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Identification of Maize Fusarium Ear Rot Resistance Genes via Integrated BSA-Seq and RNA-Seq Analysis

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Abstract: Maize ear rot, often caused by *Fusarium graminearum* (*F. graminearum*) and other fungi, is a widespread fungal disease that severely reduces grain yield and quality, while mycotoxins produced by these pathogens pose significant threats to human and animal health. However, the defense responses of maize to pathogen infection and the underlying genetic regulatory mechanisms remain largely unclear. To investigate the molecular basis of maize ear rot resistance, maize ears were artificially inoculated with *F. graminearum* at 7 days after pollination, and cytological, physiological, biochemical, and multi-omics analyses were performed between the maize resistant inbred line K0743 and susceptible inbred line K0742 at different time points after *F. graminearum* inoculation. The results showed that the resistant inbred line K0743 maintained more intact cellular structures than susceptible inbred line K0742 following inoculation and exhibited significantly higher activities of defense-related enzymes, including phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and superoxide dismutase (SOD), as well as significantly increased accumulation of osmotic regulatory substances such as proline, soluble sugars, and soluble proteins ($P < 0.05$ or $P < 0.01$). BSA-Seq identified 8 resistance-associated loci on Chr 5, 6, 7, 9, and 10, among which *qGER5* and *qGER6-2* were defined as major loci. Transcriptome analysis revealed that the resistant inbred line K0743 exhibited much higher numbers of differentially expressed genes (DEGs) than the susceptible inbred line K0742 across

three time points (72 h, 96 h, and 7 d) relative to their respective 0 h controls. Specifically, after 72 h of inoculation, the resistant inbred line K0743 had 6,030 DEGs (compared to 805 in K0742); after 96 h, 4,929 DEGs (vs. 2,212 in K0742); and after 7 d, 10,254 DEGs (vs. 3,255 in K0742). Integrative analyses of BSA-Seq and RNA-Seq, combined with weighted gene co-expression network analysis (WGCNA), identified 6 high-confidence candidate genes. These genes were distributed across two key co-expression modules: MEfloralwhite, primarily involved in oxidative stress response and ubiquitin-mediated proteolysis; and MEblue4, enriched in plant hormone signal transduction, MAPK signaling, and cell wall modification pathways. These findings provide new insights into the molecular mechanisms underlying maize resistance to *F. graminearum* and offer valuable candidate genes for resistance breeding and functional characterization.

Keywords: Maize ear rot; Physiological characteristics; Scanning electron microscopy; BSA-Seq; RNA-Seq

1 Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops worldwide and serves as a major source of food, feed, and industrial raw materials[1][2][3]. However, maize production is frequently threatened by a variety of diseases, among which maize ear rot is considered one of the most destructive fungal diseases[4][5]. *F. graminearum* is one of the predominant causal agents of Gibberella ear rot (GER), infects maize ears during flowering and grain-filling stages, resulting in substantial yield losses and deterioration of grain quality[6][7]. More importantly, infected kernels accumulate harmful mycotoxins, including deoxynivalenol (DON) and zearalenone (ZEN), which pose serious threats to food safety, animal health, and international grain trade[8]. The occurrence and severity of GER are strongly influenced by environmental conditions, particularly high humidity and moderate temperatures, and increasing climate variability has further elevated the risk of disease outbreaks in

many maize-growing regions. Consequently, improving resistance to *F. graminearum* has become an important objective in maize breeding programs worldwide[9][10].

Genetic resistance is widely regarded as the most economical, environmentally friendly, and sustainable strategy for controlling maize ear rot[11]. However, resistance to *F. graminearum* is a typical quantitative trait controlled by multiple genes and is greatly affected by environmental factors, making the genetic dissection of resistance particularly challenging[12][13][14]. Over the past two decades, numerous quantitative trait loci (QTLs) associated with GER resistance have been identified through linkage mapping and genome-wide association studies (GWAS)[15][16]. These resistance-related loci are distributed across several maize chromosomes, particularly Chr 1, 3, 4, 5, 6, and 10. Despite these advances, most reported QTLs explain only a small proportion of the phenotypic variation and often exhibit instability across genetic backgrounds and environments[17][18][19]. Moreover, only a limited number of candidate genes have been proposed, and the molecular mechanisms underlying maize resistance to *F. graminearum* remain largely unclear[20]. Therefore, the identification of stable resistance loci and functionally relevant genes remains a major challenge and a prerequisite for accelerating resistance breeding.

Recent advances in high-throughput sequencing technologies have provided unprecedented opportunities for elucidating the genetic basis of complex agronomic traits. Bulk segregant analysis (BSA-Seq) has emerged as an efficient approach for rapidly identifying genomic regions associated with target traits by combining pooled population analysis with next-generation sequencing[21][22]. Meanwhile, RNA sequencing (RNA-Seq) enables comprehensive characterization of transcriptional responses and regulatory pathways activated during pathogen infection. The integration of BSA-Seq and RNA-Seq has proven to be an effective strategy for narrowing candidate intervals and prioritizing biologically relevant genes in several crop species[23][24]. In addition to genetic regulation, plant resistance is accompanied by dynamic

70 physiological and cellular responses, including changes in tissue structure, reactive oxygen species
71 (ROS) scavenging, osmotic adjustment, secondary metabolite accumulation, and extensive
72 transcriptional reprogramming. Although some studies have individually investigated physiological
73 responses or gene expression changes during *F. graminearum* infection, comprehensive analyses
74 integrating cytological observations, physiological traits, and multi-omics data remain limited in
75 maize[25][26]. Such integrated investigations are essential for understanding the coordinated defense
76 mechanisms that contribute to disease resistance.

77 To bridge these knowledge gaps, the resistant inbred line K0743 and the susceptible inbred line
78 K0742 were investigated using an integrated approach combining scanning electron microscopy,
79 physiological and biochemical analyses, BSA-Seq, RNA-Seq, and weighted gene co-expression
80 network analysis (WGCNA). By comparing host responses at cytological, physiological, and
81 molecular levels, this study aimed to identify resistance-associated genomic regions and candidate
82 genes and to elucidate the regulatory networks underlying maize defense against *F. graminearum*. The
83 findings provide new insights into the molecular mechanisms of GER resistance and valuable genetic
84 resources for resistance breeding.

85 **2 Materials and methods**

86 **2.1 Plant materials and field trials**

87 Resistant inbred line K0743 and the susceptible inbred line K0742, which differ significantly in
88 resistance to maize ear rot, were provided by the Gansu Heheng Maize Research Institute. Field
89 experiments were conducted at the Dajiatai Experimental Station in Xigu using a randomized complete
90 block design. Each inbred line was planted in four rows, with a row length of 8 m and a row spacing
91 of 0.8 m. Thirty plants were maintained in each row after thinning. Standard agronomic management
92 practices were applied throughout the growing season.

To investigate the response of maize kernels to pathogen infection, kernel samples of susceptible inbred line K0742 and resistant inbred line K0743 were collected at six time points following inoculation: 0, 24, 48, 72, and 96 h, and 7 d post-inoculation. Three independent biological replicates were included for each treatment and sampling time point.

The fungal pathogen *F. graminearum* used in this study was obtained from the Institute of Plant Protection, Gansu Academy of Agricultural Sciences. To prepare the inoculum, the pathogen was cultured in potato sucrose broth and incubated at 25°C under natural light conditions with continuous shaking at 120 rpm for 7 d to induce conidial production. The culture suspension was filtered through two layers of sterile gauze to remove mycelial debris, and the resulting conidial suspension was adjusted to a concentration of 2×10^6 conidia mL⁻¹ using sterile distilled water. The prepared suspension was used for artificial inoculation and could be stored at 2 - 4°C for up to 3 - 4 weeks before use.

2.2 Artificial inoculation and phenotypic evaluation

Maize inoculation was performed 4 - 7 days after silk emergence and could be carried out at any time of day using the kernel-wounding injection method. Briefly, a continuous syringe fitted with a hollow metal needle was inserted through the outer husk at the upper-middle part of the ear. Once the needle penetrated the kernels, the inoculum was injected, allowing infection to occur through conidial germination and subsequent hyphal growth. Only the first ear of each plant was inoculated.

After kernel maturity, the inoculated ears were harvested, dehusked, and examined individually. The area of infected kernels was recorded, and disease severity was scored according to the established grading criteria. The total number of plants and the number of diseased plants were also recorded, and the resistance level of each germplasm accession was evaluated accordingly.

At maturity, inoculated ears were scored on a 1- to - 9 scale based on the percentage of infected ear area: 1 = 0 - 1%, 3 = 2 - 10%, 5 = 11 - 25%, 7 = 26 - 50%, and 9 = 51 - 100% (Figure 1). Each ear was independently scored 30 days after inoculation using the above five scales.

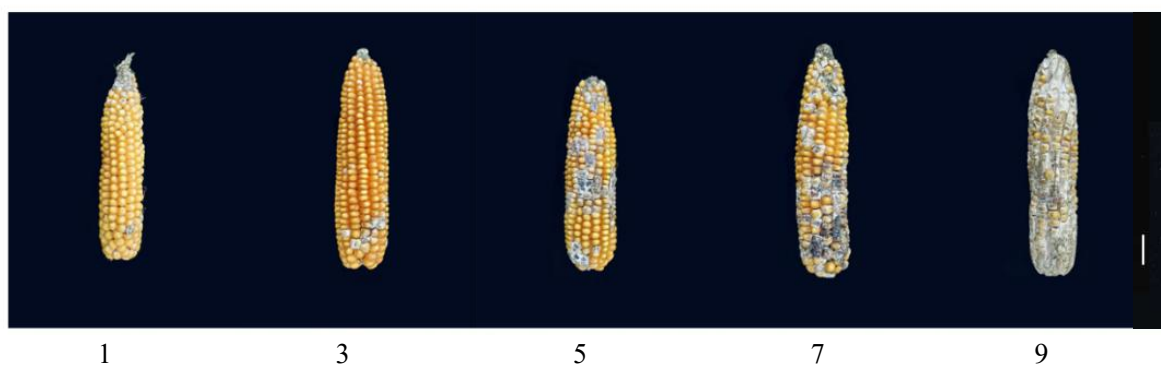


Figure 1 Phenotypic representation of resistance scale for maize ear and kernel rot

2.3 Scanning electron microscopy analysis

Maize kernels from the resistant inbred line (K0743) and susceptible inbred line (K0742) were collected at 0, 24, 48, 72, and 96 h, and 7 d post-inoculation. Samples were fixed in 2.5% glutaraldehyde at 4 °C, rinsed three times with phosphate buffer, and dehydrated through a graded ethanol series (35%, 50%, 70%, 85%, 95%, and 100%), with 15 min at each concentration. Following dehydration, samples were infiltrated with isoamyl acetate for 15 min, frozen overnight at –80 °C, and then freeze-dried. Dried samples were sputter-coated with gold and observed using a scanning electron microscope (SEM). Kernels collected at 0 h post-inoculation served as the control.

2.4 Measurement of physiological and biochemical indicators

At 0, 24, 48, 72, and 96 h, and 7 days post-inoculation, kernel samples from the resistant inbred line K0743 and the susceptible inbred line K0742 were collected for physiological and biochemical analyses. Fresh tissue (0.2 - 0.5 g) was weighed and homogenized in phosphate buffer, and crude enzyme extracts were prepared using extraction buffers with appropriate pH values according to the requirements of each assay. Enzyme activities such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and malondialdehyde (MDA) were measured according to published methods[27]. Polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) assays were conducted according to published methods[28]. All measurements were performed using three independent biological replicates.

2.5 Statistical analysis

Phenotypic data were organized and visualized using Microsoft Excel 2019. Prior to parametric analyses, all datasets were tested for normality and homogeneity of variance. Normality was assessed using the Shapiro-Wilk test, with $P > 0.05$ indicating a normal distribution. Homogeneity of variance was evaluated using Levene's test, with $P > 0.05$ indicating equal variances. After confirming that the data met the assumptions for parametric testing, one-way analysis of variance (ANOVA) was performed using SPSS 22.0. Significant differences among means were determined using Duncan's multiple range test at $P < 0.05$.

2.6 BSA-Seq analysis

2.6.1 DNA extraction and quality assessment

After evaluation of resistance to ear and kernel rot, DNA was extracted from leaf samples of 60 maize plants with extreme phenotypes selected from the F₂ population. Genomic DNA was isolated using a modified CTAB method[29]. DNA integrity was assessed by 1% agarose gel electrophoresis, with GelRed used as the DNA stain for gel visualization. DNA purity was determined using a NanoDrop 2000 spectrophotometer, and samples with an OD₂₆₀/OD₂₈₀ ratio of 1.8 - 2.0 and a DNA concentration of ≥ 20 ng/ μ L were used for subsequent analyses.

2.6.2 Library construction and sequencing

Equal amounts of qualified DNA from the 30 resistant and 30 susceptible F₂ individuals were pooled to construct the resistant bulk and susceptible bulk, respectively. DNA samples from each bulk were fragmented by ultrasonication and then subjected to end repair, A-tailing, adaptor ligation, fragment size selection, and PCR amplification to construct sequencing libraries. Library quality was assessed using an Agilent 2100 Bioanalyzer. Qualified libraries were sequenced on the Illumina NovaSeq 6000 platform by BMK Cloud Technologies Co., Ltd. (Beijing, China). Raw reads were quality-filtered using the fastp software (v 0.23.2) to remove adapters and low-quality reads for

obtaining high-quality clean reads. Clean reads were aligned to the maize reference genome (B73 RefGen_v5, https://ftp.ensemblgenomes.ebi.ac.uk/pub/plants/release-60/fasta/zea_mays/dna/, last accessed on 25 April 2026) via BWA software (v 0.7.17-r1188) with the default parameters[30]. The Picard package (picard.sourceforge.net, v1.87) was used to mark duplicates and deduplicated reads. The GATK toolkit (v 4.2.5.0) was used to detect single nucleotide polymorphisms (SNPs)/ insertion and deletion (InDels) variants with the default parameters[31]. The generated raw genotypes in vcf format for two pools were filtered with the criterion of average depth $\geq 5\times$ and quality scores ≥ 20 , to obtain high-quality variant sets. The ANNOVAR package (v 2017-07-17) was used for variant annotation.

2.6.3 Maize ear rot response gene mapping

The preliminary mapping of genes associated with resistance to maize ear rot based on the BSA-Seq strategy was implemented by DeepBSA software (v 1.6), which is a user-friendly bulked segregant analysis tools integrating the different BSA algorithms. Of them, Deep Learning (DL) and Δ (SNP-index) were used to map the loci governed maize ear rot. The regions with signals exceeding the threshold were identified as candidate intervals.

2.7 RNA-Seq analysis

2.7.1 RNA extraction and sequencing

Kernels of resistant inbred line K0743 and susceptible inbred line K0742 were collected at 0 h, 72 h, 96 h, and 7 days post-inoculation, with three biological replicates per treatment. Total RNA was extracted using Trizol reagent. RNA purity and integrity were assessed using a NanoDrop 2000 and an Agilent 2100 Bioanalyzer, respectively. Libraries were constructed following the Illumina standard workflow and sequenced on the Illumina NovaSeq 6000 platform (150 bp paired-end).

2.7.2 Library construction and sequencing

Raw reads were filtered using Trimmomatic (v0.39) to remove adapter sequences and low-quality reads ($Q > 20$). Reads with $> 10\%$ N bases or $> 50\%$ bases with $Q \leq 20$ were discarded, and the minimum read length was set to 50 bp. The average sequencing depth was approximately $20 \times$ per sample, with $> 95\%$ clean reads and $\geq 92\%$ Q30 for all samples. Raw reads were quality-controlled using the fastp (v 0.23.2) to remove adapters and low-quality sequences to generate high-quality clean reads. Clean reads were then aligned to the maize reference genome (B73 RefGen_v5) using HISAT2 software (v 2.2.1) with default parameters[32]. Read counts per gene were quantified using featureCounts software (v 2.0.3) software. Gene expression levels were normalized as FPKM (Fragments Per Kilobase of transcript per Million mapped reads). Differentially expressed genes (DEGs) were identified using R package DESeq2 (v1.30.1) with the screening criteria of $|\log_2(\text{Fold Change})| \geq 1$ and $\text{FDR} < 0.01$ [33][34][35].

2.7.3 Functional analysis of DEGs

Gene Ontology (GO) enrichment analysis was performed using TopGO (v2.50.0) with Fisher's exact test and Benjamini-Hochberg correction ($P < 0.05$). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was conducted using ClusterProfiler (v4.6.2) with $q < 0.05$.

2.7.4 Weighted gene co-expression network analysis (WGCNA)

Genes with an $\text{FPKM} \geq 1$ were used to construct a weighted gene co-expression network using the WGCNA R package (power = 12, mergeCutHeight = 0.25, minModuleSize = 30). Modules significantly associated with disease resistance were identified through module-trait association analysis, and the core interaction networks of hub genes were visualized using Cytoscape (v3.10.1).

2.7.5 Combined analysis of BSA-Seq and RNA-Seq

The two major resistance-associated loci (*qGER5* and *qGER6-2*) identified by BSA-Seq were first mapped onto the maize reference genome (B73 RefGen_v5). Differentially expressed genes ($|\log_2\text{FC}| \geq 1$, $\text{FDR} < 0.01$) from RNA-Seq were then overlapped with these QTL intervals using Bedtools

(v2.30.0) to obtain a set of core candidate genes located within the resistance-associated regions. Functional annotation of these candidates was performed using the NCBI CDD and MaizeGDB databases. Expression pattern analysis was further conducted to refine the candidate list, prioritizing genes that showed significant expression differences between resistant and susceptible inbred lines before and after inoculation. The final candidate genes were prioritized based on functional relevance to plant disease resistance, including resistance proteins, transcription factors, and defense enzymes, as well as high fold change ($|\log_2FC| \geq 1$) in the resistant line with $FDR < 0.01$, and location within QTL peak regions.

2.7.6 Validation of gene expression via RT-qPCR

To validate the reliability of the transcriptome sequencing data, we randomly selected nine differentially expressed genes (DEGs) for qPCR validation. The relative expression was calculated by calculating the $2^{-\Delta\Delta C_t}$ method, and the specific primers were designed using Primer 5.0 software with Actin internal as the reference gene. Reverse transcription was performed using the Accurate Reverse Transcription Kit. Standardize the cDNA product using RT-qPCR. The thermal cycling conditions were as follows: 15 min at 37°C, 5 sec at 85°C, followed by 40 cycles of 30 sec at 95°C, 5 sec at 95°C, and 30 sec at 60°C. Each PCR reaction was repeated at least three times.

3 Results

3.1 Cytological and physiological responses induced by *F. graminearum* infection

3.1.1 Dynamic differences in kernel structural between resistant and ear susceptible inbred lines

The ultrastructural changes in kernels of the susceptible inbred line K0742 and the resistant inbred line K0743 following *F. graminearum* infection were examined by scanning electron microscopy (SEM) at 0, 24, 48, 72, and 96 h, and 7 d post-inoculation. Kernels of both lines exhibited intact and compact cellular structures at 0 h, with starch granules tightly embedded within a dense protein matrix.

(Figure 2) Following inoculation, progressive structural deterioration was observed in the susceptible inbred line K0742. Small pores appeared on the kernel surface, accompanied by increased roughness of the starch granules at 24 h. Partial degradation of the protein matrix became evident, and the pores expanded further at 48 h. Starch granules displayed pronounced indentations and erosion, together with extensive fungal hyphal colonization at 72 h. Severe disruption of kernel tissues was observed at 96 h, when the morphology of starch granules became indistinguishable. The cellular architecture was almost completely destroyed at 7d.

Conversely, the resistant inbred line K0743 exhibited substantially less structural damage throughout the infection process. The protein matrix remained largely intact up to 72 h post-inoculation, and only slight surface modifications of starch granules were observed. Although partial degradation of kernel tissues occurred at 7 d, the starch granules remained clearly distinguishable and the overall cellular structure was largely preserved compared with susceptible inbred line K0742. These observations demonstrate that resistant inbred line K0743 is capable of maintaining kernel structural integrity during pathogen invasion, thereby limiting tissue damage caused by *F. graminearum*. The enhanced preservation of cellular architecture in the resistant inbred line K0743 suggests a stronger cytological defense response and may contribute to its higher level of resistance to maize ear rot.

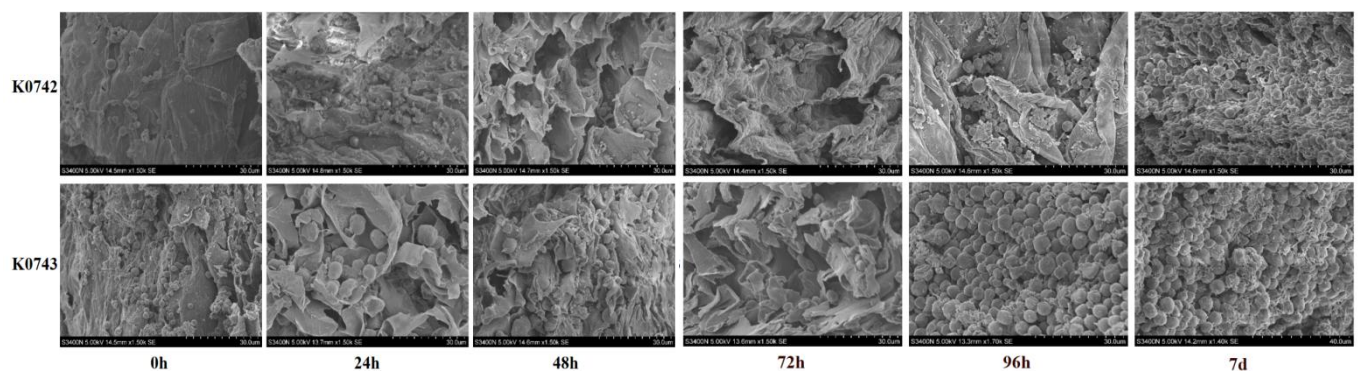


Figure 2 Scanning electron microscopy observation of structural changes in maize kernel tissues

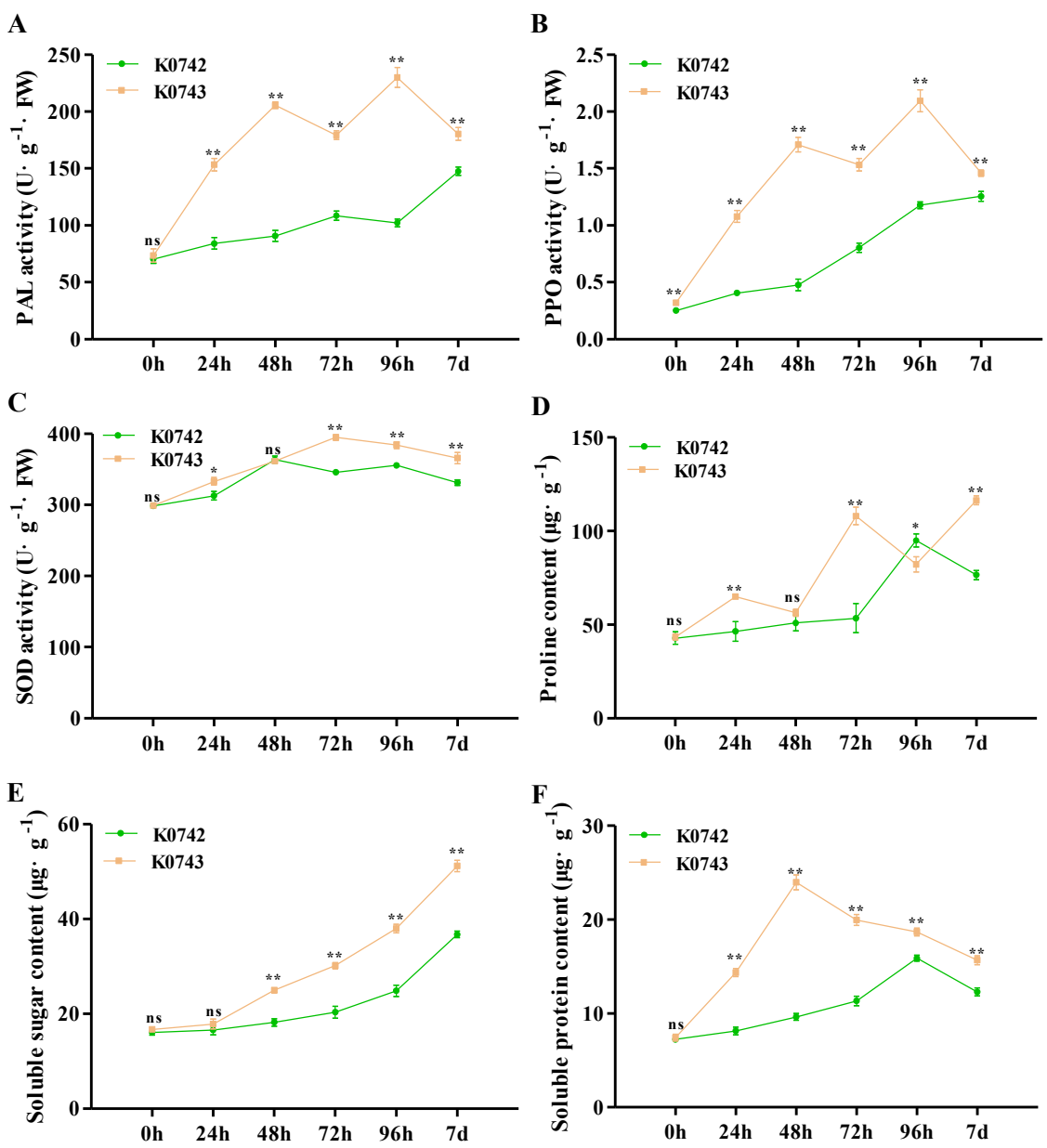
3.1.2 Temporal changes in defense enzyme activities and accumulation patterns of osmoregulatory substances in resistant and susceptible maize inbred lines

Changes in defense enzyme activities and osmoregulatory substance contents in maize kernels of the resistant inbred line K0743 and the susceptible inbred line K0742 during *F. graminearum* infection were systematically analyzed (Figure 3). In the resistant inbred line K0743, phenylalanine ammonia-lyase (PAL) activity increased continuously, reaching a peak at 96 h post-inoculation, 2.8-fold higher than at 0 h, and remained stable thereafter (Figure 3A). By comparison, PAL activity in the susceptible inbred line K0742 increased more gradually, peaking at 7 d with a 1.6-fold increase relative to 0 h; this peak was significantly lower than that in the resistant inbred line K0743 ($P < 0.05$). Polyphenol oxidase (PPO) activity followed a similar trend (Figure 3B). Superoxide dismutase (SOD) activity in the resistant inbred line K0743 peaked at 72 h (2.1-fold increase relative to 0 h) and subsequently declined gradually (Figure 3C). In the susceptible inbred line K0742, SOD activity peaked earlier at 48 h, with only a 1.3-fold increase, and decreased more rapidly. Across all time points, SOD activity in the resistant inbred line K0743 was consistently higher than in the susceptible inbred line K0742.

The accumulation of osmoregulatory substances exhibited temporal differences between the two lines. Proline (Pro) content increased continuously in both lines; however, accumulation occurred earlier and was more pronounced in the resistant inbred line K0743, reaching a 3.2-fold increase at 7 d (Figure 3D). In the susceptible inbred line K0742, Pro content peaked at 96 h (2.1-fold increase) and slightly declined thereafter. Soluble sugar content showed a comparable trend, with resistant inbred line K0743 displaying a 38.6% higher level than susceptible inbred line K0742 at 7 d ($P < 0.01$) (Figure 3E). Soluble protein content initially increased and then decreased, peaking earlier in the resistant inbred line K0743 at 48 h (1.9-fold increase), compared with a peak at 96 h in the susceptible inbred line K0742 (1.4-fold increase), with resistant inbred line K0743 levels significantly higher than susceptible inbred line K0742 ($P < 0.05$) (Figure 3F).

These results indicate that resistant inbred line K0743 rapidly activates the defense enzyme system (PAL, PPO, and SOD) and accumulates osmoregulatory substances (Pro, soluble sugars, and soluble

279 proteins) more efficiently than susceptible inbred line K0742 during *F. graminearum* infection. The
 280 sustained elevation of defense enzyme activities may enhance cell wall lignification and reactive
 281 oxygen species scavenging, while the rapid accumulation of osmolytes likely contributes to
 282 maintaining cellular osmotic balance and energy supply. Collectively, these physiological responses
 283 constitute a robust defense mechanism underlying the enhanced resistance of resistant inbred line
 284 K0743 to maize ear rot.



285

Figure 3 Changes in defense enzyme activities and osmoregulatory substance contents in two inbred lines

Note: All data represent the mean $\pm$ standard error (SE) of three independent biological replicates. Statistical comparisons between the two inbred lines (K0743 vs. K0742) were performed using an independent two-sample Student's t-test. Significant differences are denoted as follows: $P < 0.05$ (significant), $P < 0.01$ (highly significant), and ns (not significant, $P \geq 0.05$).

3.2 Genetic basis of maize resistance to ear rot

3.2.1 Identification of resistance-associated genomic regions by BSA-Seq strategy

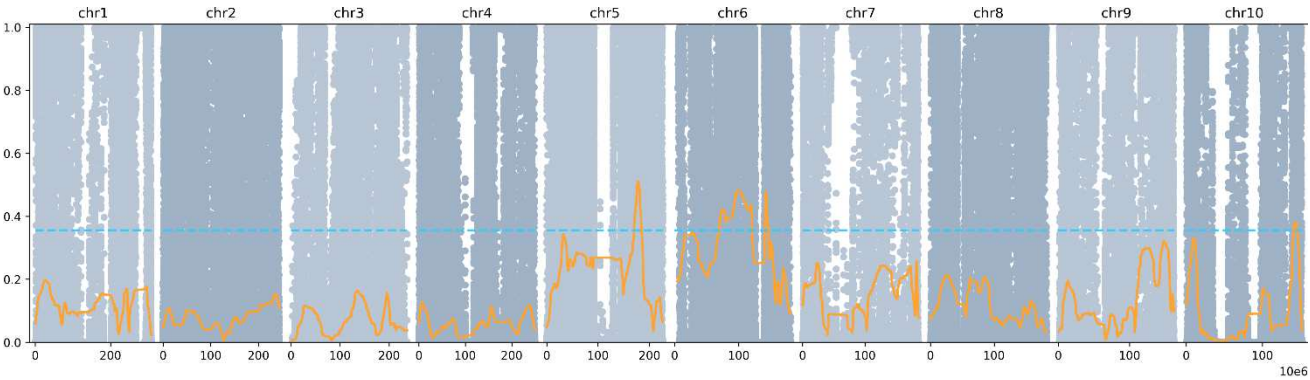
To identify genomic regions associated with maize resistance to *F. graminearum*-induced GER, bulked segregant analysis sequencing (BSA-Seq) was performed using resistant and susceptible extreme bulks, and analyzed with both DL and Δ SNP-index methods. A total of eight resistance-associated loci were detected across Chr 5, 6, 7, 9, and 10 (Table 1). Specifically, the DL approach identified four loci (*qGER5*, *qGER6-2*, *qGER6-4*, and *qGER10*) (Figure 4A), whereas the Δ SNP-index approach identified 6 loci (*qGER5*, *qGER6-1*, *qGER6-3*, *qGER7*, *qGER9*, and *qGER10*) (Figure 4B). Among these, *qGER5* was consistently detected by both methods, indicating high reliability across analytical strategies.

Based on the peak values of the DL and Δ (SNP-index) curves, which reflect the strength of association signals, two loci on Chr 5 and 6 were prioritized for further analysis. These included *qGER5* on Chr 5 (170.18-185.84 Mb; 388 genes), detected by both the DL and Δ (SNP-index) methods; *qGER6-2* on Chr 6 (68.74-120.89 Mb; 865 genes), identified exclusively by the DL method. *qGER6-2* was selected over the other three Chr 6 loci (*qGER6-1*, *qGER6-3*, and *qGER6-4*) because it exhibited the highest DL peak value, the largest physical interval, and the greatest number of genes, classifying it as a major-effect locus. In contrast, *qGER6-1*, *qGER6-3*, and *qGER6-4* displayed weaker signals or smaller intervals and were considered minor-effect loci. Subsequent analyses therefore focused on *qGER5* and *qGER6-2* for candidate gene identification, functional annotation, and preliminary exploration of resistance regulatory mechanisms.

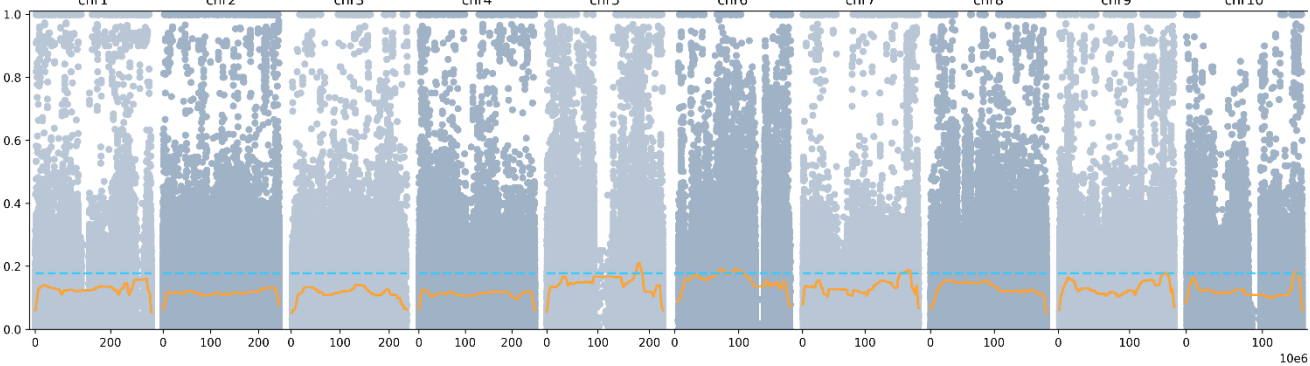
311 Table 1 Parameters associated with quantitative trait loci for GER resistance identified in F₂ populations

Loci	Chr	Start (bp)	End (bp)	Size (Mb)	Methods	Gene Number
<i>qGER5</i>	5	170, 178, 634	185, 844, 922	15.67	DL、 Δ (SNP-index)	388
<i>qGER6-1</i>	6	65, 799, 238	78, 567, 548	12.77	Δ (SNP-index)	123
<i>qGER6-2</i>	6	68, 743, 394	120, 891, 836	52.15	DL	865
<i>qGER6-3</i>	6	85, 059, 639	107, 122, 171	22.06	Δ (SNP-index)	374
<i>qGER6-4</i>	6	140, 688, 771	145, 809, 901	5.12	DL	120
<i>qGER7</i>	7	160, 327, 303	172, 872, 495	12.55	Δ (SNP-index)	411
<i>qGER9</i>	9	147, 416, 101	149, 106, 013	1.69	Δ (SNP-index)	54
<i>qGER10</i>	10	140, 770, 174	146, 176, 298	5.41	DL、 Δ (SNP-index)	214

312 A



313 B



314 Figure 4 Preliminary mapping of genes associated with resistance to ear rot via BSA-seq in maize

315 Note: A: Results obtained using the DL method; B: Results obtained using Δ SNP-index.

3.2.2 RNA-Seq reveals differential gene expression between resistant and susceptible maize inbred lines

Using samples collected at 0 h post-inoculation as controls, differentially expressed genes (DEGs) in the susceptible inbred line K0742 and the resistant inbred line K0743 were identified at 72 h, 96 h, and 7 d post-inoculation, respectively. In the susceptible inbred line K0742, a total of 805, 2,212, and 3,255 DEGs were detected at 72 h, 96 h, and 7 dpi, harboring 299, 484, and 1,026 upregulated genes, respectively (Figure 5A). The numbers of DEGs identified in the resistant inbred line K0743 reached 6,030, 4,929, and 10,254 at the corresponding time points, with 1,714, 4,453, and 2,968 genes being upregulated respectively (Figure 5B). Notably, the number of DEGs detected in the resistant inbred line K0743 at 72 h (6,030) was substantially higher than that in the susceptible inbred line K0742 (805), indicating a more rapid transcriptional response to *F. graminearum* infection in the resistant inbred line.

To further characterize transcriptional differences between the above genotypes, the samples collected at 0 h, 72 h, 96 h, and 7 d post-inoculation were used to perform the comparative analysis among the different groups by venn diagram (Figure 5C-D). Comparisons between resistant inbred line K0743 and susceptible inbred line K0742 at the same time points identified 564 constitutively differential expressed genes that were consistently detected regardless of infection status, suggesting inherent transcriptional differences between the two genotypes. In addition, 1,723 genotype-specific DEGs were identified between resistant inbred line K0743 and susceptible inbred line K0742 at 0 h, representing basal expression differences prior to pathogen infection. Following inoculation, the number of genotype-specific DEGs increased progressively and reached its maximum at 96 h.

Temporal expression analysis revealed the distinct transcriptional response patterns between these two inbred lines. In the resistant inbred line K0743, 277 DEGs were shared across all post-inoculation time points, indicating a set of core genes that responded continuously throughout the infection process.

Furthermore, the number of time-specific DEGs increased gradually over time. However, susceptible inbred line K0742 contained 2,065 DEGs that were consistently detected across all infection time points, and the number of time-specific DEGs increased markedly, reaching 5,461 at 7 d.

Collectively, these results demonstrated substantial differences in transcriptional responses between these two resistant and susceptible inbred lines during *F. graminearum* infection. Resistant inbred line K0743 exhibited an earlier and more dynamic transcriptional response, whereas the susceptible inbred line K0742 displayed extensive changes at the transcription level, particularly at the late stage of *F. graminearum* infection.

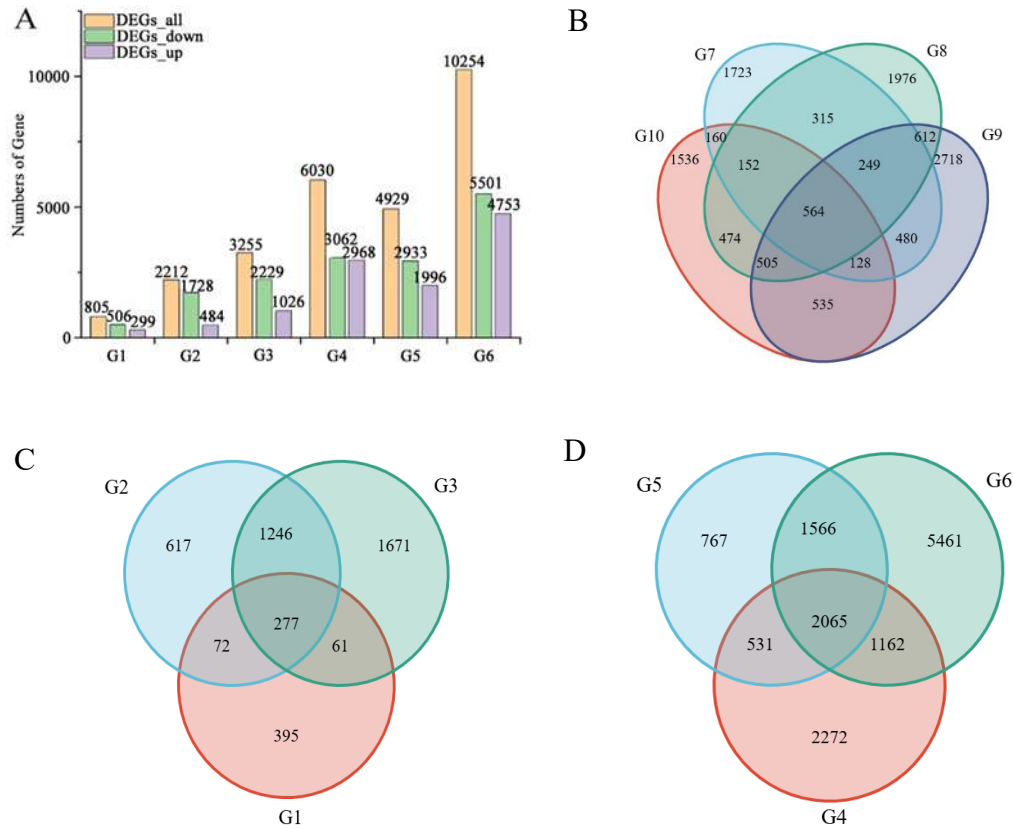


Figure 5 Number of differentially expressed genes (DEGs) and venn diagram

Note: G1: K0742_0h vs K0742_72h; G2: K0742_0h vs K0742_96h; G3: K0742_0h vs K0742_7d; G4: K0743_0h vs K0743_72h; G5: K0743_0h vs K0743_96h; G6: K0743_0h vs K0743_7d; G7: K0742_0h vs K0743_0h; G8: K0742_72h vs K0743_72h; G9: K0742_96h vs K0743_96h; G10: K0742_7d vs K0743_7d.

To further characterize the biological functions associated with the DEGs identified in the resistant inbred line K0743 and the susceptible inbred line K0742 following *F. graminearum* infection,

GO enrichment analysis was performed for the DEGs detected at 72 h, 96 h, and 7 d post-inoculation (Figure 6). The results showed that DEGs in the resistant inbred line K0743 were significantly enriched in multiple GO terms associated with plant defense responses across the three GO categories. Within the biological process category, significantly enriched terms included “response to biotic stimulus”, “defense response to fungus”, “cell wall organization or biogenesis”, “secondary metabolite biosynthetic process”, and “phenylpropanoid metabolic process”, containing 124, 67, 89, 53, and 78 DEGs, respectively. The proportions of DEGs assigned to these terms were significantly higher than those in the background gene set ($P < 0.01$). In the cellular component category, DEGs in the resistant inbred line K0743 were predominantly enriched in “cell wall”, “plasma membrane”, and “apoplast”, with 102 DEGs assigned to the “cell wall” term. In the molecular function category, significant enrichment was observed for “catalytic activity”, “binding”, “oxidoreductase activity”, and “chitinase activity”, indicating extensive activation of genes involved in enzymatic and defense-related functions. However, GO enrichment analysis of DEGs in the susceptible inbred line K0742 revealed that the significantly enriched terms were mainly associated with primary metabolic activities, including “small molecule metabolic process” and “oxidation–reduction process”. Most defense-related GO terms identified in the resistant inbred line K0743 were not significantly enriched in the susceptible inbred line K0742. Notably, the number of DEGs assigned to the term “defense response to fungus” in the resistant inbred line K0743 increased by 42.6% at 7 d compared with 72 h, suggesting a sustained transcriptional response to pathogen infection during the later stages of disease incidence. Overall, GO enrichment analysis revealed distinct functional characteristics of the transcriptional responses in these two inbred lines. Compared with the susceptible inbred line K0742, the resistant inbred line K0743 exhibited stronger enrichment of genes involved in defense-related processes, cell wall-associated functions, and secondary metabolism following *F. graminearum* infection.

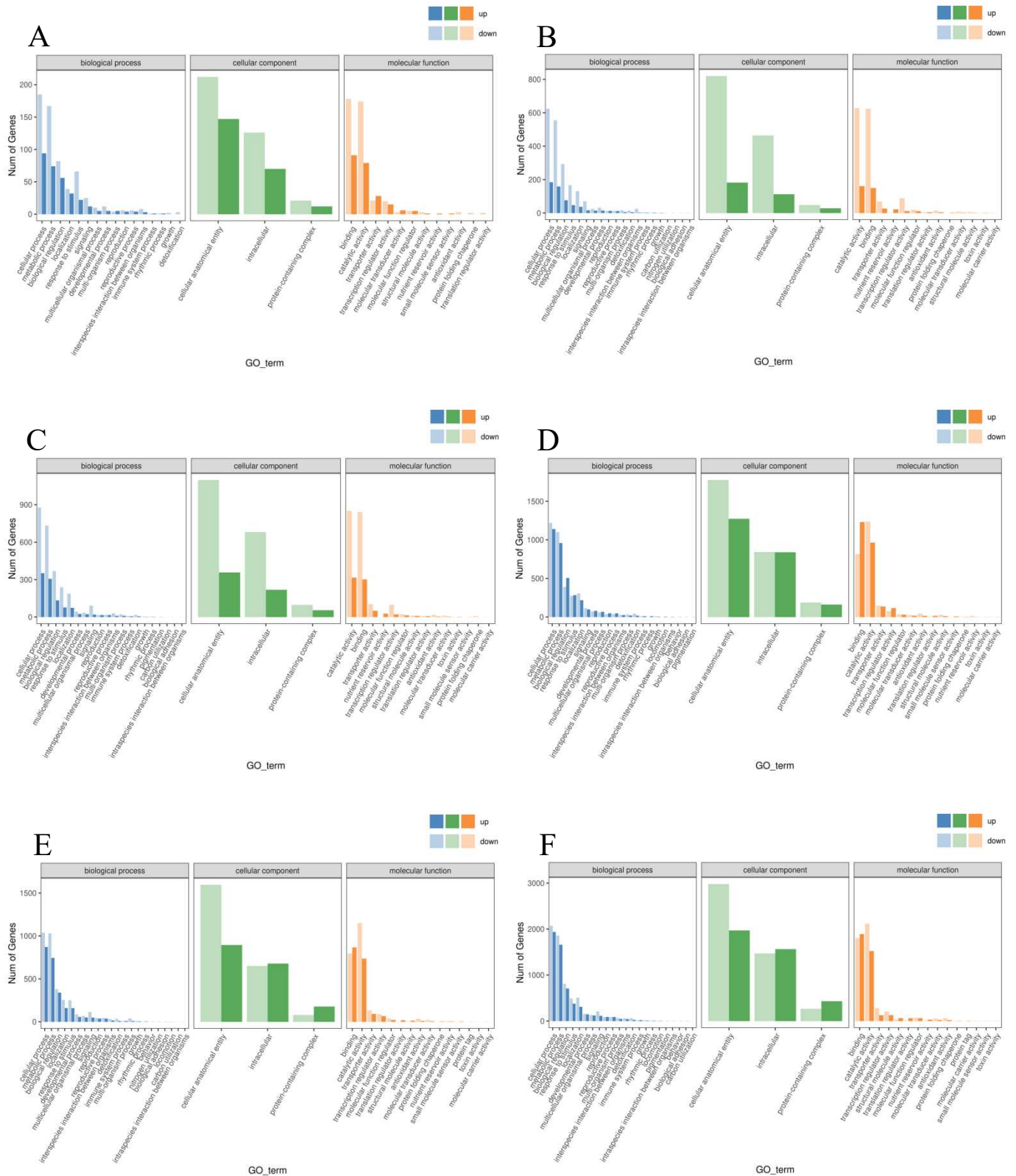


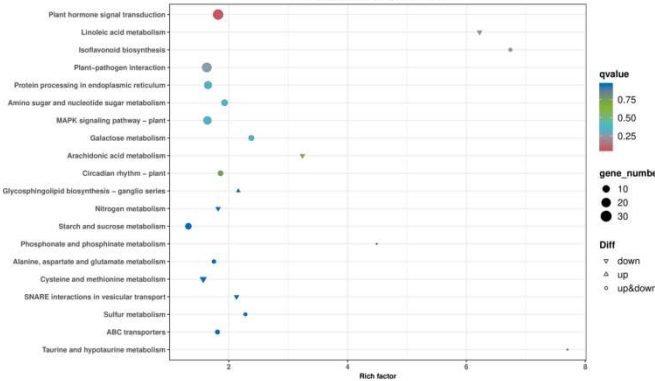
Figure 6 GO enrichment analysis of DEGs in the susceptible inbred line K0742 and resistant inbred line K0743

Note: A: GO enrichment analysis of DEGs in the susceptible inbred line K0742 between 0 h and 72 h; B: GO enrichment analysis of DEGs in the susceptible inbred line K0742 between 0 h and 96 h; C: GO enrichment analysis of DEGs in the susceptible inbred line

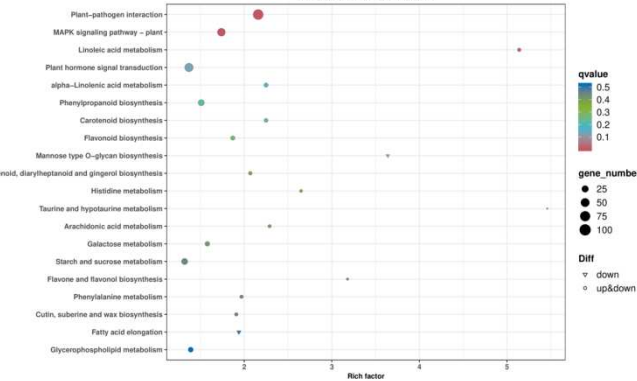
K0742 between 0 h and 7 d; D: GO enrichment analysis of DEGs in the resistant inbred line K0743 between 0 h and 72 h; E: GO enrichment analysis of DEGs in the resistant inbred line K0743 between 0 h and 96 h; F: GO enrichment analysis of DEGs in the resistant inbred line K0743 between 0 h and 7 d.

In the resistant inbred line K0743, 72 h post-inoculation revealed significant enrichment in 15 pathways, including “plant–pathogen interaction” (86 DEGs), “MAPK signaling pathway” (52 DEGs), and “plant hormone signal transduction” (67 DEGs). By 96 h post-inoculation, 12 pathways remained enriched, with additional ROS-related pathways, such as “glutathione metabolism” and “peroxisome,” included among them. Seven pathways associated with energy and primary metabolism were enriched by 7 d post-inoculation. In the susceptible inbred line K0742, fewer pathways were enriched: only the “plant hormone signal transduction” pathway was significant at 72 h (23 DEGs), and three pathways were enriched at 96 h, with DEG counts lower than in the resistant inbred line K0743 ($P < 0.05$). No disease-resistance-related pathways were enriched in the susceptible inbred line K0742 by 7 d. Notably, the “plant hormone signal transduction” pathway remained enriched throughout the observed period in the resistant inbred line K0743, with 67 DEGs, representing a 2.9-fold higher gene count relative to susceptible inbred line K0742, whereas enrichment in the susceptible inbred line K0742 was transient, observed only at 72 h. These results indicate that resistant inbred line K0743 exhibits earlier, stronger, and sustained activation of disease-resistance-related pathways than susceptible inbred line K0742.

A



B



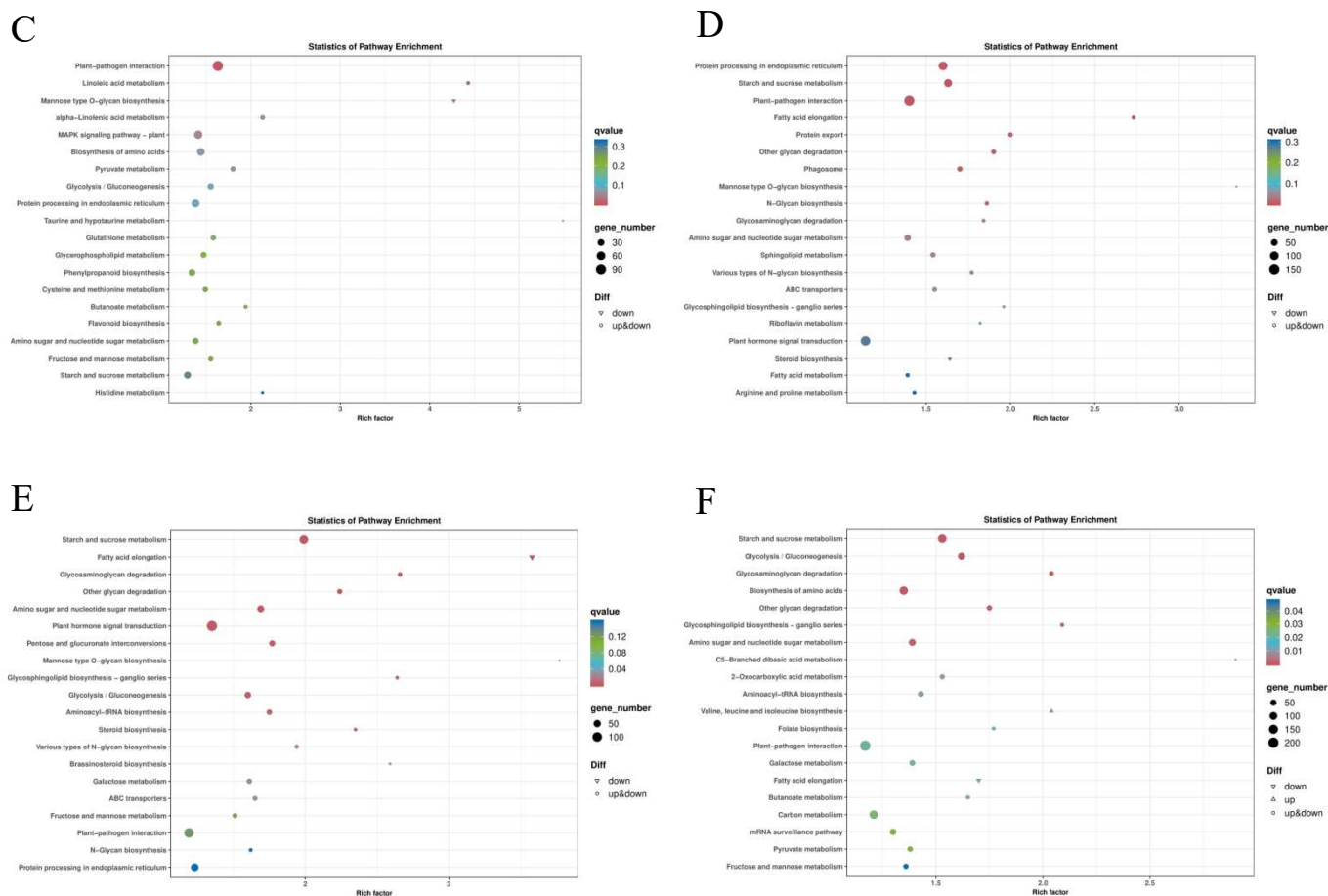


Figure 7 KEGG pathway enrichment of DEGs in the susceptible inbred line K0742 and resistant inbred line K0743

Note: A-C: Main KEGG pathways in the susceptible inbred line K0742 at 72 h, 96 h and 7 d vs. 0 h, respectively; D-F: Main KEGG pathways in the resistant inbred line K0743 at 72 h, 96 h and 7 d vs. 0 h, respectively.

3.2.3 RT-qPCR validation of transcriptome data

To validate the reliability of the transcriptome sequencing results, nine differentially expressed genes (DEGs) were randomly selected for RT-qPCR analysis. The expression patterns of these nine genes across the 12 sample groups were consistent between the RT-qPCR and RNA-Seq results (Figure 8). The reliability of the transcriptome data was further supported by a significant correlation coefficient ($r > 0.8$) between the RNA-Seq and RT-qPCR data, reinforcing the validity of the transcriptomic findings.

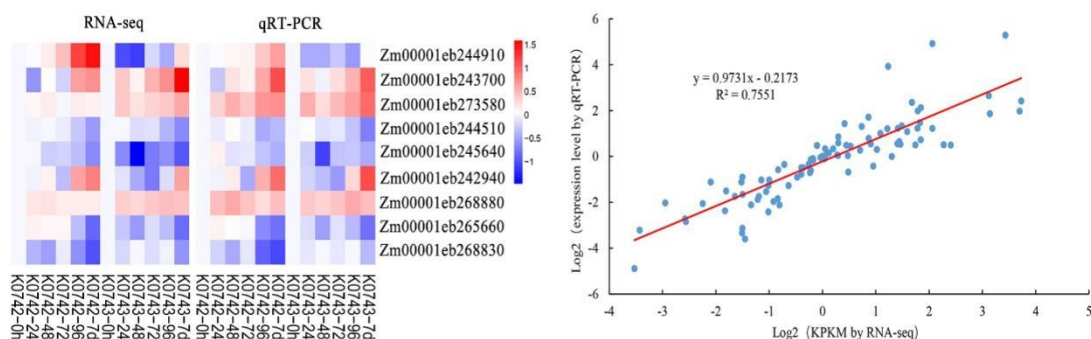


Figure 8 The expression pattern of 9 selected genes identified by RNA-seq was verified by RT-qPCR

Note: A: Heat map showing the expression changes (log₂-fold change) of each Differentially Expressed Gene (DEGs) measured by RNA-Seq and RT-qPCR after treatment at 0 h, 24 h, 48 h, 72 h, 96 h and 7 d in resistant and susceptible varieties K0742 and K0743; B: Scatter plot showing the changes in the expression (log₂-fold change) of selected genes based on RNA-Seq via RT-qPCR. Gene expression levels are indicated by colored bars.

3.2.4 Weighted gene co-expression network analysis (WGCNA)

A total of 15 co-expression modules were identified by WGCNA. Among them, two modules, MEfloralwhite and MEblue4, exhibited the strongest positive correlation with disease resistance (Figure 9). MEfloralwhite was predominantly enriched in oxidative stress response, glutathione metabolism, and ubiquitin-mediated proteolysis, indicating a role in ROS scavenging and protein turnover during infection. In contrast, MEblue4 was significantly enriched in pathways related to plant hormone signal transduction, MAPK signaling, and phenylpropanoid biosynthesis, suggesting its involvement in early defense signaling and cell wall reinforcement.

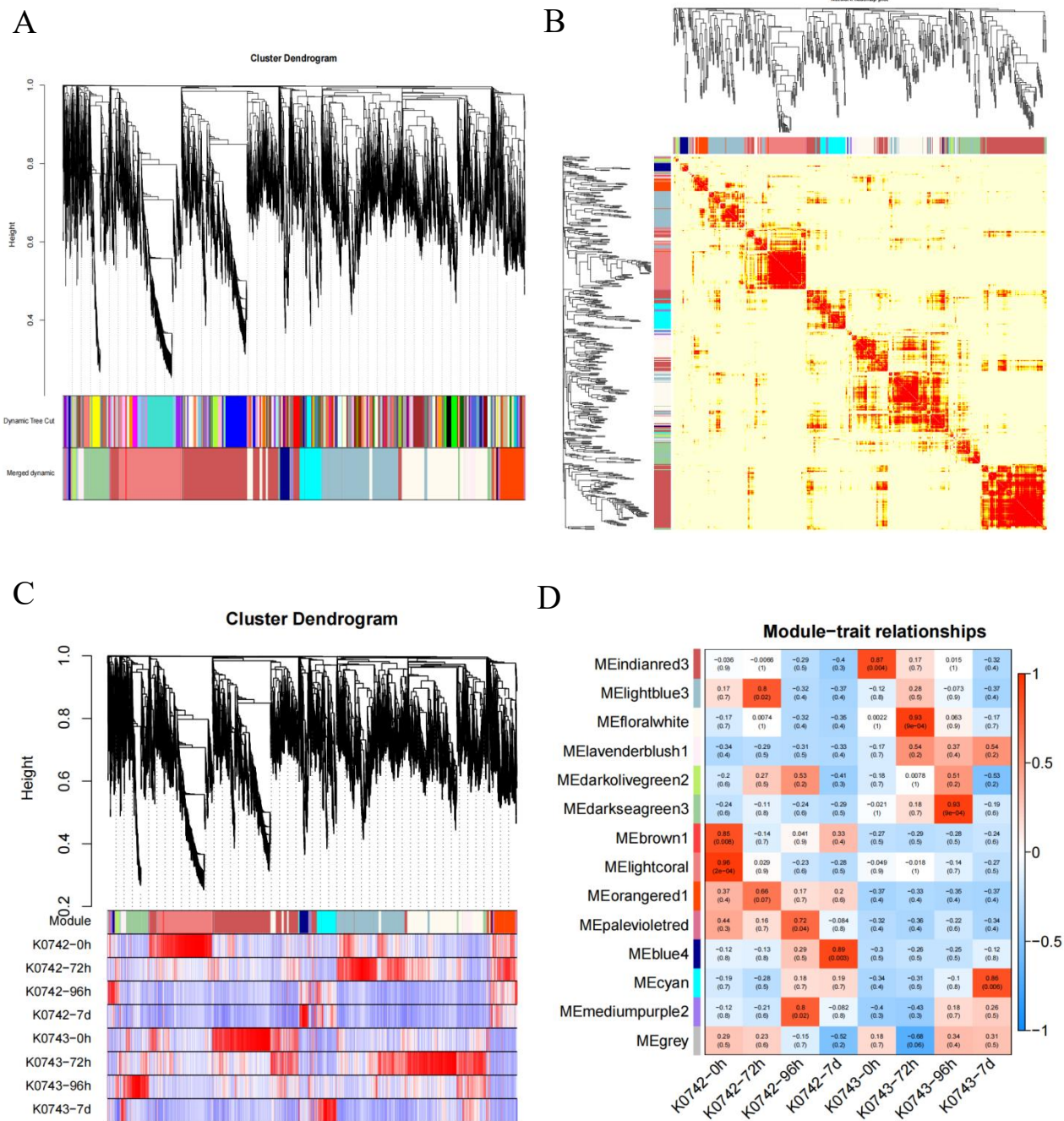


Figure 9 Construction of gene co-expression modules based on WGCNA

Note: A: Gene co-expression network modules identified by WGCNA; B: heatmap of the gene co-expression network; C: gene dendrogram combined with the trait correlation heatmap; D: heatmap showing correlations between WGCNA modules and traits.

In the MEfloralwhite co-expression network (Figure 10A) comprises three genes: *Zm00001eb242770* (Chr5), *Zm00001eb273840* (Chr6), and *Zm00001eb269150* (Chr6). These genes

are primarily associated with antioxidant defense and oxidative stress responses, consistent with the functional enrichment of the MEfloralwhite module. In contrast, the MEblue4 network consists of three genes: *Zm00001eb244910* (Chr5), *Zm00001eb269250* (Chr6), and *Zm00001eb276160* (Chr6), which are putatively involved in cell wall modification and structural reinforcement. Together, the two modules capture complementary defense mechanisms-oxidative stress management (MEfloralwhite) and structural reinforcement (MEblue4)-that contribute to maize ear rot resistance.

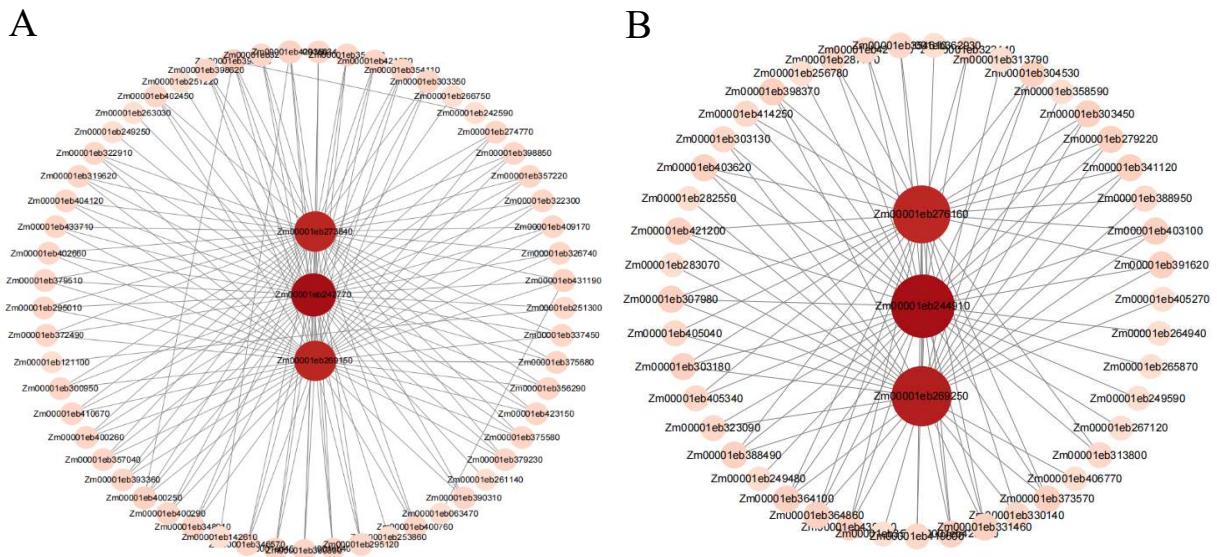


Figure 10 Interaction network analysis of hub genes identified in key WGCNA modules

Note: A: represents the gene co-expression network of the MEfloralwhite module. B: represents the gene co-expression network of the MEblue4 module.

3.2.5 Integrated analysis of BSA-Seq and RNA-Seq identifies candidate disease-resistance genes

Integration of BSA-Seq-derived QTL intervals (*qGER5* and *qGER6-2*) with RNA-Seq-based differential expression profiles identified 6 high-confidence candidate genes. These genes were located within or adjacent to the mapped loci and showed significant expression changes ($|\log_2FC| \geq 1$, $FDR < 0.01$) in the resistant inbred line K0743 compared with the susceptible inbred line K0742 following inoculation. Among them, two genes (*Zm00001eb242770* and *Zm00001eb244910*) were located on Chr 5 within *qGER5*, and four genes (*Zm00001eb273840*, *Zm00001eb276160*, *Zm00001eb269250* and

456 *Zm00001eb269150*) on Chr 6 within *qGER6-2*. Functional annotation indicated that these candidates
 457 are involved in cell wall modification, defense signal transduction, oxidative stress response, and
 458 regulation of pathogen development. Notably, all 6 candidates overlapped with hub genes identified
 459 by WGCNA in the MEfloralwhite and MEblue4 modules (*Zm00001eb242770*, *Zm00001eb273840*,
 460 *Zm00001eb269150* for MEfloralwhite; *Zm00001eb244910*, *Zm00001eb269250*, *Zm00001eb276160*
 461 for MEblue4), providing strong cross-validation between independent approaches. Thus, the
 462 integration of BSA-Seq and RNA-Seq prioritized a functionally diverse set of candidate genes that
 463 may act coordinately to confer resistance to *Gibberella* ear rot in maize.

464 Table 2 Information of candidate genes

No.	Gene ID	Gene annotation	Chr
1	<i>Zm00001eb242770</i>	Iron-sulfur cluster biosynthesis family protein	5
2	<i>Zm00001eb273840</i>	auxin response factor 17	6
3	<i>Zm00001eb269150</i>	Growth-regulating factor 6	6
4	<i>Zm00001eb244910</i>	Galactose mutarotase-like superfamily protein	5
5	<i>Zm00001eb269250</i>	ARM repeat superfamily protein	6
6	<i>Zm00001eb276160</i>	alpha/beta-Hydrolases superfamily protein	6

465

466 4 Discussion

467 4.1 Enhanced cell wall defense is associated with disease resistance in the resistant inbred line

468 K0743

469 Significant differences in kernel cellular integrity were observed between the resistant inbred line
 470 K0743 and the susceptible inbred line K0742 following *F. graminearum* infection. SEM analysis
 471 showed that, at 72–96 h post-inoculation, starch granules in the susceptible inbred line K0742 exhibited
 472 depressions and wrinkling, and cellular tissues were extensively colonized by fungal hyphae. The tissue
 473 structure of susceptible inbred line K0742 was severely disrupted at 7d. In contrast, resistant inbred

line K0743 maintained relatively intact cellular structures throughout the infection process, with starch granules largely preserved even at 7 d, suggesting enhanced resistance to pathogen invasion and spread.

Transcriptome analysis further showed that multiple cell wall modification-related genes were significantly induced in the resistant line. These included a pectinesterase inhibitor gene, which may inhibit pathogen-secreted pectinases and preserve wall integrity, as well as several glycoside hydrolases capable of releasing oligosaccharide signals to activate downstream immune responses. The coordinated activation of structural reinforcement and immune signaling likely underlies the reduced cellular damage observed in the resistant line.

4.2 Coordinated roles of the reactive oxygen species-scavenging system and oxidative stress response

The defense enzyme system of resistant inbred line K0743 was activated more rapidly and robustly than in the susceptible inbred line K0742. PAL and PPO, key enzymes in the phenylpropanoid pathway, peaked earlier in the resistant inbred line K0743 (PAL at 96 h, PPO 1.67-fold higher than susceptible inbred line K0742, $P < 0.01$), facilitating early lignin and phenolic compound accumulation to reinforce the cell wall[36][37]. SOD activity in the resistant inbred line K0743 peaked at 72 h and declined gradually, whereas susceptible inbred line K0742 showed a bimodal pattern at 48 and 96 h, with a peak approximately 10% lower than resistant inbred line K0743 ($P < 0.05$), indicating a more stable redox regulation in the resistant line.

Consistent with enzyme activity, transcriptome analysis showed significant enrichment of antioxidant-related genes in glutathione metabolism and peroxisome pathways in the resistant inbred line K0743. Among these, the monooxygenase gene (*Zm00001eb243970*) was continuously highly expressed, with a 4.2-fold higher expression than in the susceptible inbred line K0742 at 7 d ($P < 0.001$). This gene may contribute to ROS scavenging and the biosynthesis of secondary metabolites such as phenolic compounds and lignin precursors. Together, these results suggest that resistant inbred line

K0743 establishes a stable and efficient ROS-scavenging network that supports structural defense and mitigates oxidative stress during infection.

4.2 Osmotic adjustment and energy metabolism reprogramming support disease resistance

Resistant inbred line K0743 exhibited more efficient osmotic regulation than susceptible inbred line K0742. Proline accumulation was higher in the resistant inbred line K0743 at 7 d, supported by enrichment of amino acid biosynthesis pathways. Soluble sugars increased significantly from 72 h, with levels 38.6% higher than in the susceptible inbred line K0742 at 7 d ($P < 0.05$), consistent with continuous enrichment of the “starch and sucrose metabolism” pathway, suggesting sufficient energy and carbon skeleton supply for defense. Soluble protein peaked earlier in the resistant inbred line K0743 (48 h) compared with susceptible inbred line K0742 (96 h), indicating rapid conversion of storage proteins into defense-related molecules, supported by activation of “protein processing in the endoplasmic reticulum” pathways. These coordinated adjustments in proline, sugars, and proteins provide a physiological basis for enhanced disease resistance[38][39].

4.4 Resistance differences regulated by signal transduction networks

BSA-Seq analysis identified eight resistance-associated loci across Chr 5, 6, 7, 9, and 10, among which *qGER5* and *qGER6-2* were defined as major loci based on peak signal intensity and reproducibility. Notably, *qGER5* was consistently detected by both DL and Δ (SNP-index) approaches, indicating high reliability. Transcriptome and WGCNA analyses revealed that the resistant inbred line K0743 activated key defense-related pathways earlier and more strongly than the susceptible inbred line K0742. Specifically, pathways involved in plant hormone signal transduction, MAPK signaling, and plant-pathogen interactions were significantly enriched in the resistant inbred line K0743 at early infection stages. Within the MEblue4 co-expression module, genes associated with hormone signaling and cell wall modification were preferentially expressed, whereas the MEfloralwhite module comprised genes involved in oxidative stress response and ubiquitin-mediated

proteolysis. The coordinated expression of these modules suggests that upstream signal perception and transduction are tightly coupled with downstream defense execution, including ROS scavenging, cell wall reinforcement, and protein turnover. These findings provide a mechanistic basis for understanding the quantitative nature of maize ear rot resistance.

4.5 Candidate genes involved in oxidative stress and cell wall modification

Integration of BSA-Seq, RNA-Seq, and WGCNA identified 6 high-confidence candidate genes located within or adjacent to the major QTLs *qGER5* and *qGER6-2*. These genes were distributed across two co-expression modules: MEfloralwhite and MEblue4. The MEfloralwhite module (including *Zm00001eb242770*, *Zm00001eb273840*, and *Zm00001eb269150*) was predominantly associated with oxidative stress response, glutathione metabolism, and ubiquitin-mediated proteolysis. The MEblue4 module (including *Zm00001eb244910*, *Zm00001eb269250*, and *Zm00001eb276160*) was significantly enriched in plant hormone signal transduction, MAPK signaling, and cell wall modification pathways. All 6 genes showed significant expression differences between the resistant inbred line K0743 and the susceptible inbred line K0742 following inoculation, and their expression patterns were validated by RT-qPCR (Figure 8). The consistency across BSA-Seq, RNA-Seq, and WGCNA strengthens the reliability of these candidates, suggesting that resistance to *F. graminearum* in the resistant inbred line K0743 is orchestrated by a multilayered network combining oxidative stress management (MEfloralwhite) with structural and signaling defense (MEblue4). These candidates provide promising targets for functional validation and molecular breeding.

5 Conclusion

An integrated multi-omics approach revealed the cytological, physiological, and molecular basis of maize resistance to *F. graminearum*-induced Gibberella ear rot. The resistant inbred line K0743 exhibited enhanced maintenance of kernel structural integrity, earlier and stronger activation of defense enzymes (PAL, PPO, SOD), and more efficient accumulation of osmotic adjustment substances than

the susceptible inbred line K0742. BSA-Seq identified major resistance-associated loci on Chr 5 and Chr 6 (*qGER5* and *qGER6-2*), which, combined with RNA-Seq and WGCNA analyses, led to the identification of 6 high-confidence candidate genes. These genes were distributed across two co-expression modules: MEfloralwhite (involved in oxidative stress response and ubiquitin-mediated proteolysis) and MEblue4 (enriched in plant hormone signal transduction, MAPK signaling, and cell wall modification pathways). Functional annotation indicated potential roles in cell wall modification, defense signal transduction, oxidative stress response, and host-pathogen interaction processes. Collectively, these findings provide a comprehensive understanding of GER resistance mechanisms in maize and highlight a set of candidate genes and loci for future functional validation and molecular breeding aimed at improving ear rot resistance.

Data availability

The sequencing data (BSA-Seq and RNA-Seq) supporting the findings of this study are not yet publicly available as they are part of ongoing research. However, upon acceptance of this manuscript, the raw data will be deposited in a public repository (e.g., NCBI SRA) and the corresponding accession numbers will be provided in the final version. Prior to publication, the data can be obtained from the corresponding author upon reasonable request, and will be shared with editors and reviewers for verification purposes.

Author contributions

FW planned and designed the experiments. JL and WZ wrote the manuscript. JL and WZ performed the experiments and analyzed data. JL, WZ, FC, WW, CW and YS participated in the planting and identification of experimental materials and the critical reading and discussion of the manuscript. All authors contributed to the article and approved the submitted version.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at:

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