



Identification of candidate regions associated with resistance to early blight in tomato (*Solanum lycopersicum* L.) using BSA-seq

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Abstract

Early blight, caused by *Alternaria alternata*, is a key factor that reduces tomato yield and tomato product quality. Owing to the quantitative nature of resistance and the limited number of characterized loci, identification of genomic regions associated with resistance remains an urgent task for breeding programs. Here, bulked segregant analysis with whole-genome resequencing (BSA-Seq) was used to identify candidate regions for resistance to early blight in the F₂ population obtained by crossing a resistant line (Gloria×BSS335) and a susceptible cultivar (Samaladay). A total of 14 overlapping candidate genomic regions were identified on chromosome 2 by intersecting SNP- and InDel-associated regions (totaling 0.26 Mb). Functional annotation of the 33 genes in this locus revealed the involvement of membrane proteins, components of signaling pathways, transcriptional regulators, and Rab GTPase family proteins, which are potentially involved in stress and immune responses. This is the first genome-wide study of early blight resistance in tomato in Kazakhstan. These results expand our understanding of the genetic architecture of early blight resistance and may serve as a basis for the functional validation of key candidate genes and their application in marker-assisted selection (MAS).

Keywords Early blight · *Solanum lycopersicum* (L.) · BSA-Seq · Candidate gene · Resistance · Functional annotation

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Abbreviations

BSA	Bulked Segregant Analysis
COG	Clusters of Orthologous Groups
ED	Euclidean Distance
GO	Gene Ontology
InDel	Insertion–Deletion
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAS	Marker-Assisted Selection
NR	Non-Redundant Protein Sequences
QTL	Quantitative Trait Locus
SNP	Single-Nucleotide Polymorphism
SwissProt	The manually annotated and reviewed protein sequence database

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops, playing a key role in ensuring food security and nutrition of the population throughout the world. According to FAO, the annual world production of tomatoes exceeds 192.3 million metric tons (FAOSTAT

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2025). However, tomato fruit yield and quality are significantly reduced by various diseases, with early blight being among the most destructive (Adhikari and Panthee 2017; Foolad et al. 2008).

Early blight is caused by several fungal species of the genus *Alternaria*, including *A. linariae* (syn. *A. tomatophila*), *A. solani* and *A. alternata* (Panthee and Paudel 2024). Among the *Alternaria* species identified in Kazakhstan, the most aggressive tomato pathogen is *A. alternata* (Yesimseitova et al. 2025). A characteristic symptom of early blight is the formation of necrotic spots with concentric rings, which occur mainly on the leaves, as well as on the stems and, in some cases, on the fruits. This disease leads to leaf necrosis, fruit quality deterioration, and significant crop losses (Agrios 2005; Sadeghi et al. 2022; Thomma 2003). Unlike biotrophs, which extract nutrients from living cells, *Alternaria* species are necrotrophic pathogens. In plants, resistance to necrotrophs requires the activation of complex and multilevel defense mechanisms (Sadeghi et al. 2022; Zhang et al. 2013; Tan et al. 2010; Glazebrook 2005).

The main current measures to combat tomato early blight are agronomic practices, the use of fungicides, and the use of resistant varieties (Adhikari and Panthee 2017). However, agronomic measures do not completely protect plants from disease. The use of fungicides can be effective (Odilbekov et al. 2019), but is associated with a number of limitations, such as high cost and adverse effects on the environment and human health (Wightwick et al. 2010). Breeding crops for resistance is the most effective and environmentally friendly approach for disease prevention (Dodds and Rathjen 2010). However, the number of tomato varieties resistant to early blight remains limited, owing to the polygenic nature of this trait and the insufficient number of characterized resistance genes (Adhikari and Panthee 2017; Zhang et al. 2003; Ashrafi and Foolad 2015; Adhikari et al. 2023).

Although resistance has been identified in wild tomato species (*S. habrochaites*, *S. peruvianum*, *S. pimpinellifolium*), their use in breeding is difficult owing to the presence of unfavorable agronomic traits, such as late maturity, indeterminate growth, and low yield (Adhikari and Panthee 2017). Despite some success in mapping resistance loci, most of the available data are focused on other diseases (Sullenberger and Foolad 2024; Panthee et al. 2017; Chai et al. 2020; Chen et al. 2020), and the genetic architecture of resistance to early blight remains poorly understood. Further, the available molecular markers are not always reproducible in different genetic backgrounds, limiting their application in practical breeding (Collard and Mackill 2008; Wang et al. 2023; Xu et al. 2024).

Consequently, it remains urgent to identify the genomic regions associated with resistance and to clarify the localization of potential candidate genes. Bulk Segregant

Analysis combined with whole-genome resequencing (BSA-Seq) provides a promising method to achieve this by analyzing segregating populations. This method combines the classical BSA approach (Michelmore and Kesseli 1991; Giovannoni et al. 1991) with high-throughput sequencing and allows for the rapid and efficient identification of markers associated with traits, without the need to genotype the entire population and develop DNA markers (Takagi et al. 2013; Zhang et al. 2021). This method is based on crossing two parental lines with contrasting phenotypes (e.g., resistant and susceptible), obtaining an F₂ population, phenotyping the offspring, and forming two DNA pools based on extreme phenotypes. Resequencing of these pools allows us to identify the different frequencies of single-nucleotide polymorphisms (SNPs) and insertions and deletions (InDels) associated with the trait of interest (Takagi et al. 2013; Zhang and Panthee 2020). Unlike traditional quantitative trait locus (QTL) mapping, BSA-Seq requires genotyping of only individuals with extreme phenotypes, significantly reducing the labor and cost of analysis (Shen et al. 2023; Majeed et al. 2022). This approach has been successfully used to identify loci that control traits of interest in various crops including tomato (Topcu 2024; Sarwar et al. 2023), eggplant (Tassone et al. 2022), sunflower (Imerovski et al. 2019), peanut (Mo et al. 2025), and wheat (Klymiuk et al. 2022).

To the best of our knowledge, BSA-Seq has not yet been used to identify early blight resistance genes in tomatoes. Here, we applied this method to identify genomic regions and potential candidate genes associated with tomato early blight resistance based on F₂ population analysis. The main objectives were to identify associated genomic regions and to functionally annotate these candidate genes to assess their potential roles in resistance-related mechanisms. This work provides a basis for future functional validation of key candidate genes and their use in MAS of resistant tomato varieties.

Materials and methods

Plant material and population development

Two contrasting tomato parental genotypes were used to develop the mapping population. The resistant parent, Gloria×BSS335 (breeding line), and the susceptible parent, cultivar Samaladay, were both developed and maintained at the Kazakh Research Institute of Fruit and Vegetable Growing (Almaty, Kazakhstan). The resistance of Gloria×BSS335 to *A. alternata* was verified through multi-year field observations and artificial inoculation assays, where it consistently showed high levels of protection. Conversely, Samaladay

was selected due to its stable susceptible phenotype under the same conditions.

Crossing was performed between Gloria×BSS335 as the female parent (P1-2-1) and Samaladay as the pollen donor (P2-2-1) to obtain the F₁ generation. The resulting F₁ plants were subsequently self-pollinated to produce an F₂ population used for bulked segregant analysis sequencing (BSA-seq). All plant materials were grown at the Kazakh Research Institute of Fruit and Vegetable Growing during the 2023 and 2024 growing seasons.

Phenotyping of F₂ population

Seeds of the 291 F₂ population, resistant and susceptible parents were sown in 4.5×4.5 cm plastic trays and grown under greenhouse conditions in a 2:1 peat: vermiculite substrate supplemented with micro- and macroelements (Kekkilä Professional, Finland). After 12 days of germination, the seedlings were transplanted into 9×9 cm plastic pots. Plant placement was performed using a completely randomized design to eliminate the influence of microclimatic factors. A pure culture of *Alternaria alternata* was obtained as previously described (Yessimseitova et al. 2025). The *A. alternata* isolate used in this study was identified by sequence analysis of the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and histone H3 (H3) regions. The sequences were deposited in the NCBI GenBank database under accession numbers PQ881903, PV012141, and PV402132, respectively. Inoculation was carried out on six-week-old plants by uniform spraying with a suspension of *A. alternata* spores at 1×10⁷ sporangia/ml+0.1% Tween 20. After treatment, the plants were covered with a polyethylene film to maintain high humidity and were incubated at 25 °C for 48 h. The cover was then removed, humidity was reduced to 90%, and the plants were grown at 25 °C under a 16:8 h photoperiod (light: dark). Phenotypic assessment of the degree of early blight infection was conducted individually for each plant on days 14 and 21 after inoculation using a five-point scale (0, no symptoms; 5, complete leaf death) (Pandey et al. 2003). Based on the average values, the 30 plants with the lowest damage scores (resistant pool) and 30 with the highest damage scores (susceptible pool) were selected and used for BSA-Seq analyses.

DNA extraction and construction of segregating pools

Genomic DNA was extracted from the fresh leaves of the two parental and F₂ plants using the CTAB method (Doyle 1991). The quality of the extracted DNA was assessed using 1% agarose gel electrophoresis and DNA was quantified

using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Four DNA samples were prepared in this study: two parental samples (P1-2-1 and P2-2-1) and two F₂ pools (RGS1, a resistant pool, and SGS2, a susceptible pool). The F₂ pools were formed by combining equal amounts of DNA from 30 resistant and 30 susceptible plants selected based on phenotypic data. A standard DNA mass of 1.5 µg was used for each sample.

Library preparation and sequencing

DNA samples of the parental genotypes and pools of resistant and susceptible plants were sent to Biomarker Technologies Co., Ltd (Hong Kong, China) for library preparation, whole-genome sequencing, and data processing. Libraries were prepared individually for each sample. After quality assurance, the DNA was fragmented by ultrasonic disruption to an average fragment length of 350 bp. Standard library preparation steps were then performed, including fragment end alignment, 3'-tagging, adapter ligation, fragment selection, and PCR amplification of libraries. Libraries that successfully passed quality control were sequenced on an Illumina NovaSeq platform with 150 bp paired-end reads (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. The average sequencing depth was 20× for parental samples and 30× for pools.

Bulked segregant analysis (BSA)

Raw reads were obtained via sequencing. To remove low-quality reads and reads containing adapter sequences, the raw data were processed using fastp v0.21.0. During filtering, the error rate for each base (based on the Phred and Qphred quality scores), GC content, and level of sequence duplication in the cleaned data were calculated. All subsequent analyses were performed using only high-quality clean data. The clean reads were aligned to the tomato reference genome SL4.0 (https://solgenomics.net/ftp/tomato_genome/, accessed December 31, 2024) using bwa-mem2 v2.2 (Li and Durbin 2009). Alignment to the reference genome was performed with the parameters “bwa-mem2 mem -t 4 -M.” Using SAMtools v1.9 software (Danecek et al. 2021), the alignment files were sorted, and the sequencing depth and genome coverage indices were calculated. GATK v3.8 (McKenna et al. 2010) was used to detect SNP and InDel variants. The obtained variants were filtered using the following parameters: Quality Depth<2.0, Mapping Quality<40.0, Fisher Strand>60.0, and quality score<30.0. After filtering, the variation sites were annotated using SnpEff (Cingolani et al. 2012) to predict their functional effects. For linkage analysis, high-quality SNPs were selected, excluding those with multiple alleles, coverage depth<4, the same genotype

in all pools, those that were homozygous and identical in both parents, and those that did not correspond to the expected type of inheritance from a recessive or dominant parent. SNP and InDel variants identified from the sequencing data were then used for linkage analysis. The Euclidean Distance (ED) algorithm (Hill et al. 2013) was used to estimate differences in allele frequencies between the resistant and susceptible pools separately for SNPs and InDels. The Δ SNP-index (Takagi et al. 2013; Fekih et al. 2013) was calculated to quantitatively compare allele frequencies between the two pools. Genomic regions potentially associated with the trait were identified using fitted Δ SNP-index and Δ InDel-index curves calculated with a sliding-window approach. Significance thresholds were estimated based on simulation-derived confidence intervals. As no genomic regions exceeded the stringent linkage threshold, candidate regions were subsequently defined by selecting windows with fitted Δ SNP- and Δ InDel-index values within the upper 99% of the genome-wide fitted index distributions. Regions consistently detected by both Δ SNP- and Δ InDel-based analyses were prioritized for further analysis.

Candidate region gene functional annotation

Functional annotation of candidate genes located in the identified regions was performed using BLAST (Altschul 1997). The non-redundant protein sequences (NR, <http://ftp://ftp.ncbi.nih.gov/blast/db/>) (Deng et al. 2006), a manually annotated and reviewed protein sequence (SwissProt, <http://www.uniprot.org/>) (Deng et al. 2006), Gene Ontology (GO; <http://www.geneontology.org/>) (Ashburner et al. 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) (Kanehisa et al. 2004), and Clusters of Orthologous Groups (COG, <http://www.ncbi.nlm.nih.gov/COG/>) (Tatusov et al. 2000) databases were used for functional annotation and to identify candidate genes potentially involved in early blight resistance.

To evaluate the statistical significance of functional categories and to reduce potential bias caused by differences in annotation richness across the genome, hypergeometric tests were applied for GO and KEGG enrichment analyses. Statistical significance was assessed based on adjusted *P*-values (*Q*-values) obtained from the enrichment analysis. All bioinformatic analyses were performed using the integrated pipeline provided by the BMKCloud platform (<http://www.biocloud.net/>).

Statistical analysis

Descriptive statistics, including mean, standard deviation, skewness, kurtosis, and coefficient of variation (CV%), were calculated for disease severity scores in the F_2 population

Table 1 Phenotypic variation of F_2 population

F_2 population					
Range	Mean	SD	Skewness	Kurtosis	CV, %
0–5	2.62	1.04	−0.049	0.633	39.6

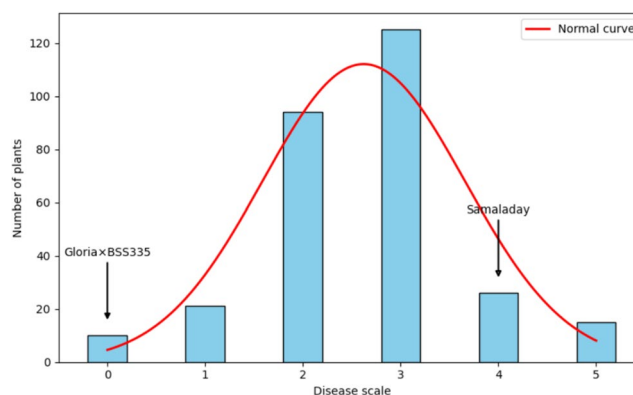


Fig. 1 Frequency distribution of disease severity scores in the F_2 population of tomato. The histogram shows the number of plants scored on a 0–5 scale based on symptoms of early blight caused by *A. alternata*. The red curve represents the fitted normal distribution, and arrows indicate resistant and susceptible parents

using Microsoft Excel and Python 3.11. A histogram with a fitted normal distribution curve was generated using Matplotlib v3.8.

Results

Phenotypic analysis

Phenotypic evaluation of the infected tomato plants revealed early blight symptoms as necrotic spots with concentric rings on the leaves; in the most susceptible individuals, these progressed to complete defoliation. The parental lines showed a distinct contrasting reaction in terms of damage scores: the resistant Gloria×BSS335 scored zero, whereas the susceptible Samaladay line scored 4. In the F_2 population, the damage scores varied from 0 to 5, averaging 2.62 (Table 1).

The phenotypic distribution of the F_2 population exhibited a weak negative skewness (−0.049), suggesting a tendency toward duplicate gene action governing resistance. The excess kurtosis value (0.633) indicates a distribution close to normal, reflecting the broad phenotypic variation and the polygenic nature of the trait. The high coefficient of variation (39.6%) further supports the quantitative inheritance of early blight resistance, making the population suitable for BSA-seq analysis (Fig. 1). Based on the average scores, plants with minimum and maximum damage scores (resistant and susceptible pools, respectively; $n=30$ per group) were selected for using BSA-seq.

Table 2 Summary of Clean Reads and Alignment Statistics for BSA-Seq Samples

Sample	Total reads	Q20 (%)	Q30 (%)	GC (%)	Mapped (%)	Average depth (x)	Coverage (%)
P1-2-1	148,386,562	98.32	95.21	36.80	99.65	28	95.11
P2-2-1	153,010,638	98.40	95.49	36.34	98.80	28	92.31
RGS1	170,444,258	98.28	95.10	35.15	99.54	32	97.07
SGS2	182,516,950	98.12	94.60	35.92	99.46	34	97.03

Table 3 Summary statistics of SNP calling in parental lines and bulks

Sample	SNP number	Transition	Transversion	Ti/Tv	Heterozygosity	Homozygosity	Het-ratio (%)
P1-2-1	269,420	154,187	115,233	1.33	97,270	172,150	36.1
P2-2-1	628,636	352,287	276,349	1.27	136,066	492,570	21.64
RGS1	648,983	366,170	282,813	1.29	513,586	135,397	79.13
SGS2	650,964	367,267	283,697	1.29	515,622	135,342	79.2
Total	715,888	405,855	310,033	1.3	-	-	-

BSA-seq analysis

After sequencing and filtering the raw reads, we obtained 148386562, 153010638, 170444258, and 182516950 clean read pairs, respectively, from four samples (parental samples P1-2-1 and P2-2-1 and mixed-pool samples RGS1 and SGS2). Q20 ranged from 98.12% to 98.40%, Q30 from 94.60% to 95.49%, and GC content from 35.15% to 36.80%. The clean reads were aligned to the tomato reference genome SL4.0, with an average percentage of mapped reads of 99.36% and average coverage depth of $30.5\times$ (Table 2). These results confirm the high quality of the data obtained and their suitability for subsequent analysis using BSA-Seq.

SNP analysis revealed significant differences in the number and nature of variations among the samples studied. In total, 715,888 SNPs were detected. The average transition-to-transversion ratio was 1.3, corresponding to the expected value for high-quality data and indicating the high reliability of SNP calling. The heterozygosity levels varied from 21.64% to 79.2%. The mixed-pool samples (SGS2 and RGS1) exhibited a significantly higher proportion of heterozygous SNPs (79%), typical of segregating populations. The parental lines, and P2-2-1 in particular, were characterized by higher homozygosity, reflecting their stable genotype. These differences confirmed genetic segregation in the F₂ population and highlighted the suitability of the data for subsequent linkage analysis and identification of SNPs associated with the resistance trait (Table 3).

Differences in SNPs and InDels between the parent and mixed pools are presented using a Venn diagram (Fig. 2A, B). The SNP and InDel annotation results for all accessions were visualized using a pie chart (Fig. 2C, D). Of the 715,888 SNPs identified, 57.09% were nonsynonymous coding mutations and 71.57% were located in the intergenic regions. Among the InDels, 79.61% were associated with putative reading frameshifts and 60.54% were intergenic. Because such mutations can significantly alter protein

structure, they are expected to have a high probability of affecting gene function and may be involved in the development of early blight resistance in tomatoes.

For linkage analysis, 377,577 SNPs and 44,690 InDels meeting the established quality criteria were selected from the total set of identified variants. The sequencing depth of the differential SNPs in each mixed pool was used to calculate the Euclidean Distance (ED) in each genomic region. To minimize the influence of background noise caused by minor variations, the obtained ED values were raised to a given power. Additionally, the number of SNPs per window (SNPNUM) was processed to refine the ED curve fitting and more accurately identify linked genomic regions. The median plus three standard deviations (Median + 3SD) of fitted ED values was used as the linkage threshold (Hill et al. 2013); this threshold was chosen because of its ability to effectively separate true signals from background noise, allowing for highly accurate identification of candidate genomic regions and minimizing false-positive associations. The SNP-based analysis initially identified 67 potential regions with a total length of 64.00 Mb, while the InDel-based analysis revealed 19 additional regions spanning 26.21 Mb. Based on simulation-based confidence interval analysis (Takagi et al. 2013), a stringent linkage threshold corresponding to the 90% confidence interval was first applied to the Δ SNP-index; however, no genomic regions exceeded this threshold, indicating the absence of strongly linked loci. Therefore, to identify potential candidate regions, a relaxed criterion was subsequently applied by selecting regions with relatively elevated fitted Δ SNP-index and Δ InDel-index values, corresponding to the upper 99% of the fitted index distributions. Using this criterion, 33 potential regions with a total length of 7.14 Mb were identified on chromosomes 2 and 4. Similarly, analysis of the Δ InDel-index identified four associated regions with a total length of 0.34 Mb, all of which were localized on chromosome 2. Final candidate regions were defined based on the spatial overlap (intersection) between SNP- and InDel-associated regions identified using a sliding-window

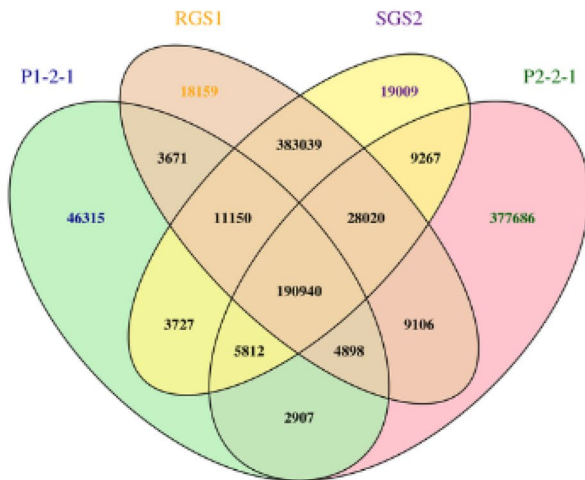
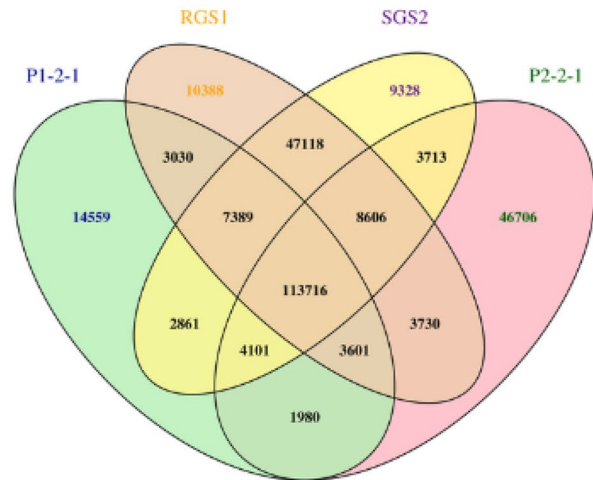
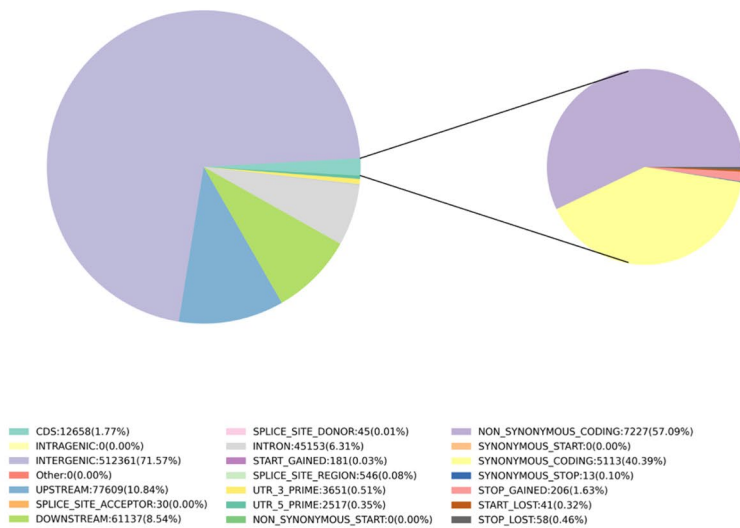
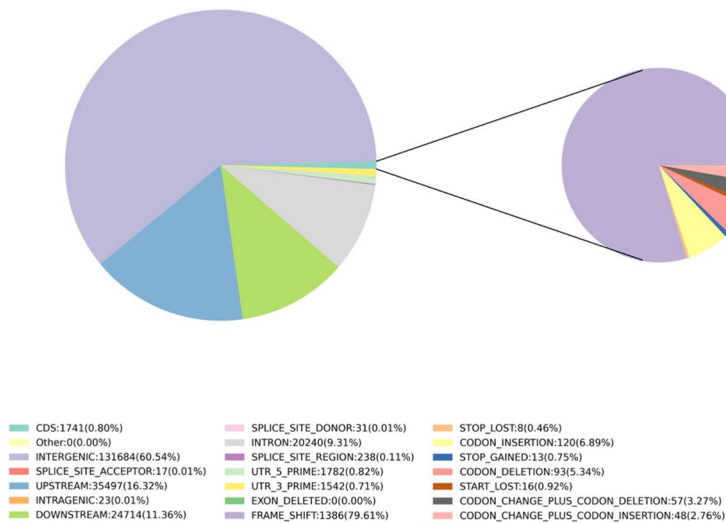
A**B****C****D**

Fig. 2 Summary of SNP and InDel distribution and overlap between resistant and susceptible bulks. **A** Venn diagram on SNP between samples. **B** Venn diagram of InDels between samples. **C** Pie chart of SNP. **D** Pie chart of InDel

approach. Using this intersection criterion, 14 overlapping candidate regions were detected, all of which were localized on chromosome 2, effectively filtering out the non-overlapping regions on chromosome 4. These 14 candidate regions formed four contiguous genomic blocks spanning a total of 0.26 Mb. The genomic coordinates of these blocks were 38,502,714–38,508,637 bp, 38,559,718–38,572,056 bp, 38,587,350–38,594,989 bp, and 38,596,696–38,826,575 bp (Fig. 3, Table S1).

Comparative analysis of parental lines identified 45 non-synonymous SNPs and one InDel causing a frameshift. Between the two mixed pools, one nonsynonymous SNP was identified, while no InDel variants were detected. Given their localization in associated regions and their potential impact on protein structure, these variants are likely associated with the formation of the target trait.

Functional annotation and enrichment

Genes located in candidate regions were annotated using the non-redundant protein sequences (NR), manually annotated and reviewed protein sequence (SwissProt), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups (COG) databases, using the BLAST algorithm. In total, 33 genes in the candidate regions were annotated; 17 of these exhibiting nonsynonymous mutations and one had a frameshift mutation (Table S2).

All 33 genes were successfully annotated using the NR database, and 25 of them had high-confidence functional descriptions in the manually curated SwissProt database. Among these 25 genes, 11 contained non-synonymous mutations, suggesting potential functional relevance to early blight resistance.

Gene Ontology (GO) annotation was performed, classifying the genes into three main categories: biological processes, molecular functions, and cellular components (Fig. 4A). The most frequently represented aspects of biological processes includes metabolic and cellular processes, whereas catalytic activity was the predominant molecular function represented. Within the cellular component category, the highest level of functional enrichment was observed for the term *integral component of membrane* (GO:0016021), which included 12 of the 18 annotated candidate genes. Among the molecular functions, the most pronounced enrichment was found for the term *purine nucleoside transmembrane transporter activity* (GO:0015211), which included 6 of the 21 genes, along with significant enrichment for *serine-type*

peptidase activity (GO:0008236) (Table S3). Although not all terms reached the threshold for statistical significance, the observed trends suggest that membrane proteins, including purine nucleoside transporters and serine peptidases, may play an important role in pathogen resistance. These proteins are presumably involved in the regulation of membrane permeability, signal transduction, metabolic processes, and proteolysis, thereby contributing to effective cellular protection against *Alternaria alternata* penetration.

Among the genes annotated by KEGG within the final candidate regions, the gene Solyc02g071100.3, which encodes the Ras-related protein Rab-6 A, was highlighted. According to the KEGG annotation (KO: K07893), this protein may be involved in vesicular transport and stress responses, indicating its possible role in resistance mechanisms (Fig. 4B).

According to the COG annotation results, several of the identified candidate genes encode proteins involved in vesicular transport (*Solyc02g070870.4*), ion exchange (*Solyc02g071140.3*), signal transduction (*Solyc02g071220.3*, *Solyc02g071260.3*), transcription (*Solyc02g071250.3*), translation (*Solyc02g150130.1*, *Solyc02g071280.3*), and post-translational modification and proteolysis (*Solyc02g071190.4*, *Solyc02g076690.3*) (Fig. 4C). These results indicate that such genes participate predominantly in the regulatory and signaling processes critical for adaptive responses.

Hence, based on functional annotation, we assume that the identified candidate genes associated with tomato resistance to early blight are involved in membrane defense mechanisms, regulation of signaling pathways, and activation of immune responses such as vesicular transport of defense compounds, regulation of transcription, and post-translational modification of proteins. The identified changes in the structure and function of these proteins presumably contribute to the effective defense against *Alternaria alternata*, enhancing plant resistance and reducing tissue damage.

Discussion

Early blight remains one of the most significant tomato diseases, causing significant yield losses (Adhikari and Panthee 2017; Habib et al. 2021; Bessadat et al. 2017). Despite previous efforts to map QTL for early blight resistance in tomato, most studies have focused on *A. linariae* or *A. solani* (Adhikari and Panthee 2017; Zhang et al. 2003; Ashrafi and Foolad 2015; Adhikari et al. 2023), and no known functional resistance genes have been characterized for *A. alternata* to date. In this context, the present study represents the first genome-wide identification of candidate

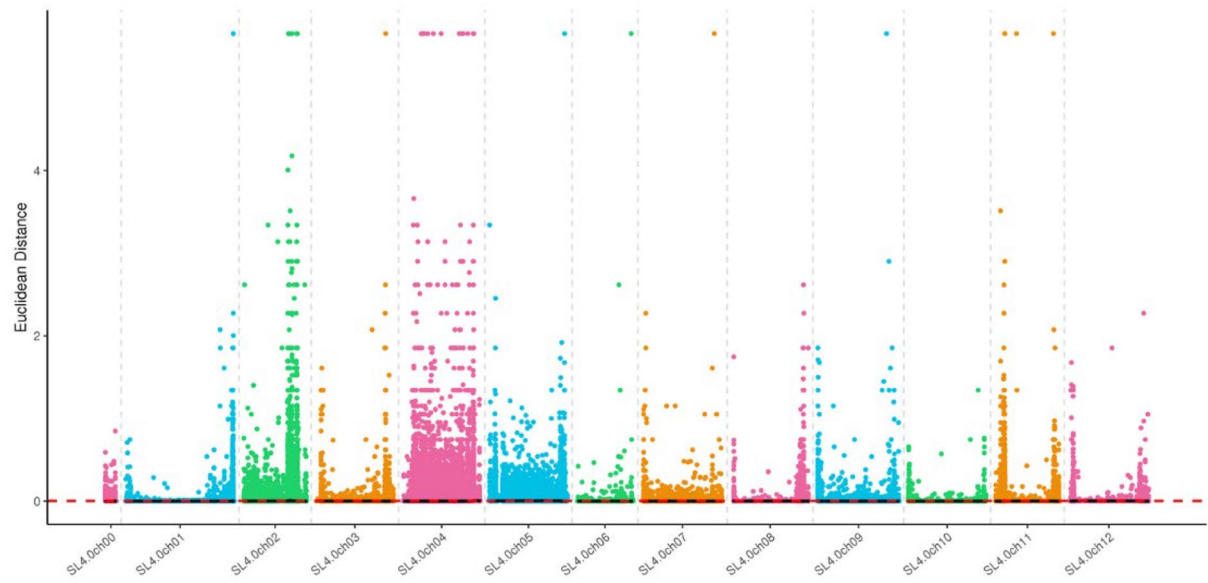
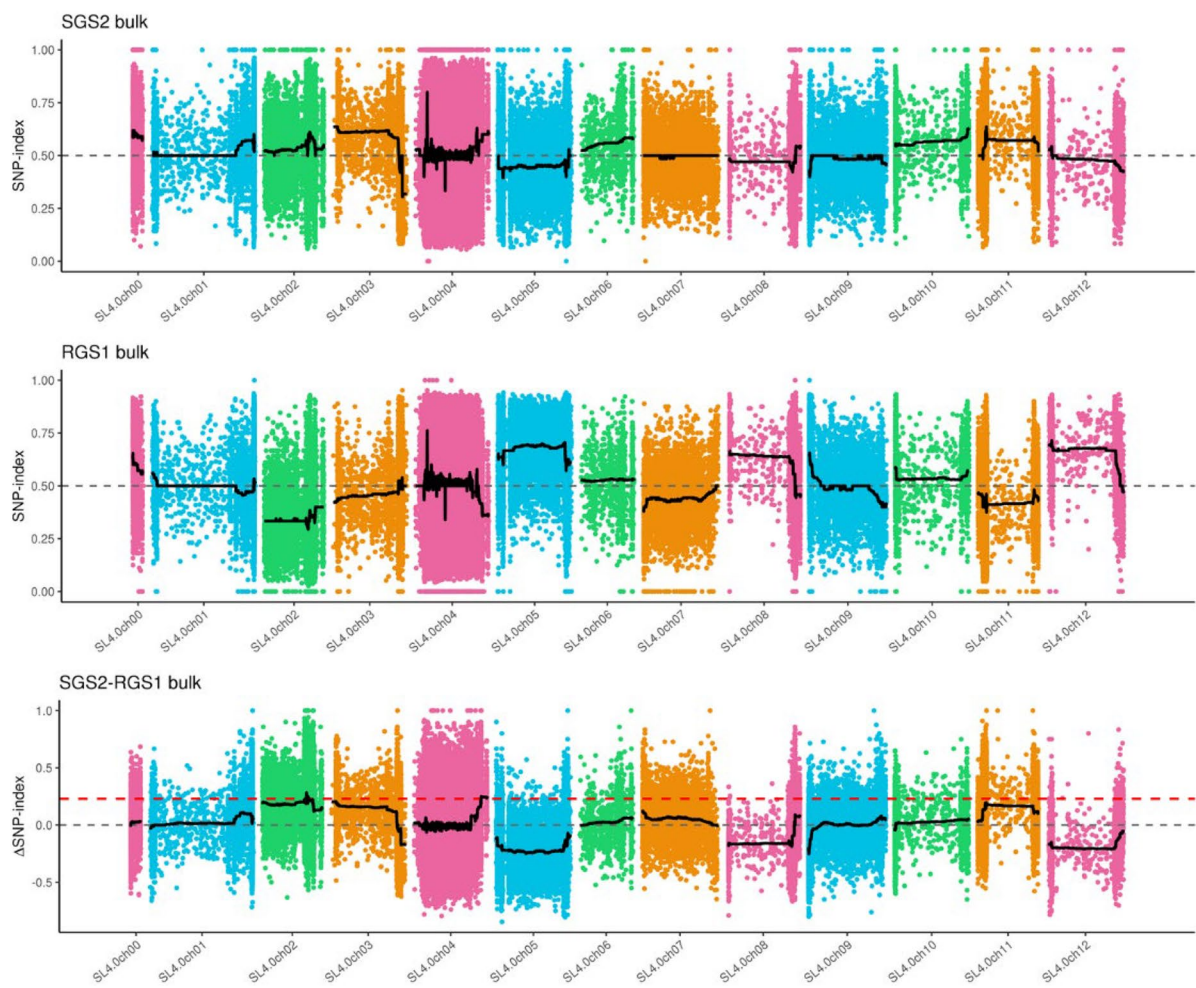
A**B**

Fig. 3 Association analysis of SNP and InDel markers using ED and (Δ)index methods. **A, C** Genome-wide distributions of ED-based linkage values across chromosomes. **B, D** Genome-wide distributions of SNP-/ Δ SNP-index and InDel-/ Δ InDel-index across chromosomes. Colored points indicate per-site values; the black line shows the fitted trend calculated using a sliding-window approach; the red dashed line denotes the 90% confidence interval estimated by simulation-based interval analysis

genes associated with resistance to *A. alternata* in tomato using a BSA-seq approach.

Here, several genomic regions potentially associated with tomato resistance to early blight were identified via BSA-seq. Of these, one region localized on chromosome 2 was consistently detected based on the overlap of SNP- and InDel-based signals between the contrasting pools. This 0.26 Mb locus, comprising 14 candidate regions, is presumably involved in plant defense mechanisms. Together, these results provide insights into the genetic architecture underlying tomato resistance to *A. alternata* and form a foundation for future functional analyses of the identified candidate genes.

Early blight resistance is a polygenic trait, and previous studies have identified multiple QTLs distributed across different chromosomes. Most of these QTLs were identified in studies focusing on *A. solani*. For example, Zhang et al. (2003) reported six QTLs, including four on chromosomes 5, 8, 10, and 11 and two on chromosomes 3 and 8, while Ashrafi and Foolad (2015) identified four QTLs on chromosomes 2, 5, 6, and 9. More recently, studies targeting *A. linariae* have also reported several resistance-associated loci; Anderson et al. (2021) identified three QTLs on chromosomes 1, 5, and 9, and Adhikari et al. (2023) characterized three QTLs located on chromosomes 2, 8, and 11, with pronounced effects on early blight resistance.

Despite these advances, direct empirical evidence demonstrating that resistance loci in tomato differ among *Alternaria* species remains limited, and it is currently unclear whether QTLs identified primarily for *A. solani* or *A. linariae* are fully effective against *A. alternata*.

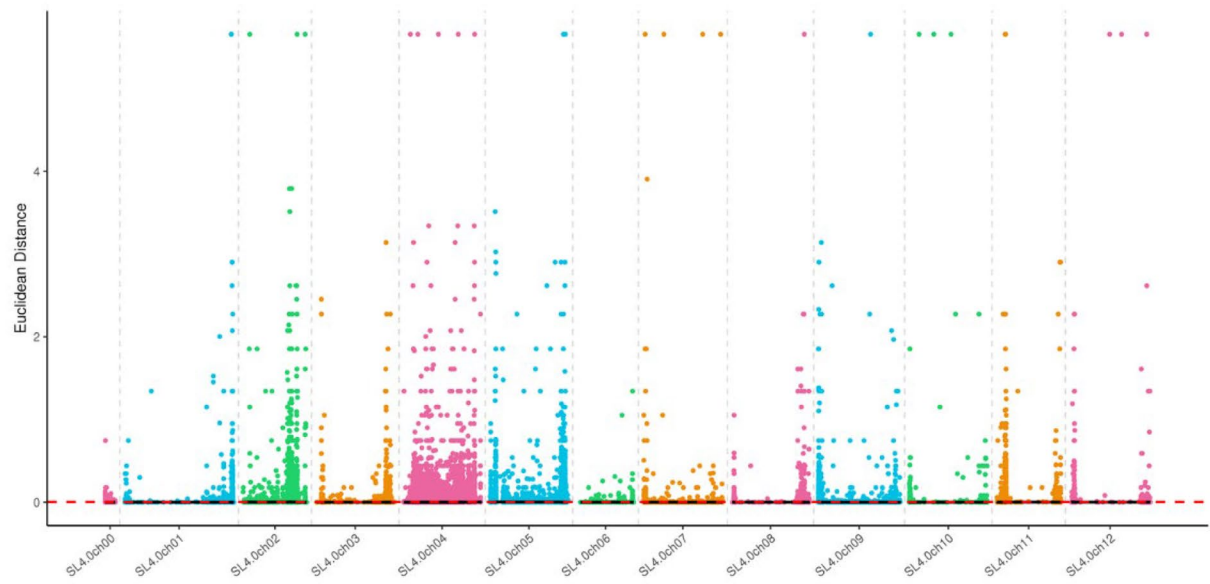
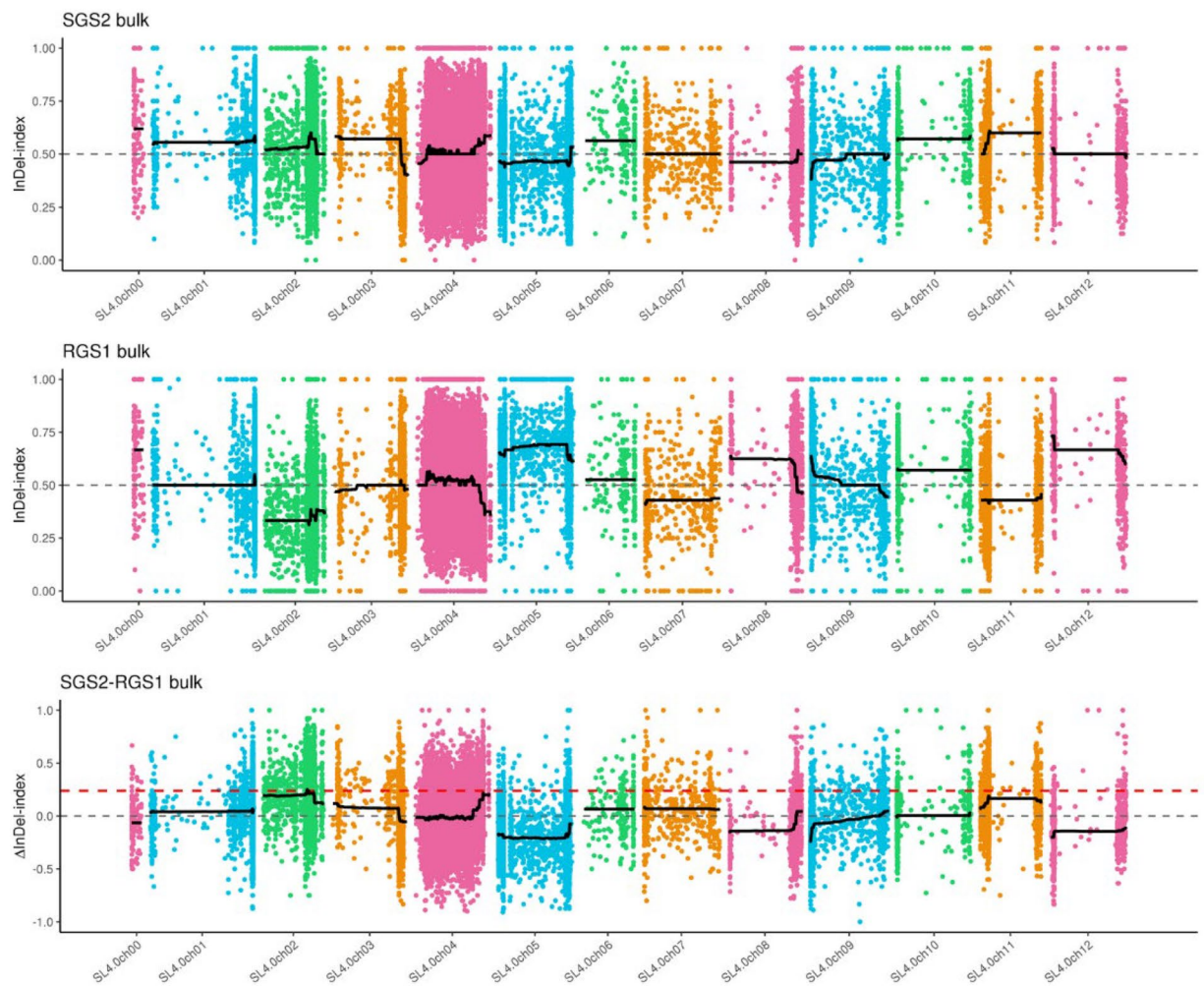
Notably, in the abovementioned studies, QTL mapping was performed based on genetic maps generated using molecular markers. Here, in contrast, a physical map of the tomato genome (SL4.0 version), providing more accurate positioning of the resistance locus, was used. Although direct comparison of localizations determined from different types of maps is difficult owing to differences in recombination levels and marker density, the interval we identified had a high resolution. This allowed us to significantly narrow the search area, to prioritize a set of 14 candidate genomic regions, and to perform functional annotation of the 33 localized genes. Moreover, the localization of resistance genes and activation of signaling pathways in plants

can vary depending on the pathogen and environmental factors (Panthee and Paudel 2024; Akhtar et al. 2019).

Within the trait-linked region detected here, nonsynonymous mutations were detected in 17 genes, and one gene contained a frameshift mutation, indicating its potentially high functional significance. NR and SwissProt databases enabled reliable functional annotation of candidate genes. While NR annotated all 33 genes, Swiss-Prot provided high-confidence matches for 25, including 11 with non-synonymous mutations, suggesting their potential roles in defense against *Alternaria alternata*. GO functional annotation analysis revealed many of the corresponding genes participate in biological processes associated with metabolism and cellular activity, with molecular functions including catalytic activity. Enrichment was especially pronounced for terms related to membrane structures: “membrane” and “membrane part,” potentially indicating the involvement of these components in the formation of structural and signaling barriers that limit pathogen penetration and activate the immune response (Dodds and Rathjen 2010; Couto and Zipfel 2016; Zhou and Zhang 2020). Our results are consistent with those of Jiménez-Gómez and Maloof (2009), who found that genes involved in adaptation to stress exhibit an increased frequency of nonsynonymous polymorphisms, whereas those responsible for basic cellular functions are highly conserved. Thus, membrane-bound and signaling proteins exhibiting structural changes may play a key role in improving resistance to *A. alternata* and warrant further functional validation and use in genetic breeding.

KEGG annotation analysis supported the role of Rab-GTPases in plant immune responses, highlighting the functional significance of the *Solyc02g071100.3* gene, annotated as Rab-6 A. Rab-GTPases participate in vesicular transport of protective PR proteins to the plasma membrane and in regulating oxidative and salt stress in tomatoes (Wang et al. 2016; Soto et al. 2024). Some Rab proteins, such as RabG3b, enhance autophagy and programmed cell death (PCD), thus limiting the spread of pathogens (Rodríguez-Furlan et al. 2023).

COG database analysis confirmed the involvement of the annotated genes in key biological processes such as translation, post-translational modification, transcription, and signal transduction, processes previously characterized as important in plant immune responses (Meng and Zhang 2013). For instance, in a network analysis study, genes involved in translation, proteolysis, signal transduction, and transcriptional regulation were predicted to be central regulators of tomato resistance to early blight (Tominello-Ramirez et al. 2024), partially consistent with our findings. *Solyc02g150130.1* and *Solyc02g071280.3*, associated with translation, as well as *Solyc02g071190.4* and *Solyc02g076690.3*, related to post-translational

C**D****Fig. 3** (continued)

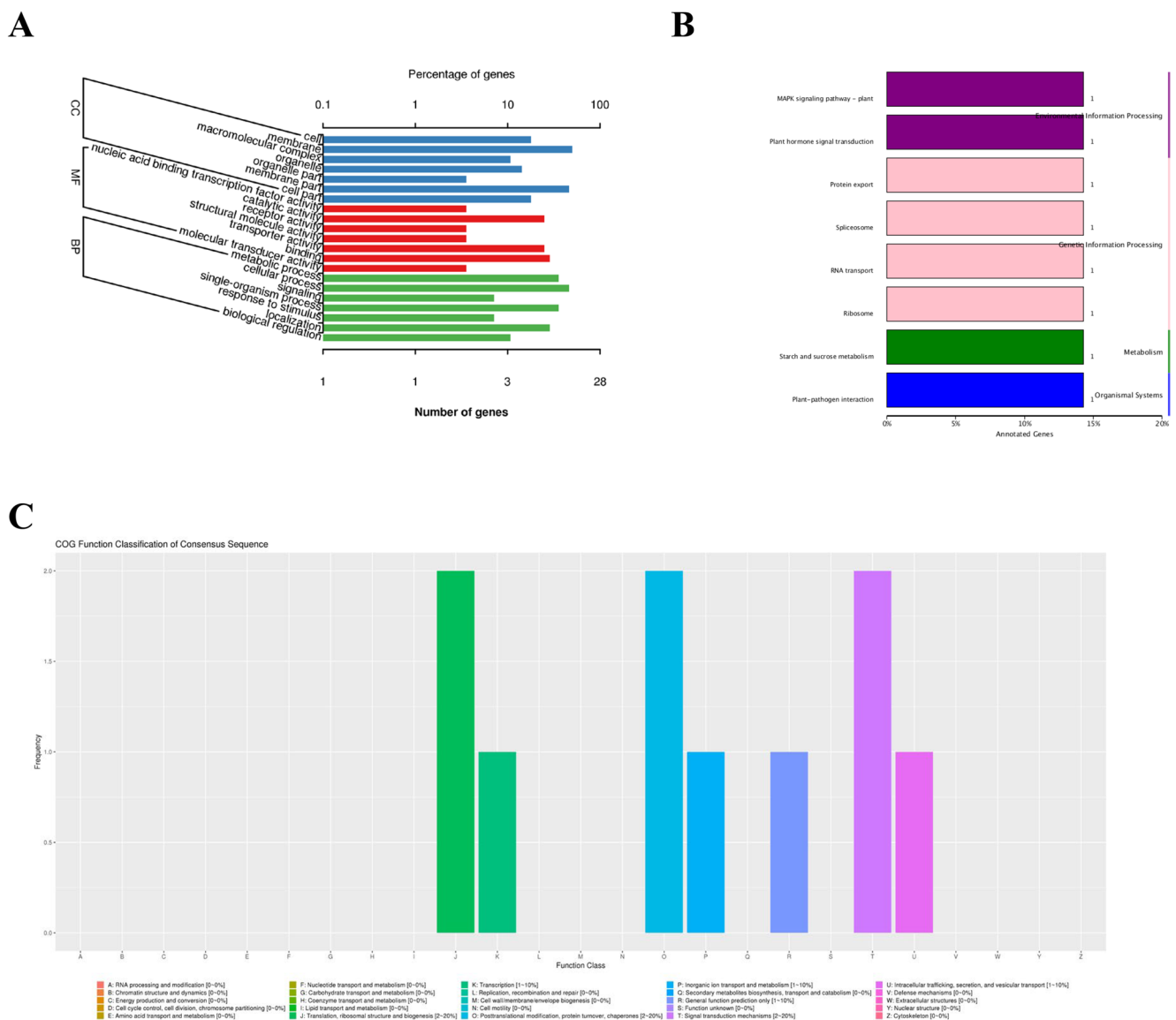


Fig. 4 Functional enrichment analysis of 33 candidate genes associated with early blight resistance. Gene Ontology (GO) classification of genes based on three categories: **A** cellular component (CC, blue),

molecular function (MF, red), and biological process (BP, green). **B** KEGG pathway enrichment, **C** COG functional category classification

modification, reflecting the activation of biosynthetic and proteolytic mechanisms involved in the rapid stress response. *Solyc02g071250.3*, *Solyc02g071220.3*, and *Solyc02g071260.3*, annotated as transcriptional regulators and components of signal transduction pathways, overlap functionally with the transcription factors of ERF, WRKY, and NAC proteins, key modulators of resistance. These results indicate that the identified loci may play important roles in the development of resistance to necrotrophic pathogens such as *Alternaria alternata* (Mengiste 2012; Cho 2015).

Taken together, the GO, KEGG, and COG enrichment analyses indicate that the candidate genes identified in this study participate in interconnected layers of the tomato

immune response. Enrichment of membrane-associated GO terms suggests a role in pathogen perception and early defense signaling at the cell surface, consistent with current models of pattern-triggered immunity (Zipfel 2014). These early signaling events are functionally linked to intracellular signal transduction and vesicle trafficking processes, including Rab-GTPase-mediated transport of defense-related proteins, as highlighted by KEGG pathway analysis (Rodriguez-Furlan et al. 2023). In parallel, COG-based annotation points to the involvement of transcriptional regulation, post-translational modification, and signal transduction, reflecting downstream regulatory reprogramming following pathogen recognition (Meng and Zhang 2013). Together, these results support a model in which resistance to *Alternaria alternata*

is mediated by coordinated interactions between membrane-associated perception, intracellular signaling, vesicle trafficking, and transcriptional regulation, consistent with the polygenic nature of early blight resistance.

Here, for the first time, BSA-Seq was used to analyze the genetic architecture of tomato resistance to early blight in Kazakhstan. Unlike other studies that have focused mainly on other *Alternaria* species (*A. linariae* and *A. solani*), this study provides new information on the genetic mechanisms underlying resistance to *A. alternata*. While phenotyping was conducted under greenhouse conditions, future studies under field environments may help validate the stability of the observed resistance. In the future, functional validation of the most promising candidate genes, via expression analysis, transgenesis, and genome editing methods, will be necessary.

Conclusions

Based on BSA-Seq of the F₂ population, a locus comprising 14 final candidate regions was identified on chromosome 2. Functional annotation of the 33 genes in this locus revealed the participation of membrane proteins, signaling molecules, and transcription factors in the plant immune response. The detection of nonsynonymous SNPs and reading frameshifts indicates the potential functional significance of these variations in the development of resistance. These findings enhance our understanding of the molecular mechanisms underlying tomato resistance to *Alternaria alternata* and provide a foundation for the future use of the identified genomic regions in marker-assisted selection of resistant cultivars.

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Data availability The data presented in this study are available on request from the corresponding author.

Declarations

Conflict of interest The authors declare no conflict of interest.

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