^2 = \left(\frac{MSG - MSE}{rep} \right), \sigma_P^2 = \left(\frac{MSG - MSE}{rep} \right) + MSE, H^2 = \frac{\left(\frac{MSG - MSE}{rep} \right)}{\left(\frac{MSG - MSE}{rep} \right) + MSE} \quad (2)$$

2.4. SNP Genotyping and Genome-Wide Association Analysis

Genomic DNA was isolated from young leaf tissues collected from the 330 soybean germplasm accessions 10 days after emergence using the cetyltrimethylammonium bromide (CTAB) method [22]. DNA concentration and purity were measured using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only DNA samples with A260/A280 ratios between 1.8 and 2.0 were selected for subsequent genotyping.

SNPs with a minor allele frequency (MAF) below 0.05 or missing rates greater than 10% were excluded. Population stratification was characterized by principal component

analysis (PCA) implemented in GAPIT version 3 [23]. Genome-wide association analysis was performed using GAPIT 3.0 in R version 4.5.2. Both the Fixed and random model Circulating Probability Unification (FarmCPU) and the mixed linear model (MLM) were applied to identify SNPs associated with resistance to *F. solani*. Quantile–quantile (QQ) plots were generated to compare the observed and expected distributions of p -values. The genomic inflation factor (λ_{GC}) was calculated as the median of the observed chi-square statistics divided by the expected median of a chi-square distribution with one degree of freedom. SNPs with $-\log_{10}(p) \geq 4.0$ were considered putative resistance-associated SNPs and were retained for downstream candidate gene analysis [24]. The selection of GWAS significance thresholds requires a balance between controlling false-positive associations and retaining potentially meaningful loci with small or moderate effects [24]. Given the quantitative nature of *F. solani* resistance and its likely involvement of multiple loci with small effects, $-\log_{10}(p) \geq 4.0$ was used as a suggestive threshold to avoid missing biologically relevant associations. More stringent genome-wide thresholds, including those based on Bonferroni correction, FDR correction, permutation testing, or the effective number of independent markers, may reduce false-positive associations. However, they may also make small-effect loci more difficult to detect at the discovery stage [25,26]. Signals detected by the two models were compared, and closely linked or duplicated SNPs representing the same local association signal were collapsed into representative loci for downstream candidate gene analysis.

2.5. Candidate Gene Identification, Annotation, and Enrichment Analysis

A 200 kb window was defined around each GWAS-associated SNP, extending 100 kb upstream and 100 kb downstream. Genes within this region were retained for subsequent candidate gene analysis [27]. This fixed ± 100 kb window was applied consistently across representative SNPs, and locus-specific LD blocks were not reconstructed in this study. Gene information and functional annotation were obtained from the Williams 82 soybean reference genome assembly Wm82.a2.v1 in Phytozome [28].

Candidate gene prioritization was performed using a sequential, non-weighted procedure. Genes were prioritized when: (i) a resistance-associated SNP was located within a potentially functional region of the gene, particularly an exon or promoter; (ii) the alternative SNP alleles were associated with a significant difference in DSI; and (iii) the predicted gene function was related to plant defense, signaling, transcriptional regulation, redox homeostasis, protein metabolism, or lipid metabolism based on genome annotation, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, and the published literature. Genes satisfying all three criteria were retained for experimental expression analysis. Public expression databases were not used as a primary prioritization criterion. The annotated candidate genes were analyzed for enrichment of GO terms and KEGG pathways and visualized using bubble plots. In the bubble plots, enrichment level was represented by $-\log_{10}(p)$.

2.6. RNA Extraction and qRT-PCR Analysis

Expression profiles of the selected genes were examined in the resistant accession HF47 and the susceptible accession SN17 after *F. solani* challenge. Seven-day-old seedlings were treated with a conidial suspension of the pathogen. Control seedlings were treated with sterile water. Root samples were harvested at 72 h post-inoculation, a time point selected with reference to a recent study of soybean resistance to *Rhizoctonia solani* root rot in which candidate-gene expression responses were detected at 72 h after inoculation [29]. Total RNA was reverse-transcribed with the PrimeScript™ RT Reagent Kit containing gDNA Eraser (Takara Bio Inc, Kusatsu, Shiga, Japan) in accordance with the manufacturer's

protocol. Gene-specific primers used for qRT-PCR analysis were designed using the Primer-BLAST tool available on the NCBI website (accessed on 11 March 2026). Primer-BLAST. Supplementary Table S6 provides the primer sequences, expected amplicon sizes, and amplification efficiencies for all qRT-PCR primer pairs.

Quantitative expression assays were conducted with SYBR Green Master Mix (Toyobo, Osaka, Japan). Amplification started with denaturation at 94 °C for 5 min. The PCR program consisted of 45 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s. Primer amplification efficiencies were determined from standard curves generated using five-point, five-fold serial dilutions of pooled cDNA. Amplification specificity was verified by melting-curve analysis over a temperature range of 55–100 °C. Representative melt curves for all primer pairs are shown in Supplementary Figure S4. *GmActin4* (*Glyma.12g063400*) was selected as the internal reference gene. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Three independent biological replicates were included for each treatment, with three technical replicates per biological sample. Results are expressed as the mean $\pm$ standard error (SE). For each gene, relative expression levels among the four genotype–treatment combinations were compared using Tukey’s HSD test. Different lowercase letters indicate significant differences among the four combinations at an adjusted $p < 0.05$. The pairwise adjusted p values from Tukey’s HSD test are provided in Supplementary Table S7.

2.7. KASP Marker Development and Preliminary Evaluation

SNPs were chosen for KASP marker development based on the strength of their GWAS signals, their significant allelic effects on DSI, and their locations within candidate genes. Flanking sequences for each target locus were retrieved from the soybean reference genome and used to design the primers.

Each assay comprised two allele-specific forward primers, F1 and F2, together with a common reverse primer (R). The 3′-terminal base of each forward primer corresponded to one of the two alleles at the target SNP. Standard FAM- and HEX-compatible tails were added to F1 and F2, respectively, for fluorescence-based allelic discrimination. The common reverse primer was positioned within a conserved region near the SNP. Primer specificity was examined against the soybean genome using Primer-BLAST. Primer pairs were discarded when nonspecific amplification was predicted or when additional polymorphisms occurred within the primer-binding sites.

Genotyping was performed on an ABI 7500 Real-Time PCR System using KASP V4.02 $\times$ Master Mix, following the manufacturer’s protocol. Sequence alignments around S13_37431242 and S13_37529208 are presented in Supplementary Figure S3. The corresponding primer sequences are provided in Supplementary Table S8. Genotypes were assigned as homozygous or heterozygous according to the fluorescence cluster profiles.

The marker genotypes were compared with the disease phenotypes obtained after artificial inoculation with *F. solani*. Accessions with DSI values ≤ 40 were classified as resistant, whereas those with values > 40 were considered susceptible. The favorable homozygous genotype was considered a positive marker prediction. The proportion of resistant accessions among those carrying the favorable genotype was expressed as the positive predictive value (PPV). Following Tan et al. [30], marker sensitivity and specificity were calculated as sensitivity = $TP/(TP + FN)$ and specificity = $TN/(TN + FP)$, respectively, where TP, FP, FN, and TN represent true-positive, false-positive, false-negative, and true-negative predictions.

3. Results and Analysis

3.1. Phenotypic Variation in Resistance to *Fusarium solani* Among Soybean Germplasm Accessions

Resistance to *F. solani* was assessed in a panel of 330 soybean accessions. The disease severity index (DSI) for each accession is presented in Supplementary Table S1. DSI scores varied from 4.04 to 90.36, showing marked differences in disease response among the tested materials. The trait displayed a broad, approximately unimodal distribution (Figure 1). This continuous distribution suggests that resistance to *F. solani* is likely a quantitative trait controlled by multiple loci. The broad-sense heritability (H^2) of DSI was 0.77. The high heritability indicates that genetic factors contributed substantially to the observed variation in DSI.

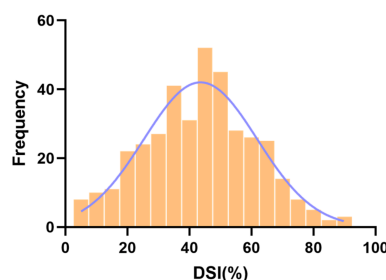


Figure 1. Distribution of DSI among 330 soybean germplasm accessions following *F. solani* inoculation. Orange bars indicate the frequency distribution of DSI values, and the purple curve represents the fitted normal distribution.

This level of variation provided a suitable basis for the subsequent genome-wide association study. None of the accessions met the criterion for immunity. Thirty-nine accessions fell into the highly resistant category, whereas 94 were assigned to the resistant group. These two classes represented 11.82% and 28.48% of the population, respectively. In contrast, 125 accessions were classified as susceptible and 72 as highly susceptible, representing 37.88% and 21.82% of the panel, respectively (Table 1). Overall, 133 accessions with $DSI \leq 40$ were considered resistant, while the remaining 197 accessions were susceptible to *F. solani* to varying degrees.

Table 1. Evaluation of resistance to *F. solani* among 330 soybean germplasm accessions.

Resistance Category	DSI	Soybean Accessions	Percentage (%)
Immune, I	DSI = 0	0	0.00%
Highly Resistant, HR	$0 < DSI \leq 20$	39	11.82%
Resistant, R	$20 < DSI \leq 40$	94	28.48%
Susceptible, S	$40 < DSI \leq 60$	125	37.88%
Highly Susceptible, HS	$DSI > 60$	72	21.82%

3.2. Genome-Wide Association Analysis for Resistance to *Fusarium solani*

The 330 soybean germplasm accessions were analyzed using SNPs generated from resequencing. SNPs with a minor allele frequency below 0.05 or more than 10% missing genotypes were removed. A total of 627,436 SNPs passed the filtering criteria and were retained for subsequent analyses. Their distribution and density across the 20 soybean chromosomes are summarized in Supplementary Table S5. Examination of the first ten principal components revealed a clear inflection point at PC3 in the scree plot (Supplementary Figure S2a). PC1, PC2, and PC3 explained 9.22%, 4.99%, and 3.39% of the total variance, respectively, together accounting for 17.60%. Based on the inflection point, the first three principal components were included as covariates in the GWAS models to account for the

major axes of population structure. The three-dimensional PCA plot and pairwise kinship matrix are shown in Supplementary Figure S2b,c. Pairwise kinship coefficients among the 330 soybean accessions were generally low, indicating relatively weak genetic relatedness within the association panel.

GWAS was subsequently conducted using the FarmCPU and MLMs to identify SNP loci associated with DSI following *F. solani* inoculation. In the QQ plots, the observed and expected $-\log_{10}(p)$ values showed close agreement across most quantiles, with deviations from the diagonal occurring mainly in the upper tail (Figure 2B,D). The genomic inflation factors (λ GCs) were 0.94 for FarmCPU and 1.06 for MLM. These values indicated slight deflation in the FarmCPU analysis and slight inflation in the MLM analysis, with no substantial systematic deviation in either model. Together, the QQ plots and λ GC values suggested that population structure and kinship effects were largely accounted for in both association analyses. SNPs with $-\log_{10}(p) \geq 4.0$ were regarded as putative resistance-associated SNPs and retained for subsequent analysis. Based on this criterion, FarmCPU detected 120 putative SNPs distributed across seven chromosomes (Figure 2A). Most association signals were clustered on chromosome 13, with additional signals detected on several other chromosomes, including chromosomes 6, 16, and 18. The MLM analysis identified 169 putative SNPs on chromosomes 3, 6, 10, 13, 16, 17, and 18 (Figure 2C). Similar to the FarmCPU results, the strongest association peak detected by MLM was located on chromosome 13. A total of 120 SNPs were consistently identified by both FarmCPU and MLM, indicating substantial agreement between the two models. Detailed information on the resistance-associated SNPs is provided in Supplementary Table S2.

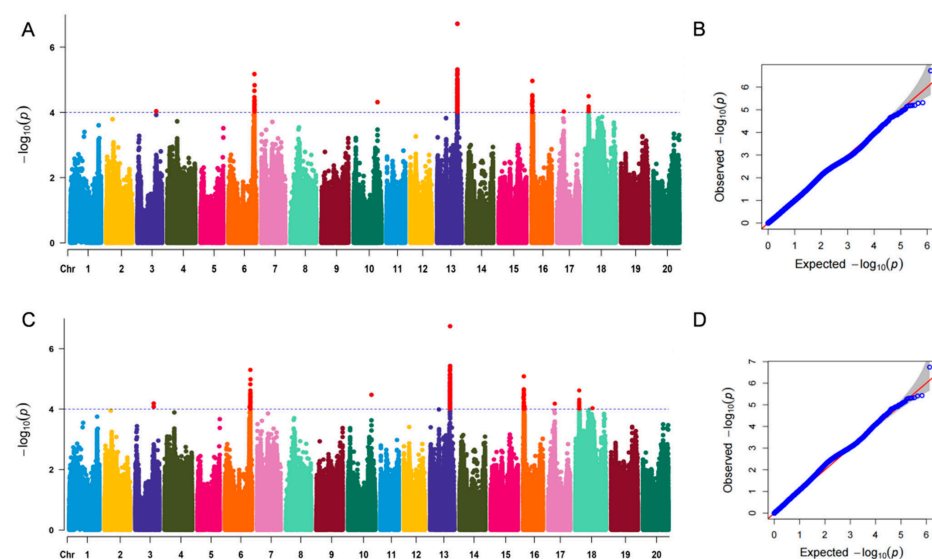


Figure 2. Manhattan plots and quantile–quantile (QQ) plots of FarmCPU (A,B) and MLM (C,D) analyses for the DSI following *F. solani* inoculation. The horizontal blue dashed line indicates the suggestive association threshold of $-\log_{10}(p) = 4.0$, and SNPs above this threshold are shown in red. Different colors represent different chromosomes. The gray shaded areas in the QQ plots represent the confidence intervals.

3.3. Functional Annotation and Enrichment Analysis of Candidate Genes

Screening of the genomic intervals surrounding the GWAS-associated SNPs yielded 199 candidate genes, the complete list of which is provided in Supplementary Table S3. Functional annotation revealed that many of these genes were associated with functions related to signal transduction, transport activity, stress responses, and secondary metabolism. GO enrichment analysis identified overrepresented terms related to lipid metabolic processes, calcium ion transport, the extracellular region, and oxidoreductase activity among the se-

lected genes. These functions may contribute to the soybean defense response (Figure 3A). KEGG pathway analysis further indicated enrichment in metabolic and biosynthetic pathways, particularly phenylpropanoid biosynthesis, secondary metabolite biosynthesis, and nicotinate and nicotinamide metabolism (Figure 3B), all of which are known to contribute to plant defense and adaptation under biotic stress.

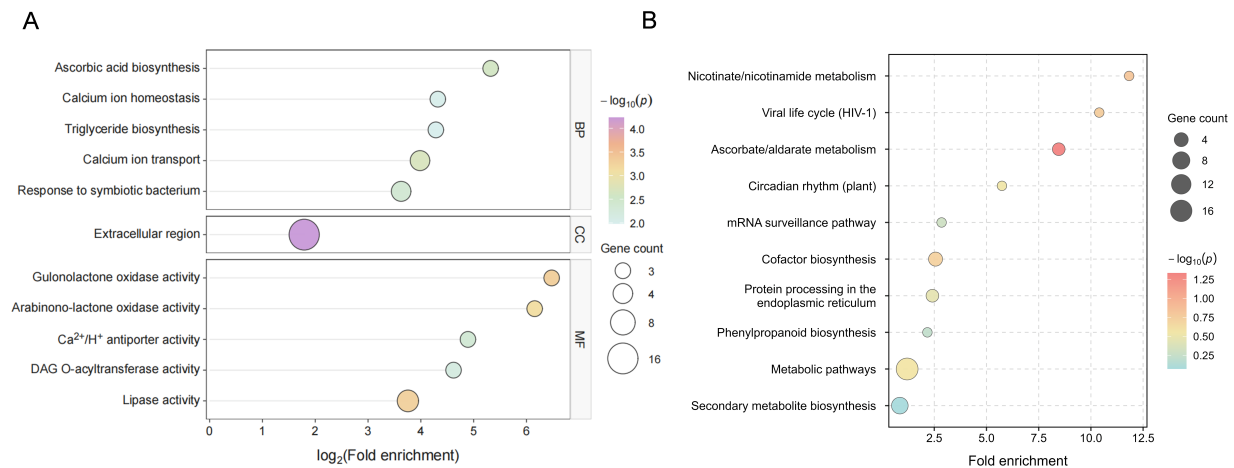


Figure 3. Functional enrichment analysis of genes located near resistance-associated SNPs. (A) Enriched GO terms. (B) Enriched KEGG pathways. Bubble size reflects the number of genes assigned to each term or pathway, whereas the color scale represents enrichment level expressed as $-\log_{10}(p)$.

Based on SNP allelic-effect analysis and functional annotation, eight candidate genes were selected for further investigation: *Glyma.06g291000*, *Glyma.10g210600*, *Glyma.13g271700*, *Glyma.13g272000*, *Glyma.13g272300*, *Glyma.13g273600*, *Glyma.18g076800*, and *Glyma.18g076900*. These genes were prioritized because each contained a resistance-associated SNP within a potentially functional region, showed a significant allele-dependent difference in DSI, and had a predicted function potentially related to plant defense or stress responses. Seven of the selected SNPs were located in exons, whereas S18_7297549 was located in the promoter of *Glyma.18g076900*. Neighboring genes within the candidate intervals were not prioritized when they did not meet the same combination of positional, allelic-effect, and functional criteria. Detailed functional annotations of the eight prioritized candidate genes are summarized in Supplementary Table S4. Their annotations suggest roles in nodulin-related transport, auxin signaling, TCP-mediated transcriptional regulation, calcium-dependent ion exchange, protein–protein interactions, ubiquitin-mediated protein turnover, and lipid metabolism, highlighting their potential contributions to soybean responses to *F. solani* infection. The eight genes were subsequently subjected to qRT-PCR analysis. Their expression responses were used as experimental support for candidate gene prioritization rather than as an initial selection criterion among the 199 candidate genes.

3.4. Allelic Effects of Resistance-Associated SNPs on Disease Severity

Significant differences in disease severity were observed among soybean accessions carrying different alleles at the identified SNP loci (Figure 4). Eight SNPs located in candidate genes showed significant associations with DSI values (Table 2). Most of these SNPs were located in exon regions, whereas S18_7297549 was identified in the promoter region of *Glyma.18g076900*. Significant allele-dependent differences in DSI were detected at several loci. At each locus, the favorable allele was associated with lower disease severity than the alternative allele (Figure 4). The alleles associated with lower DSI values were T at S06_47975172-T/G, C at S10_44287403-G/C, C at S13_37376513-C/T, G at both

S13_37395974-G/C and S13_37431242-G/T, A at S13_37529208-A/T, C at S18_7296291-T/C, and A at the promoter locus S18_7297549-T/A. The observed allele effects indicate that these loci are associated with variation in the soybean response to *F. solani*. They also represent promising genomic regions for marker development and resistance-oriented breeding.

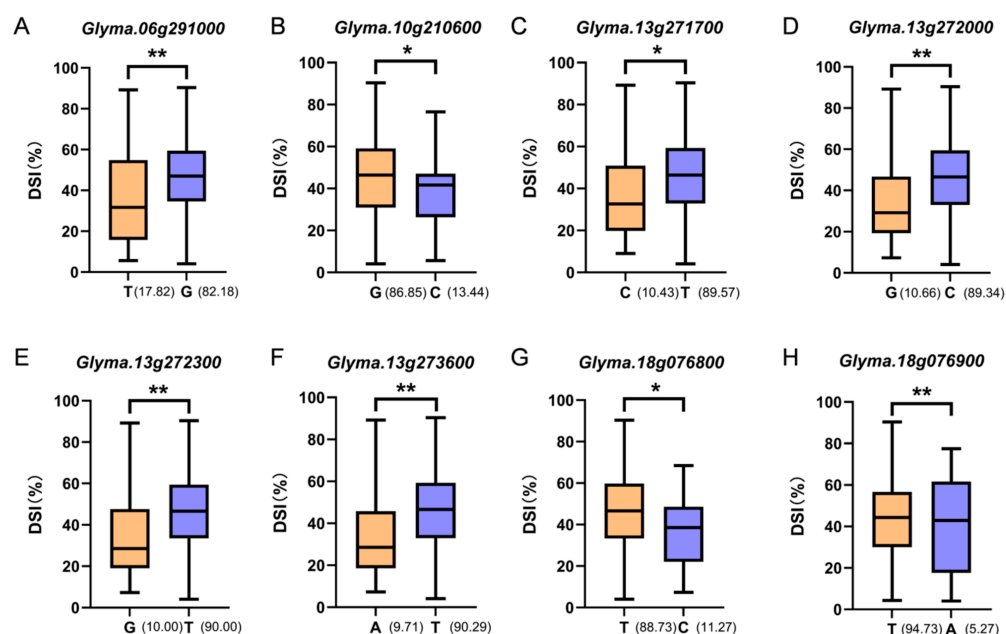


Figure 4. Effects of resistance-associated SNP alleles on DSI in soybean following *F. solani* inoculation. Panels (A–H) correspond to the eight SNP loci listed in Table 2. The orange and purple boxes represent the DSI distributions of the two alleles at each SNP locus, as indicated by the allele labels below the x-axis. Lower DSI values indicate higher resistance. Percentages shown in parentheses below the corresponding allele labels represent allele frequencies (AFs). AFs were calculated using accessions with valid genotype and phenotype data at each locus. For AF calculation, heterozygous genotypes contributed one copy of each allele, but heterozygous accessions were excluded from the boxplot comparisons. Asterisks indicate significant differences between alleles according to *t*-test (* $p < 0.05$, ** $p < 0.01$).

Table 2. Significant SNP-associated candidate genes related to resistance to *F. solani* in soybean.

SNP	Chr.	Position (bp)	Alleles	Candidate Genes	Region
S06_47975172	6	47975172	T/G	<i>Glyma.06g291000</i>	Exon
S10_44287403	10	44287403	G/C	<i>Glyma.10g210600</i>	Exon
S13_37376513	13	37376513	C/T	<i>Glyma.13g271700</i>	Exon
S13_37395974	13	37395974	G/C	<i>Glyma.13g272000</i>	Exon
S13_37431242	13	37431242	G/T	<i>Glyma.13g272300</i>	Exon
S13_37529208	13	37529208	A/T	<i>Glyma.13g273600</i>	Exon
S18_7296291	18	7296291	T/C	<i>Glyma.18g076800</i>	Exon
S18_7297549	18	7297549	T/A	<i>Glyma.18g076900</i>	Promoter

3.5. Expression Analysis of Prioritized Candidate Genes by qRT-PCR

The transcript levels of eight genes prioritized on the basis of SNP position, significant allele-dependent effects on DSI, and functional annotation were examined by qRT-PCR in the resistant accession HF47 and the susceptible accession SN17 after *F. solani* infection. Root samples were collected at 72 h after *F. solani* inoculation. Compared with the mock-treated plants, most candidate genes showed altered expression after pathogen challenge (Figure 5).

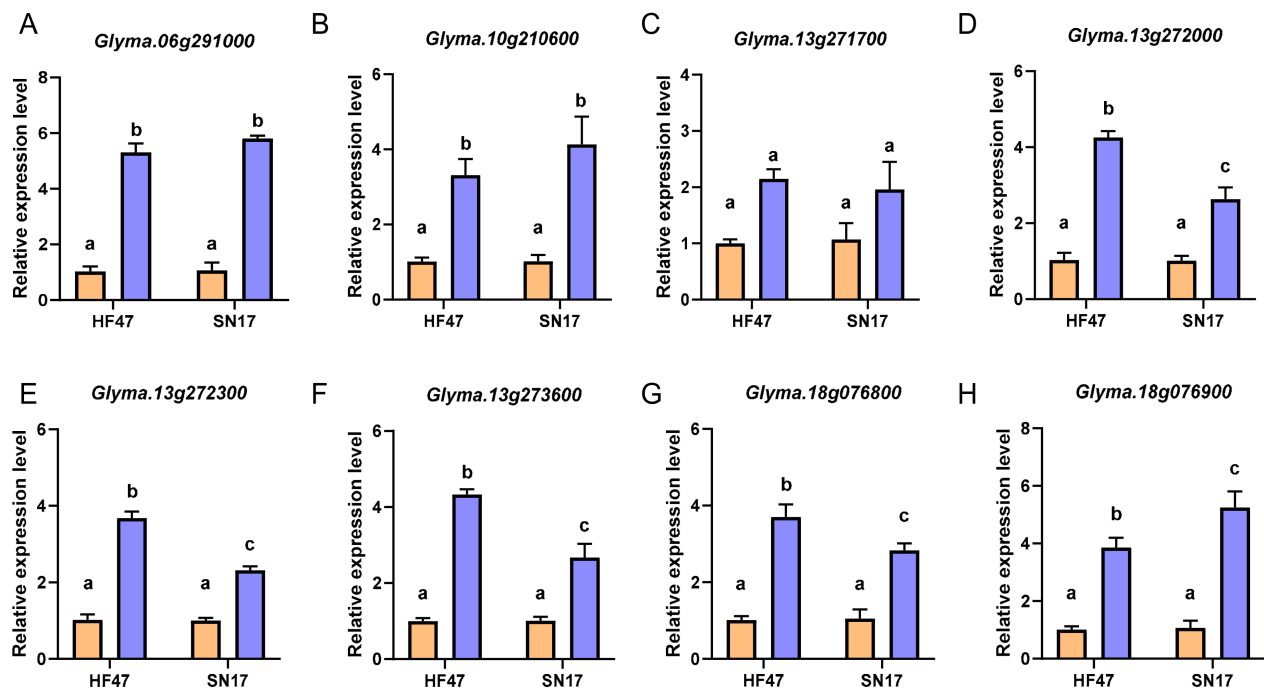


Figure 5. Relative transcript abundance of eight prioritized candidate genes in the resistant accession HF47 and the susceptible accession SN17 at 72 h after mock treatment or *F. solani* inoculation. Panels (A–H) correspond to the genes indicated above each panel. Light-orange and purple bars represent mock-treated and *F. solani*-inoculated plants, respectively. Values are means $\pm$ SE of three biological replicates. For each gene, different lowercase letters indicate significant differences among genotype–treatment combinations based on Tukey’s HSD test at adjusted $p < 0.05$. Pairwise adjusted p values are provided in Supplementary Table S7.

Most of the tested genes were induced in both accessions, but the magnitude of induction differed between HF47 and SN17. *Glyma.13g272000*, *Glyma.13g272300*, *Glyma.13g273600*, and *Glyma.18g076800* showed stronger induction in HF47 than in SN17 (Figure 5D–G). In contrast, *Glyma.18g076900* showed a higher expression level in SN17 than in HF47 after *F. solani* inoculation (Figure 5H), indicating accession-dependent transcriptional responses to infection. *Glyma.06g291000* and *Glyma.10g210600* were also induced in both accessions after inoculation, although the differences between HF47 and SN17 were less pronounced (Figure 5A,B). *Glyma.13g271700* showed only a slight increase after inoculation, and no significant difference was detected among the four genotype–treatment combinations (Figure 5C). Together, these results support the GWAS-based candidate gene selection and indicate that several of the selected genes are transcriptionally responsive to *F. solani* infection.

3.6. Development and Preliminary Evaluation of KASP Markers Associated with Resistance to *Fusarium solani*

Based on the GWAS results and candidate gene analysis, two resistance-associated SNPs on chromosome 13 were selected for KASP marker development. KASP-S13_37431242 targeted S13_37431242-G/T located in *Glyma.13g272300*, whereas KASP-S13_37529208 targeted S13_37529208-A/T located in *Glyma.13g273600*. Both assays generated clear allele discrimination patterns, separating the tested accessions into distinct genotype clusters (Figure 6A,B). These results indicated that the two SNPs were successfully converted into KASP markers suitable for genotype determination.

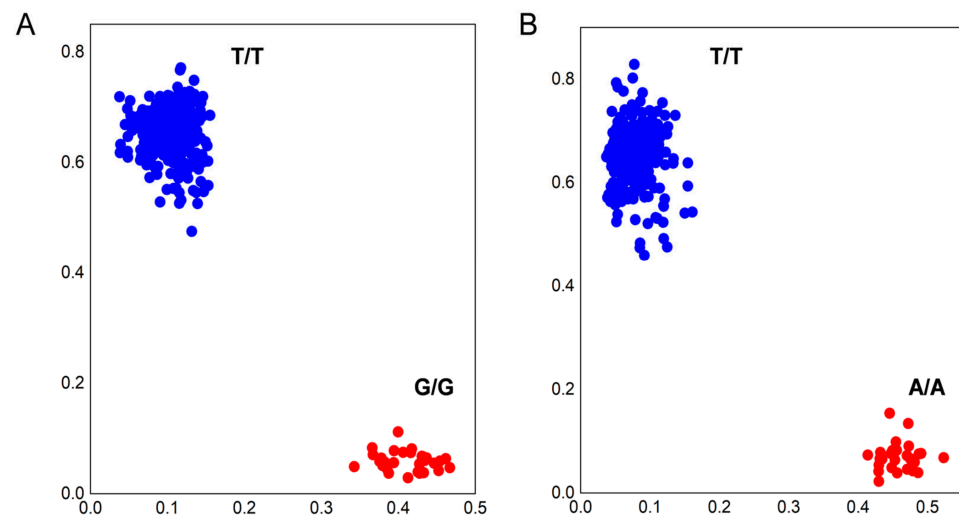


Figure 6. Allelic discrimination plots of two KASP markers associated with *F. solani* resistance. (A) KASP-S13_37431242 targeting the G/T polymorphism at S13_37431242. The blue and red clusters represent the T/T and G/G homozygous genotypes, respectively. (B) KASP-S13_37529208 targeting the A/T polymorphism at S13_37529208. The blue and red clusters represent the T/T and A/A homozygous genotypes, respectively. No distinct heterozygous clusters were observed.

The positive predictive value of the two markers was evaluated using artificial inoculation results as the phenotypic reference. The favorable G/G genotype at KASP-S13_37431242 identified 29 candidate accessions, of which 19 were resistant, giving a positive predictive value of 65.52%. The favorable A/A genotype at KASP-S13_37529208 identified 27 candidate accessions, including 18 resistant accessions, with a positive predictive value of 66.67% (Table 3). The sensitivity and specificity were 14.29% and 94.92% for KASP-S13_37431242 and 13.53% and 95.43% for KASP-S13_37529208, respectively. Both positive predictive values were higher than the overall proportion of resistant accessions in the association panel, suggesting that the favorable genotypes were enriched for resistant germplasm. Together, these results support the use of KASP-S13_37431242 and KASP-S13_37529208 for preliminary enrichment and tracking of favorable chromosome 13 alleles, particularly in combination with phenotypic evaluation, while further validation is required before routine marker-assisted selection.

Table 3. Predictive performance of favorable KASP genotypes for *F. solani* resistance.

Marker/Genotype	TP	FP	FN	TN	Sensitivity (%)	Specificity (%)	PPV (%)
KASP-S13_37431242_G/G	19	10	114	187	14.29	94.92	65.52
KASP-S13_37529208_A/A	18	9	115	188	13.53	95.43	66.67

4. Discussion

Soybean root rot can result from infection by various fungal and oomycete pathogens [31], and resistance in cultivated soybean is usually partial and dependent on the pathogen species or isolate. The *F. solani* species complex includes pathogens responsible for severe soybean root rot. Some members are also linked to sudden death syndrome in soybean [3]. Because infection and symptom development occur mainly below ground, reliable phenotyping is especially important for genetic analysis. *F. solani* has been isolated from soybean and from other hosts, including tobacco [32] and zucchini [33], and previous studies have used seed soaking [34], stem puncture [35], rolled-towel assays [36], and sorghum-grain inoculation [37]. The sorghum-vermiculite inoculation system maintained consistent infec-

tion pressure and caused little mechanical damage to soybean roots. This made it suitable for large-scale germplasm screening. DSI scores varied widely, ranging from 4.04 to 90.36, and displayed an approximately unimodal distribution. Among the 330 accessions examined, 133 were classified as resistant. The continuous phenotypic variation indicates that resistance to *F. solani* is likely governed by multiple genes rather than by a single major resistance locus. The use of a single pathogenic isolate provided uniform disease pressure and facilitated consistent phenotypic evaluation. Therefore, the loci identified in this study should primarily be interpreted as being associated with seedling resistance to the tested *F. solani* isolate under the controlled greenhouse conditions used here. Because *F. solani* belongs to a species complex with pathogenic and genetic diversity, resistance responses may vary among isolates or pathogen populations. In addition, environmental conditions, plant developmental stage, and pathogen isolate diversity may influence disease expression and the effects of individual resistance loci. Therefore, further evaluation under field conditions and with additional representative isolates would help assess the stability and broader applicability of the identified resistant sources.

The GWAS findings further supported the polygenic nature of resistance. FarmCPU and MLM yielded broadly similar association profiles. The strongest signals were located on chromosome 13, with additional association signals on chromosomes 6, 10, and 18. These regions were therefore selected for subsequent candidate-gene analysis. To further evaluate the association signals, we also applied Bonferroni- and FDR-adjusted thresholds. These more conservative criteria did not identify genome-wide significant SNPs in this panel. Given the continuous variation in seedling resistance to *F. solani* and the expected involvement of multiple loci with modest effects, the suggestive threshold was used to define putative resistance-associated regions for candidate-gene prioritization and marker development. Earlier studies of soybean sudden death syndrome and *Fusarium* root rot have likewise shown that resistance is controlled by a complex set of loci [38–40]. Recent soybean GWASs identified a major *F. proliferatum* resistance QTL on chromosome 11 [13] and found no common candidate genes across soybean responses to four *Fusarium* species [15], whereas the strongest signals in the present study were concentrated on chromosome 13. These comparisons suggest that some genomic regions may contribute to relatively broad root-rot responses, whereas others are likely specific to particular *Fusarium* species, isolates, or soybean genetic backgrounds. The regions identified here, however, showed only limited overlap with previously reported loci. This variation may reflect differences among studies in population structure, pathogen isolates, inoculation procedures, disease assessment, marker coverage, and statistical models. The present loci are therefore best interpreted as resistance-associated regions supported in this germplasm panel, rather than as universally effective loci across all soybean backgrounds and *F. solani* isolates. On chromosome 13, the four prioritized SNPs were clustered between 37.38 and 37.53 Mb, spanning approximately 152.7 kb. Because these SNPs are physically close to one another, the clustered signals may be influenced by local linkage disequilibrium and may not represent four fully independent causal loci. However, locus-specific LD blocks and haplotypes were not reconstructed in the present study. Fine-scale LD and haplotype analyses would help determine whether these signals represent multiple causal variants or linked markers associated with a common underlying locus.

Despite the insights gained, this study has several limitations. Disease resistance was evaluated under controlled greenhouse conditions using artificial inoculation with a single *F. solani* isolate. Although this experimental system provided consistent infection pressure and facilitated phenotypic assessment, it did not fully represent the complexity of field environments, where disease development may be influenced by soil properties, fluctuating temperature and moisture, microbial communities, pathogen pressure, and

genotype-by-environment interactions. Therefore, the loci identified in this study should be interpreted as being associated with seedling resistance to the tested isolate under the greenhouse conditions used here, rather than as loci conferring broad field resistance. Further evaluation of these markers across multiple field environments, years, soil conditions, and additional *F. solani* isolates would help determine their stability and potential value for breeding applications.

The 199 genes surrounding the representative loci were enriched for functions related to ion transport, lipid metabolism, oxidoreductase activity, and secondary metabolism. Eight genes were prioritized by integrating SNP position, significant allele-dependent effects on DSI, and functional annotation, and their responses to *F. solani* infection were subsequently evaluated by qRT-PCR. *Glyma.13g272000* and *Glyma.13g272300* encode sodium/calcium-exchanger-related proteins; soybean EF-hand calcium-binding proteins are known to participate in environmental and nutritional stress signaling [41,42], suggesting that altered calcium flux and root ion homeostasis may be involved in the response to infection. *Glyma.13g271700* encodes a TCP transcription factor, a family responsive to developmental, hormonal, and stress signals in soybean [43], whereas *Glyma.10g210600* is related to ARF16 and may link auxin-regulated root development or tissue remodeling with infection [44]. *Glyma.13g273600* encodes a pentatricopeptide-repeat protein involved in organellar RNA metabolism [45], and *Glyma.06g291000* encodes a phytycyanin-like protein associated with electron transfer and redox responses [46]. *Glyma.18g076800*, a TRAF-like gene, is of particular interest because TRAF proteins regulate immune signaling, ROS homeostasis, and stress responses [47], and this gene was more strongly induced in the resistant accession. *Glyma.18g076900* is an acyl-CoA-synthetase-related gene. Membrane-associated proteins can influence disease resistance in soybean, as shown for PSS1 [48]; long-chain acyl-CoA synthetases also affect membrane lipid remodeling and stress responses [49], and mutation of Arabidopsis LACS2 changes susceptibility to different pathogens [50]. The stronger induction of *Glyma.18g076900* in the susceptible accession may therefore reflect infection-driven lipid remodeling rather than a direct resistance function. The functional diversity of the prioritized genes suggests that some loci may also be associated with physiological processes beyond disease response. Such associations may reflect pleiotropy or tight linkage with neighboring genes, indicating that the candidate regions may have broader biological relevance. More generally, the qRT-PCR analysis provided expression-level support for the GWAS-prioritized candidate genes, suggesting that these candidates are associated with transcriptional changes during *F. solani* infection. These expression data can therefore be regarded as preliminary evidence supporting candidate-gene prioritization, whereas the specific biological functions of these genes warrant further investigation. Because the expression analysis was performed at 72 h post-inoculation using two genotypes with contrasting resistance responses, future expression analyses at multiple post-inoculation time points would further clarify the dynamic transcriptional responses of these genes during *F. solani* infection. Future functional studies, such as overexpression, gene knockout or editing, gene silencing, or soybean hairy-root transformation, together with disease phenotyping, would provide more direct evidence for the biological roles of these candidate genes in *F. solani* resistance.

To further evaluate the potential use of resistance-associated SNPs in germplasm screening, two SNPs were converted into KASP markers. KASP assays are inexpensive, scalable, and widely used for marker-assisted selection in soybean and other crops [51]. In this panel, the G/G genotype at KASP-S13_37431242 and the A/A genotype at KASP-S13_37529208 showed positive predictive values of 65.52% and 66.67%, respectively, compared with an overall resistant proportion of 40.30%. These values indicate useful enrichment of resistant accessions among lines carrying the favorable genotypes, whereas the

relatively low sensitivities indicate that the two markers identify only a subset of resistant accessions. Based on the current results, these markers can be regarded as preliminary screening tools for prioritizing accessions carrying favorable genotypes during large-scale germplasm evaluation, particularly when used together with phenotypic confirmation through artificial inoculation. Further validation using broader soybean germplasm, independent breeding populations, and representative environmental conditions, together with additional *F. solani* isolates, would help evaluate the robustness of these markers and support their future routine application in breeding programs. The current performance estimates should therefore be interpreted in the context of the association panel used in this study. Integrating these markers with other resistance-associated loci and phenotypic evaluation may further improve their usefulness in resistance-oriented germplasm screening.

Taken together, the GWAS signals, allelic effects, functional annotations, and qRT-PCR results indicate that soybean seedling resistance to *F. solani* may involve multiple defense-related processes, including calcium and hormone signaling, transcriptional regulation, organellar RNA metabolism, redox homeostasis, and lipid metabolism. Among the prioritized genes, *Glyma.13g272300* and *Glyma.13g273600* warrant further attention. Their favorable alleles were associated with reduced DSI, and the corresponding SNPs were converted into KASP markers. These two genes and their linked markers therefore provide useful targets for follow-up validation. Future studies should examine their expression patterns at additional infection stages, quantify pathogen biomass, and assess haplotype variation in broader soybean germplasm. Their potential roles in resistance should also be tested using soybean hairy-root transformation, gene editing, overexpression, or gene-silencing assays. These results provide a practical basis for follow-up validation of the candidate genes and continued evaluation of the KASP markers in resistance breeding.

5. Conclusions

Resistance to *F. solani* was characterized across 330 soybean accessions. The continuous variation in disease severity index (DSI) was consistent with quantitative control of this trait. Genome-wide association analyses were conducted with 627,436 high-confidence SNPs using FarmCPU and MLM. Putative GWAS signals were detected across seven chromosomes. Candidate-gene analysis based on SNP position, allelic effects, and functional annotations prioritized eight genes, and qRT-PCR analysis showed that several of them were transcriptionally responsive to *F. solani* infection. Among these candidates, *Glyma.13g272300* and *Glyma.13g273600* were highlighted by favorable allelic effects at the corresponding chromosome 13 SNPs, providing the basis for marker development.

Two chromosome 13 SNPs were converted into KASP markers and designated KASP-S13_37431242 and KASP-S13_37529208. Both assays generated clearly separated genotype clusters. Resistant accessions occurred more frequently among lines carrying the favorable genotypes. The two markers showed positive predictive values of 65.52% and 66.67% and specificities of 94.92% and 95.43%, respectively, although their sensitivities were 14.29% and 13.53%. These results indicate that the markers may support preliminary enrichment and tracking of favorable chromosome 13 alleles, particularly when combined with phenotypic evaluation or additional resistance-associated markers. Overall, this study provides candidate genomic regions, genes, and KASP markers for further validation and the development of multi-locus strategies to improve soybean resistance to *Fusarium* root rot.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16151503/s1>: Figure S1. *F. solani* root rot resistance scoring standard; Figure S2. Genome-wide SNP-based population structure and genetic relationships among 330 soybean accessions; Figure S3. Sequence alignments surrounding the two KASP marker

loci; Figure S4. Melting-curve analysis of the qRT-PCR amplification products for eight candidate genes and the reference gene *GmActin4*. Tables S1–S8. Phenotypic, SNP, candidate-gene, and primer information.

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