Exploring the influence of a single-nucleotide mutation in EIN4 on tomato fruit firmness diversity through fruit pericarp microstructure

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Summary

Tomato (*Solanum lycopersicum*) stands as one of the most valuable vegetable crops globally, and fruit firmness significantly impacts storage and transportation. To identify genes governing tomato firmness, we scrutinized the firmness of 266 accessions from core collections. Our study pinpointed an ethylene receptor gene, *SlEIN4*, located on chromosome 4 through a genome-wide association study (GWAS) of fruit firmness in the 266 tomato core accessions. A single-nucleotide polymorphism (SNP) (A → G) of *SlEIN4* distinguished lower (AA) and higher (GG) fruit firmness genotypes. Through experiments, we observed that overexpression of *SlEIN4^{AA}* significantly delayed tomato fruit ripening and dramatically reduced fruit firmness at the red ripe stage compared with the control. Conversely, gene editing of *SlEIN4^{AA}* with CRISPR/Cas9 notably accelerated fruit ripening and significantly increased fruit firmness at the red ripe stage compared with the control. Further investigations revealed that fruit firmness is associated with alterations in the microstructure of the fruit pericarp. Additionally, *SlEIN4^{AA}* positively regulates pectinase activity. The transient transformation assay verified that the SNP (A → G) on *SlEIN4* caused different genetic effects, as overexpression of *SlEIN4^{GG}* increased fruit firmness. Moreover, *SlEIN4* exerts a negative regulatory role in tomato ripening by impacting ethylene evolution through the abundant expression of ethylene pathway regulatory genes. This study presents the first evidence of the role of ethylene receptor genes in regulating fruit firmness. These significant findings will facilitate the effective utilization of firmness and ripening traits in tomato improvement, offering promising opportunities for enhancing tomato storage and transportation capabilities.

Introduction

Tomato (*Solanum lycopersicum*) is a high-value crop with worldwide markets and uses (Vincent *et al.*, 2013). The tomato fruit industry faces a critical hurdle in postharvest deterioration, contributing to 50% of harvest losses (Thole *et al.*, 2020). The firmness of tomato fruits has been identified as a critical characteristic for determining fruit maturity and storability. The decline in fruit firmness is because of the breakdown of cell wall polymers by cell wall hydrolases (Su *et al.*, 2022). Currently, enzymes such as polygalacturonase (PG), pectin lyase (PL), pectinesterase (PE), pectin methylesterase (PME) and gibberellin 2-oxidase (GA2ox) have been determined to have a role in fruit softening. PG exhibits the highest activity among these cellular hydrolases and was the first to be discovered. Employing antisense down-regulation of PG has effectively diminished softening and water loss in apples and strawberries (Atkinson *et al.*, 2012; Shi *et al.*, 2021). Silencing of the *PL* gene in tomatoes results in harder fruit without a significant effect on ripening (Brummell and Harpster, 2001; Wang *et al.*, 2019). PE has been reported in many plant tissues and implicated in many developmental processes, including cellular adhesion, stem

elongation (Micheli, 2001) and fruit ripening (Brummell and Harpster, 2001). In addition, studies have shown that PG-mediated pectin degradation is enhanced by PME-mediated pectin demethylation (Wakabayashi *et al.*, 2003). A mutation in the tomato FIRM SKIN 1 (*FIS1*) gene, which encodes a GA2 oxidase, has been shown to enhance the biosynthesis of cutin and wax, thereby increasing the firmness of the fruit (Li *et al.*, 2020). Several quantitative trait loci (QTLs) linked to firmness traits have been confirmed in tomatoes (Alexander and Grierson, 2002; Ma *et al.*, 2014). For instance, six QTLs located on chromosomes 1, 3, 4, 6, 9 and 11 have been determined in the Peruvian tomato *Lycopersicon parviflorum1706* (LA1706) (Fulton *et al.*, 1997), as well as three QTLs for increasing fruit firmness which were identified in the Pannelli tomato LA1657 (Frary *et al.*, 2004). A QTL for fruit firmness was mapped to tomato chromosome 2 using the Zamir *Solanum pennellii* interspecific introgression lines (ILs) and fine-mapped in a population consisting of 7500 F2 and F3 lines from IL 2–3 and IL 2–4. This firmness QTL contained five distinct subpeaks, Firs.p.QTL2.1 to Firs.p.QTL2.5. An ethylene response factor (ERF) underlying Firs.p.QTL2.2 and a region containing three PME genes underlying Firs.p.QTL2.5 were nominated as QTL candidate genes (Chapman *et al.*, 2012).

Fruit firmness is tightly linked to fruit ripening (Hu *et al.*, 2020; Monsalve *et al.*, 2022; Usenik *et al.*, 2008). It has been documented that ethylene plays a critical role in regulating the ripening process of fruit (Huang *et al.*, 2022; Oetiker and Yang, 1995). The ethylene production commences with methionine, which undergoes a transition to S-adenosine-L-methionine. Subsequently, it transforms into 1-aminocyclopropane-1-carboxylic acid (ACC). Furthermore, ACC undergoes oxidation, leading to the formation of ethylene, cyanide and carbon dioxide (Wang *et al.*, 2002; Yang and Hoffman, 1984). Several plant species have well-established signal transduction pathways involving ethylene (Gapper *et al.*, 2013; Hua and Meyerowitz, 1998). Based on earlier research, the perception of the ethylene signal involves ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2 and ETHYLENE INSENSITIVE4 (EIN4) in *Arabidopsis* (Adams-Phillips *et al.*, 2004; Alonso *et al.*, 1999; Stepanova and Ecker, 2000). Tomato exhibits seven ethylene receptor group representatives, including *SlETR1*, *SlETR2*, *SlETR3* (NEVER-RIPE), *SlETR4*, *SlETR5*, *SlETR6* and *SlETR7*. According to protein structure classification, SlETRl-3 belongs to subfamily 1, while SlETR4-7 belongs to subfamily 2. All these members have a vital part to play in the fruit ripening process (Chen *et al.*, 2020; Kevany *et al.*, 2007, 2008; Mubarok *et al.*, 2019; Wilkinson *et al.*, 1995). SlETR1 and SlETR2 are consistent with the AtETR1 sequence structure of *Arabidopsis*. SlETR3 and AtERS1 have similar sequences without response regulatory domains. The SlETR4-7 structure is similar to AtEIN4 and AtETR2 (Chen *et al.*, 2020; Klee and Tieman, 2002; Tieman *et al.*, 2001). In *Arabidopsis*, *EIN4* belongs to the *ETR2* subfamily, and its absence, along with *ETR1*, significantly diminishes the reaction of *Arabidopsis* to ethylene (Liu and Wen, 2012). Few studies of *EIN4* have been reported on tomatoes so far. Despite this crucial role, most studies have primarily concentrated on investigating the impact of ethylene receptors on ethylene response and fruit ripening, with only a limited amount of reports addressing the direct regulation of fruit firmness by ethylene receptors.

The advent of genome-wide association studies (GWAS) has revolutionized the investigation of complex traits and locus interactions in plants, offering a potent approach to unravelling the genetic underpinnings of these phenomena (Nordborg and Weigel, 2008). In our research, we conducted GWAS to explore fruit firmness traits across 266 diverse tomato germplasms. Through this study, we successfully identified an ethylene receptor, *SlEIN4*, located on chromosome 4, which exhibited a single-nucleotide polymorphism (SNP) (A → G) distinguishing lower (AA) and higher (GG) fruit firmness materials. Further experimentation revealed that the overexpression of *SlEIN4* (AA) significantly delayed tomato fruit ripening and reduced fruit firmness considerably. The SNP (A → G) on *SlEIN4* causes different genetic effects on fruit firmness. Instead, gene editing of *SlEIN4* using CRISPR/Cas9 noticeably accelerated fruit ripening, and markedly increased fruit firmness compared with the control. Our findings first reported that *SlEIN4* influences fruit firmness and ripening, promoting the effective use of firmness and ripening traits in tomato improvement.

Results

Sequencing, variants and population structure of natural tomato populations

For this investigation, we employed 266 tomato resources for genotyping, followed by GWAS analysis. The resources used for this study (listed in Table S1) comprised 149 large tomatoes (*S. l. lycopersicum*, BIG), 103 cherry tomatoes (var cerasiforme, CER) and 14 currant tomatoes (*Solanum pimpinellifolium*, PIM), all of them were genotyped in a previous study (Lin *et al.*, 2014). Cluster analysis was conducted using genome-wide SNPs on 266 tomato materials to uncover tomato population structure. We genotyped these materials using 5 485 483 high-quality SNPs, and the heterozygosity of the two SNP markers fell within the acceptable range (<25%). Consistent with the previous report, these materials were separated into three groups (Figure S1a), further supporting our group structure analysis (Figure S1b,c). We conducted principal components analysis (PCA) and model-based cluster analysis based on whole-genome SNPs for BIG, CER and PIM accessions to determine the genetic changes across the three groups (Figure S1b–d). The largest principal component (PC1) was responsible for 70.53% of the domestication-related variation, and the second component (PC2) accounted for 18.47% of the variation. By comparison, the third component (PC3) elucidated 11% of the variation (Figure S1c). Our admixture analysis further confirmed the genetic structure (Figure S1b). Population structure and PCA upheld the three biological classifications. However, BIG and CER could not be distinguished entirely due to their complicated genetic background.

GWAS identification of *SlEIN4* as a candidate gene for firmness determination

The experimental population was cultivated at two locations: open-field cultivation at Huazhong Agricultural University (HZ) and greenhouse cultivation at Zhongdou Seed Company (ZD). Initially, we analysed the survey data of tomato fruit firmness. We observed good repeatability in the results of the two replicates. The fruit firmness values of the 266 natural populations ranged from 3 to 12 kg/cm^2 (Table S1), with a coefficient of variation of 21.8% among the population materials. Fruit firmness remained stable across the two experiment locations (R = 0.44), and the trait exhibited a normal distribution within the panel of accessions (Figure 1a,b). We employed re-sequencing technology to screen 5.5 million high-quality SNPs. The GWAS analysis on the determined fruit firmness values was conducted using the genome-wide efficient mixed-model association (GEMMA) model. We identified 50 sites that showed significant associations with fruit firmness (Table S2). During GWAS, several significant SNPs linked to fruit firmness were identified, and no inflation was observed throughout the *P*-values distributed at the optimal step of the model (Figure 1c,d). This indicates that the population structure was effectively controlled in the study. Five clear peaks were distributed across chromosomes 4, 5, 7, 9 and 11, with the peak value on chromosome 4 being the highest (Figure 1c). Therefore, we focused on the most significant peak SNP site ch04_55155437 (release SL2.40) on chromosome 4 (*P*-value = 4.73 × 10^{-11}).

In our subsequent analysis, the pairwise linkage disequilibrium (LD) estimates (rs^2) for the genomic location were utilized to narrow down the genomic interval and search for potential candidate genes near the peak SNP (Figure 1e). We determined the physical positions of the SNPs using the tomato genomic sequence, version SL2.40, which can be obtained from the website (http://solgenomics.net/). By conducting a genetic linkage analysis of SNP locus ch04_55155437 (with a *P*-value = 4.73 × 10^{-11}), we obtained a local view of the extent of LD decay within the region of 55 127 814–55 157 228.
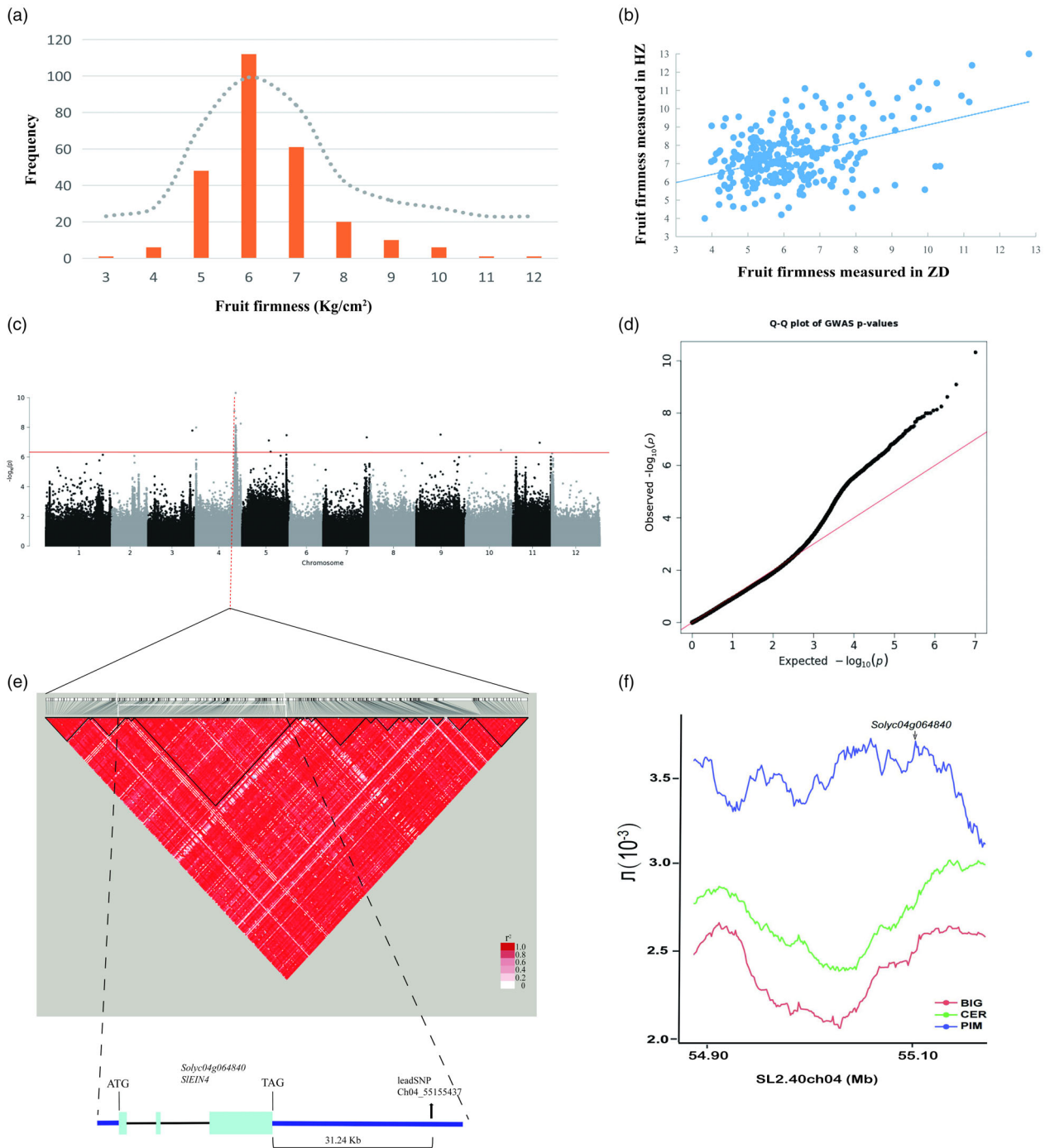
Figure 1 Fruit firmness correlation analysis results. (a) Distribution of adjusted fruit firmness values in the collection of 266 tomato samples. (b) Correlation between fruit firmness values at two different sampling sites from the 266 materials. (c) Manhattan chart displaying the genome-wide association study (GWAS) results of fruit firmness (MLM, $N = 266$). The y-axis values represent the negative logarithm of the association P-value, with the significance threshold indicated by a horizontal line (SL2.40 version). (d) Quantile-quantile plot for fruit firmness in the GWAS population. (e) Pairwise r2 values (a measure of LD) among the polymorphic sites of ch04_55155437 (release SL2.40). (f) Genomic distribution of PIM, CER and BIG nucleotide diversity in domestication sweeps containing SlEIN4 on chromosome 4 (SL2.40 version).

Within this region, an ethylene receptor, a MADS-box transcription factor, a transmembrane protein 34 gene, and two pathogenesis-related proteins were contained (Table S3). Among them, only the ethylene receptor showed a non-synonymous SNP mutation. Through gene expression profiling on the website (http://ted.bti.cornell.edu/), it was discovered that the ethylene receptor was expressed explicitly in fruit, the MADS-box transcription factor was expressed in all tissues, while others showed minimal expression in fruit. Notably, the π values indicated a significant reduction in the ethylene receptor interval

for the BIG and CER groups compared with the PIM group (Figure 1f), providing evidence that it was indeed under selection. By blasting the sequence in NCBI (https://www.ncbi.nlm.nih.gov/), it was shown to be an EIN4-like ethylene receptor with a histidine kinase domain in the amino acid sequence (Figure S2), and we named it *SlEIN4*.

Identification of a critical base mutation in *SlEIN4* tightly associated with tomato fruit firmness

The genetic structure of *SlEIN4* was thoroughly analysed through Gene Structure Display Server, GSDS (http://gsds.cbi.pku.edu.cn/), revealing a deoxyribonucleic acid (DNA) length of 2841 bp consisting of three exons and two introns. Its cDNA length was 546 bp, encoding a protein of 181 amino acids (Figures S2 and S3). Amino acid sequence alignment of SlEIN4 and SlETR1-SlETR7 evolutionary tree analysis showed that SlEIN4 and SlETR7 had relatively higher homology (Figures S4 and S5). During our investigation, a base mutation (A-G) was detected in the third exon of the *SlEIN4* DNA sequence among the 266 materials (release SL2.40) (Figure 2a, Table S1). Haplotype AA was predominantly detected in the BIG group, while haplotype GG was exclusively found in the PIM group (Figure 2b). This mutation led to an amino acid change in the sequence from lysine (Lys) to glutamate (Glu). Among the 35 high-firmness varieties with firmness greater than 8 kg/cm^2, haplotype GG accounted for 60.000%, haplotype AA accounted for 37.143%, and AG accounted for 2.857%. Conversely, in the 231 low-firmness varieties with firmness less than or equal to 8 kg/cm^2, haplotype AA accounted for 77.056%, haplotype GG accounted for 17.316%, and AG accounted for 5.628%. The gene sequencing results also revealed that all the haplotype GG was identified in the top 15 high-firmness cultivars. By comparison, all the haplotype AA was identified in the top 15 low-firmness cultivars within the natural population (Figure 2c). Among the 252 homozygous genotypes of *SlEIN4* in the natural population, two major haplotypes, on account of the leader SNP (SL2.40ch04_55126611), were relevant to high-firmness and low-firmness properties observed in tomatoes, respectively. The SNP, accompanied by the most elevated correlation with fruit firmness, accounted for 15.36% of the total variance (Figure 2d). According to these compelling findings, *SlEIN4* emerged as a prime candidate gene for determining fruit firmness.

SlEIN4 was primarily expressed in tomato fruit

We delved into its subcellular localization and expression pattern for a more comprehensive understanding of *SlEIN4's* function. Our findings revealed that SlEIN4 was situated on the cell membrane, indicating it functions as a membrane protein (Figure 3a). Through quantitative RT-PCR (RT-qPCR) analysis of different tissues, including root, stem, leaf, flower and different fruit stages, we observed notably higher expression levels of *SlEIN4* in fruit compared with other tissues, with the highest expression in mature green fruits (Figure 3b). To further explore its expression *in situ*, we generated transgenic tomatoes overexpressing the glucuronidase synthase (GUS) gene under the control of the *SlEIN4* promoter. GUS staining suggested that the *SlEIN4* gene is expressed in the leaf bud, fruits at the mature green (MG) stage, breaker (BR) stage, yellow ripening (YR) stage and red ripening (RR) stage (Figure 3c). Notably, the GUS staining was significantly darker in fruit than in other tissues, suggesting that the *SlEIN4* gene primarily plays a critical role in fruit development.

To examine the role of *SlEIN4* in fruit development, we generated both *SlEIN4* (AA) gene editing lines and overexpression lines. For this purpose, Micro Tom (MT) was used as the genetic background material due to its low firmness and haplotype AA of *SlEIN4*. Two targets for the CRISPR/Cas9 recombinant vector were identified in Figure 3d (See primers in Table S4). Subsequently, two gene editing lines of *SlEIN4*, namely CR-11 and CR-13, were identified through gene sequencing (Figures S6 and S7). CR-11 exhibited a 7-bp deletion near the second sgRNA target, while CR-13 showed a 5-bp deletion near the first sgRNA target in *SlEIN4* (Figure 3e). Furthermore, two overexpression lines, OE-18 and OE-19, with elevated expression levels of *SlEIN4*, were chosen for additional analysis (Figure 3f). Interestingly, we observed a distinct decrease in the expression of *SlEIN4* in the two gene-edited lines (Figure 3f).

SlEIN4 negatively regulates tomato fruit ripening by affecting ethylene downstream signalling

Monitoring the fruit development of transgenic and wild-type (WT) plants from 27 days after flowering, the results revealed no notable difference in appearance between WT and OE lines (Figure 4a,b). Moreover, the ripening of CR lines was remarkably accelerated, occurring probably 2–3 days earlier than WT. Conversely, the ripening of OE lines was dramatically delayed by around 3 days compared with the control (Figures 4a,c and S8). The ethylene release and ACC content of MT, CR and OE transgenic materials were detected at the same time on Days 31, 34, 37, 39 and 41 after flowering, respectively. The ethylene release of CR is earlier than that of MT, while the ethylene release of OE is later than that of MT (Figure 4d). As a precursor of ethylene synthesis, ACC content in fruit directly determines the level of ethylene release. It was found that the peak of ACC content in CR appears the earliest, while OE appears the latest among WT and transgenic lines (Figure 4e). These data were consistent with the results of ethylene production. Considering the functional redundancy of ethylene receptors, as well as the unclear relative contribution of each receptor to ethylene signalling (Chen *et al.*, 2020), we investigated the expression levels of several genes associated with ethylene signal pathways, including Constitutive Triple Response1 (*CTR1*), Ethylene Insensitive 2 (*EIN2*), *ETR5* and *EIN3*, in mature green fruits. The expression of *CTR1* was dramatically increased in OE (Figure 4f), while the transcription of both *ETR5* and *EIN2* was significantly increased in CR (Figure 4g,i). However, the mRNA level of *EIN3* was significantly increased in CR lines, whereas it was dramatically decreased in OE lines (Figure 4h).

SlEIN4 negatively regulates tomato firmness by affecting the fruit pericarp microstructure and pectinase activity

To uncover the role of *SlEIN4* on fruit firmness determination, we measured the fruit firmness across different stages in WT, OE and CR lines. At the BR and YR stage, the fruit firmness of both WT, OE and CR lines remained high, with no significant difference (Figure 5a,b). Notably, the fruit firmness of OE lines was dramatically lower than the control, while it was markedly higher in CR lines compared with the control at the RR stage (Figure 5c). These results indicate that *SlEIN4* negatively regulates tomato fruit firmness. We also assessed the fruit weight and quality in the RR stage of WT and transgenic lines. The fruit weight and sugar-acid ratio showed no apparent difference between WT and transgenic lines (Figure S9b,c). However, the total soluble solids in CR fruits were notably lower than in WT and OE lines, with no
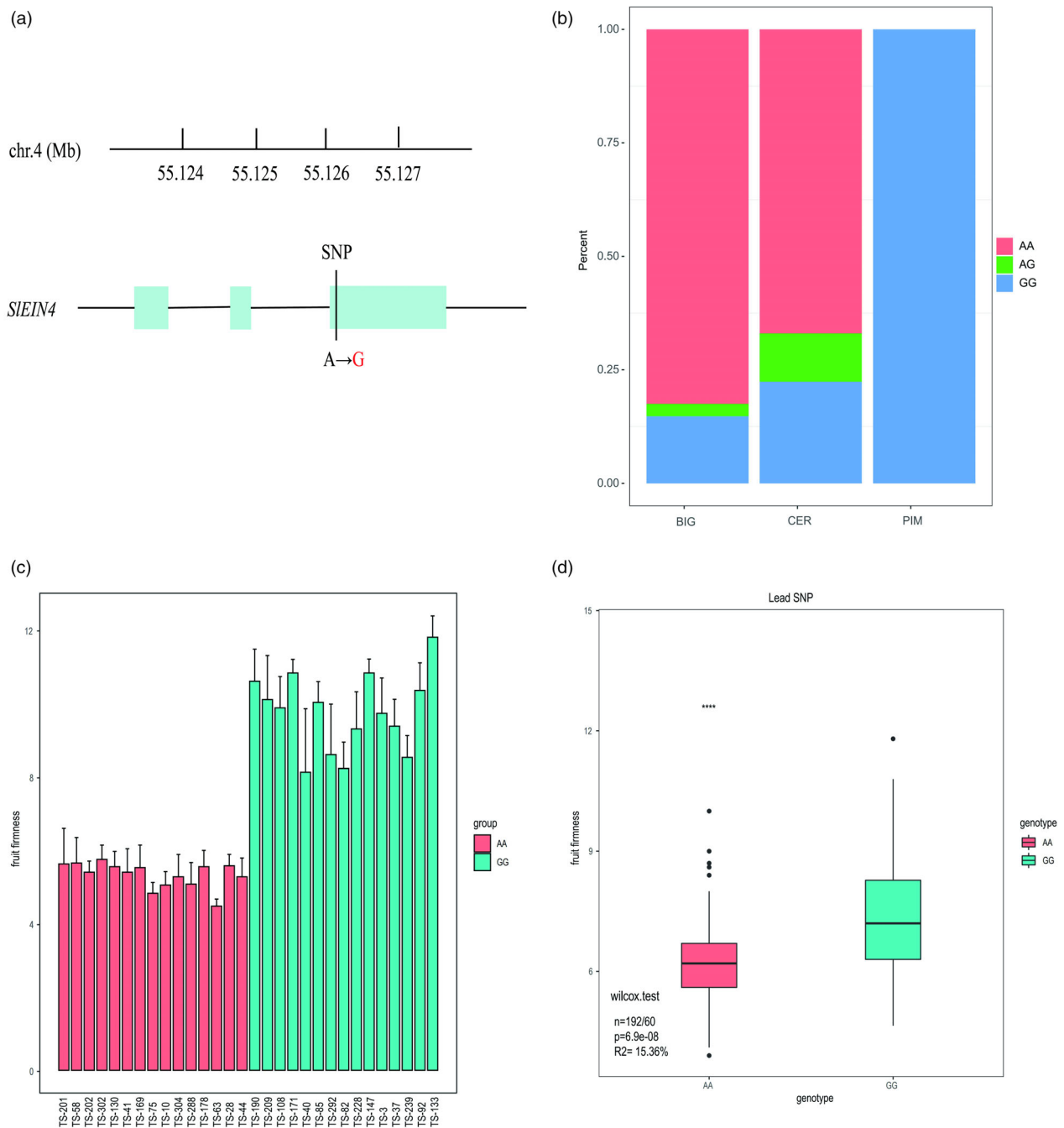
Figure 2 Lead single-nucleotide polymorphism (SNP) in SlEIN4. (a) The gene structure of the ethylene receptor (SlEIN4) is strongly associated with an SNP in the third exon, with genomic coordinates from SL2.40. (b) Distribution of strongly associated SNPEIN4 among the PIM, CER and BIG groups. (c) The SNPs of the SlEIN4 gene in 15 high-fruit firmness and 15 low-fruit firmness cultivars. (d) Box plot of stomatal densities in tomato accessions with different alleles (A or G) at SNP ch04_55126611. For each box plot, the horizontal line in the box indicates the median value, the box height indicates the 25th to 75th percentiles of the total data, the whiskers indicate the interquartile range, and the outer dots indicate outliers.

difference between WT and OE fruits (Figure S9d). Furthermore, to investigate whether the increased firmness extended the shelf life of the fruit, we conducted postharvest behaviour assessments using WT, CR-11 and CR-13 fruits harvested at the RR stage. CR-11 and CR-13 fruits displayed increased resistance to postharvest deterioration, as indicated through measurement of water and weight loss (Figure 5d,e). Compared with the control,

the content of protopectin was significantly increased, and the activity of pectinase was significantly decreased in CR lines (Figure 5f,g).

To further understand the mechanism of *SlEIN4* in regulating fruit firmness, we observed the pericarp tissue microstructure of WT and transgenic fruits. It can be found intuitively that the cell size of WT, CR-11 and OE-18 lines differed in the fruit pericarp tissue
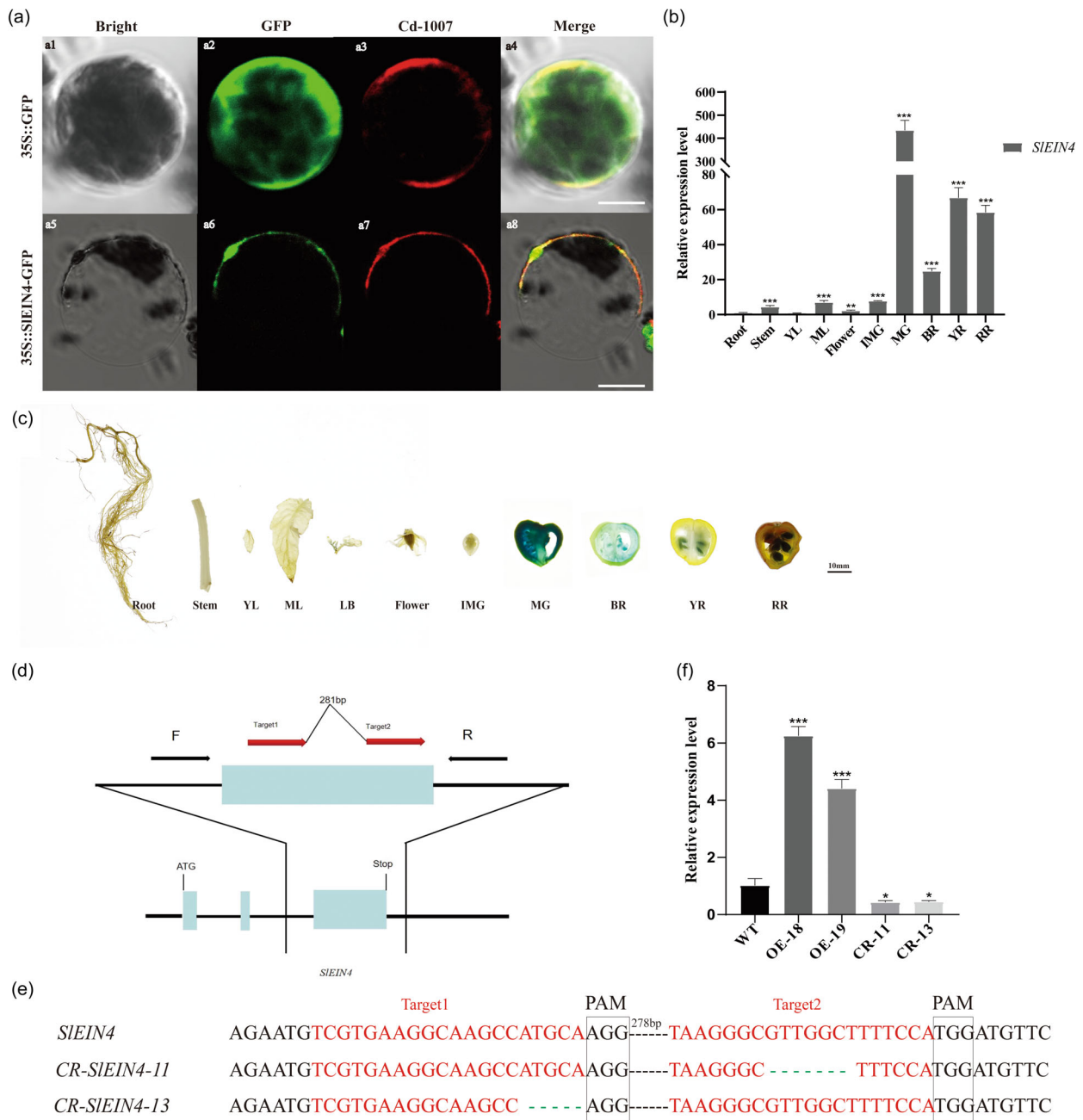
Figure 3 Subcellular localization and expression of the SlEIN4 gene and characterization of overexpression and CRISPR/Cas9-engineered mutation lines. (a) The subcellular localization of SlEIN4 in tobacco protoplast. (a-1 to a-4) Fluorescence signals derived from 35 S::GFP and mCherry in tobacco protoplast. (a-5 to a-8) Fluorescence signals derived from 35 S::SlEIN4-GFP and mCherry in tobacco protoplast. Scale bars = 20 μm. (b) Relative expression of SlEIN4 in different Micro Tom (MT) tissues by qPCR. Root, roots; Stem, stems; YL, young leaves; ML, mature leaves; Flower, flower; IMG, immature green fruit; MG, mature green fruit; BR, breaker fruit; YR, yellow ripening fruit; RR, red ripening fruit. The quantitative fluorescence results are shown from three biological and three technical replicates. The Actin gene (Solyc11g005330) was used as the internal control. Data are presented as means ± SD ($n = 3$). Asterisks indicate the significant difference between root and other tissues revealed by *t*-test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (c) The SlEIN4 promoter drives GUS gene expression in MT. Tissues of transgenic tomato plants were stained with X-Gluc. Root, roots; Stem, stems; YL, young leaves; ML, mature leaves; LB, leaf bud; Flower, flower; IMG, immature green fruit; MG, mature green fruit; BR, breaker fruit; YR, yellow ripening fruit; RR, red ripening fruit. Scale bars = 10 mm. (d) Schematic illustration of the two sgRNA target sites (red arrows) in SlEIN4. Black arrows represent the locations of PCR genotyping primers. (e) CR-SlEIN4 alleles were identified from two T0 mutant lines. Allele sequences, as determined by sequencing, are shown. Both CR-SlEIN4-11 and CR-SlEIN4-13 had base deletions near the sgRNA target site. Red text indicates the sgRNA target sequence, and black boxes indicate protospacer-adjacent motif (PAM) sequences. (f) The relative expression level of SlEIN4. Values are presented as means ± SD ($n = 3$). Asterisks indicate the significant difference between wild-type and transgenic plants revealed by *t*-test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
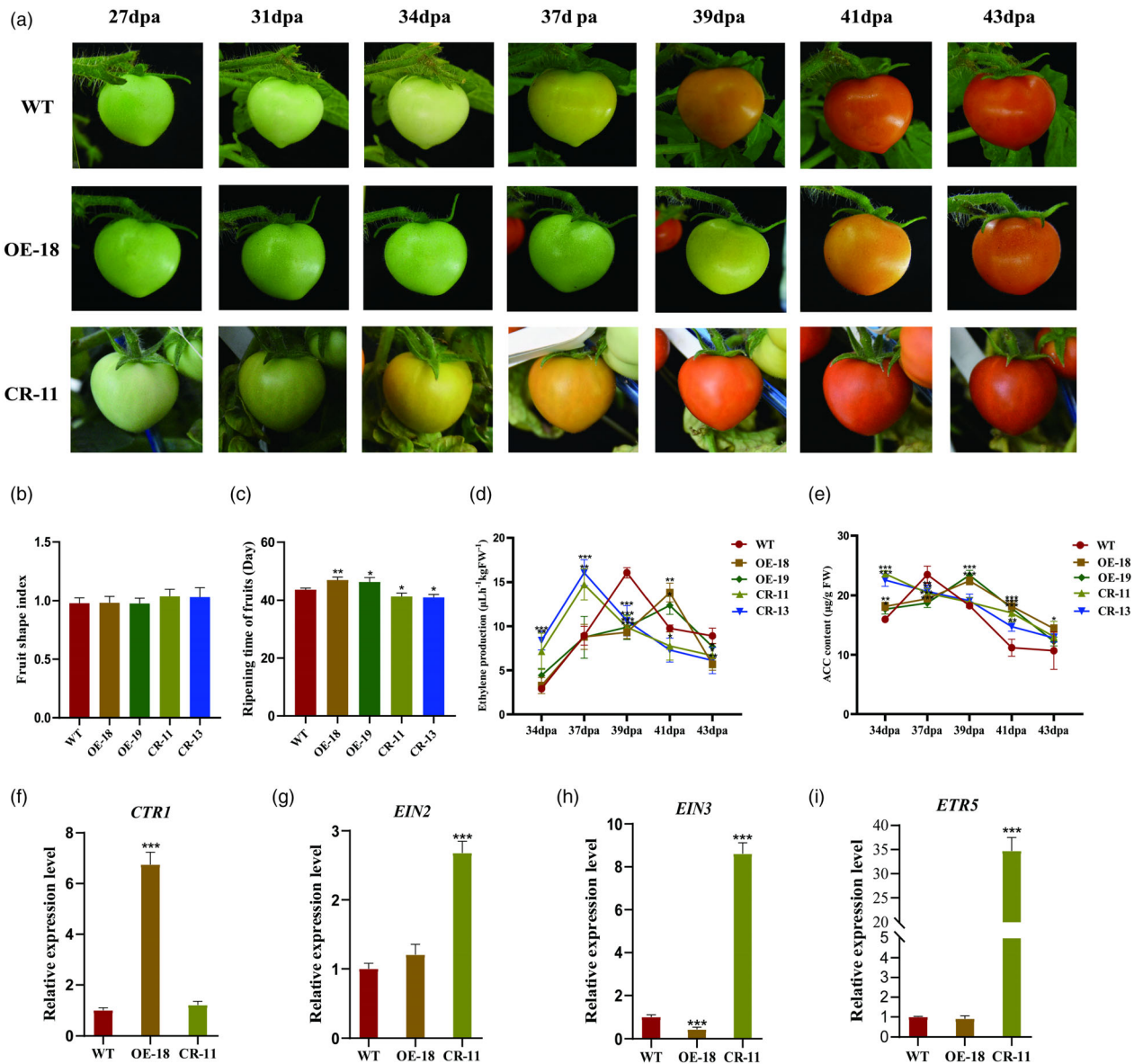
Figure 4 Fruit ripening time, shape index, 1-aminocyclopropane-1-carboxylic acid (ACC) content, and ethylene production of wild-type (WT) and transgenic lines. (a) Representative fruits of WT, OE-18 and CR-11 from 27 dpa to 43 dpa. (b-c) Fruit shape index and ripening time of WT and transgenic lines. (d-e) ethylene production and ACC content of WT and transgenic fruits at 34 dpa, 37 dpa, 39 dpa, 41 dpa and 43 dpa. (f-i) The relative expression levels of CTR1, EIN2, EIN3 and ETR5 in WT, OE-18 and CR-11 fruits at the mature green fruit (MG) stage. Values are presented as means ± SD ($n = 3$). Asterisks indicate the significant difference between WT and transgenic plants revealed by t-test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(Figure 5h). The average cell size of CR-11 lines was significantly smaller than that of WT, while the OE-18 line exhibited a significant increase compared with WT (Figure 5i). Moreover, the cell density of CR-11 was significantly higher than that of the control. At the same time, OE-18 showed a decrease with no significant difference from the control (Figure 5j). Meanwhile, the fruit cross-sectional area analysis revealed no noticeable difference among WT, OE and CR lines (Figures 5k and S9a). Lastly, we measured the pericarp thickness of WT and transgenic lines. The result demonstrated that the pericarp thickness of OE-18 decreased, while the pericarp thickness of CR-11 increased compared with the control level. No clear difference between WT and transgenic lines was observed, but OE and CR lines showed a significant difference from each other (Figure 5l).

SNP (A → G) on *SlEIN4* cause different genetic effects

To validate the SNP (A → G) effect on SlEIN4-mediated fruit firmness, we overexpressed haplotype AA and GG SlEIN4 in MT tomato by transient transformation. Fruit transformation with empty pHellsgate8 was used as a control. Both were over-expressed significantly in the fruits (Figure 6b,c). Compared with the control, the fruits injected with pHellsgate8-SlEIN4^AA and pHellsgate8-SlEIN4^GG had a certain degree of delay in ripening (Figure 6a). The expression level of ERF genes was detected on the 14th day after transformation, and they decreased significantly in both pHellsgate8-SlEIN4^AA and pHellsgate8-SlEIN4^GG fruits com-pared with the control (Figure 6d,e). The result was consistent with the phenotype of overexpressed transgenic materials.
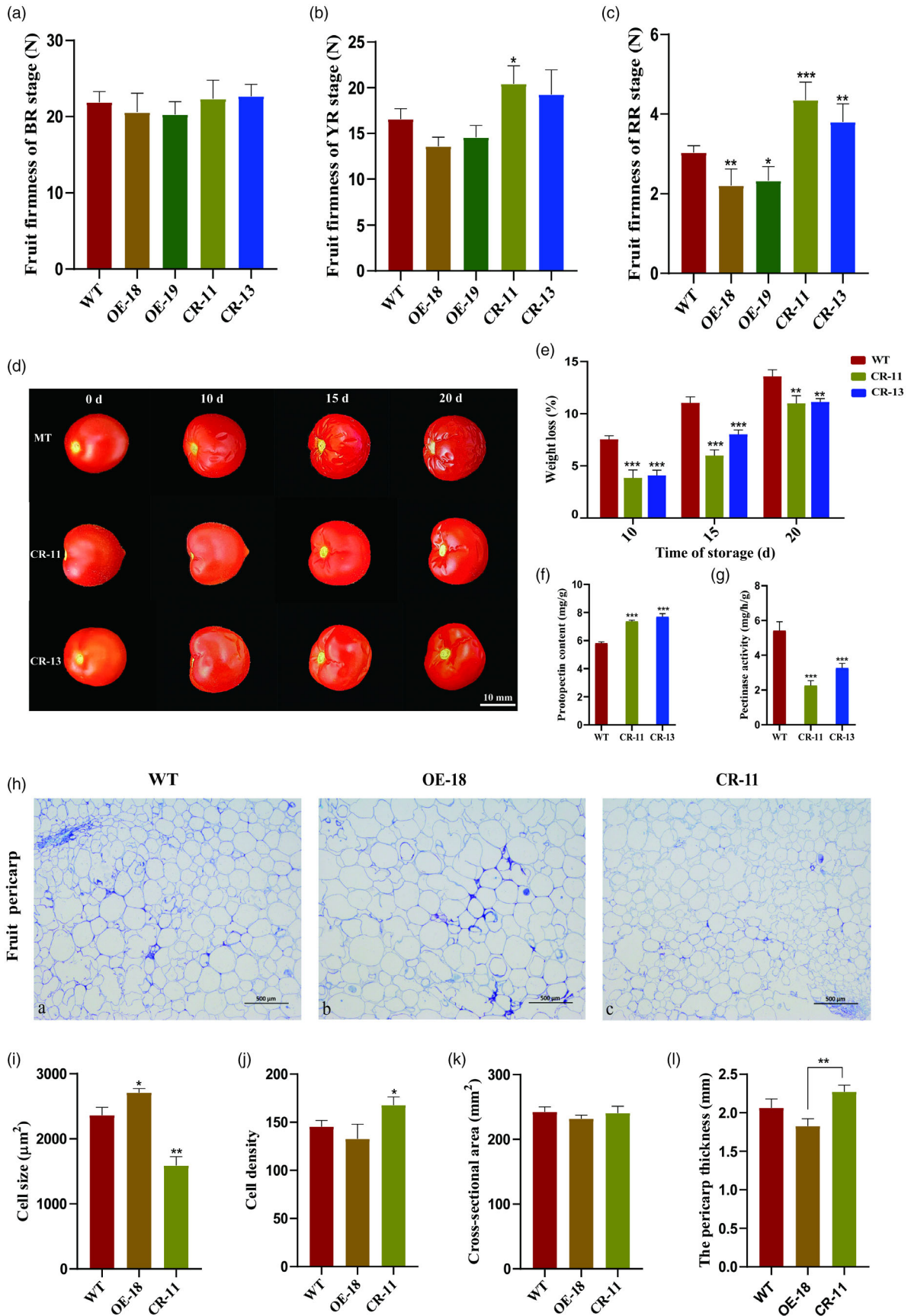
Figure 5 Fruit firmness, storage and pericarp microstructure of wild-type (WT) and transgenic lines. (a-c) Fruit firmness of WT and transgenic fruits at BR, YR and RR stages. (d) Fruits at the RR stage were harvested from WT, CR-11 and CR-13 plants and stored at room temperature for 20 days. Scale bar, 10 mm. (e) Physiological water loss (weight loss %) in WT, CR-11 and CR-13 fruits during different storage stages. The weight loss per fruit was calculated 10, 15 and 20 days after storage. (f) Protopectin content of WT, CR-11 and CR-13 fruits. (g) Pectinase activity of WT, CR-11 and CR-13 fruits. (h) Histological analysis of transverse sections of WT, OE-18 and CR-11 fruit pericarps at the red ripe stage. Scale bars = 500 μm. (i, j) Cell size and density of WT, OE-18 and CR-11 fruit pericarps. (k, l) Fruit cross-section area and pericarp thickness of WT, OE-18 and CR-11 fruits. Values are presented as means ± SD (n = 10). Asterisks indicate the significant difference between WT and transgenic plants revealed by t-test: *P < 0.05, **P < 0.01, ***P < 0.001.

In addition, the physiological characteristics of fruits were detected at the RR stage. Compared with the control, the firmness of pHellsgate8-SlEIN4[AA] fruits decreased significantly, the pectinase activity was significantly increased, and the content of protopectin was significantly reduced (Figure 6f–h). Over-expression of *SlEIN4*[GG] significantly increased fruit firmness, decreased pectinase activity and increased protopectin content (Figure 6f–h). The expression of pectinase genes in pHellsgate8-EIN4[AA] fruits was increased, while these genes were decreased in pHellsgate8-EIN4[GG] fruits compared with the control (Figure 6i,j). The above data suggested that the SNP (A → G) on SlEIN4 causes different genetic effects.

SlEIN4 gene editing impacts the expression of pectinase and ethylene regulatory pathway-related genes

Transcriptome sequencing was conducted for both fruits at 39 dpa of WT and gene-edited lines. The analysis revealed 1248 differentially expressed genes (DEGs), 585 were up-regulated, and 663 were down-regulated (Figure S10). The top 15 Gene Ontology (GO) pathways, distributed across biological processes, cellular components and molecular functions, are depicted in Figure 7a. Furthermore, the 1248 DEGs were enriched into five metabolic pathways, with the differential genes primarily enriched in pathways such as photosynthesis, photosynthesis-antenna proteins, cutin, suberine and wax biosynthesis, fatty acid biosynthesis and phenylpropanoid biosynthesis (Figure 7b).

From the set of DEGs, we identified 16 candidate genes related to tomato firmness and ripening (Table S9). Among them, nine genes were associated with cell wall degradation, including two pectate lyase, two PE, one pectin acetylesterase, one PL-like superfamily protein, one chitinase, one calmodulin binding protein-like and one xyloglucan endotransglucosylase/hydrolase. The expression levels of these DEGs were predominantly down-regulated in CR compared with WT (Figure 7c). Additionally, seven genes were related to ethylene regulatory pathways, including one ethylene-responsive transcription factor, two abscisic acid receptors, one gibberellin-regulated protein, two small auxin up-regulated RNA, one receptor-like protein kinase, all of which were found to participate in ethylene signal transduction or other hormonal pathways related to fruit development and maturation. The expression levels of these genes were up-regulated mainly in CR compared with WT (Figure 7c). The expression levels of pectinase genes and ethylene, GA or ABA regulatory pathway-related genes were further confirmed using qRT-PCR. The results were consistent with the findings from the transcriptome sequencing (Figure 7d).

Discussion

Tomato fruit firmness plays a crucial role in its storage and transportation. Recent research has increasingly concentrated on understanding the regulation of fruit firmness, contributing to

identifying several genes in this process. For instance, Yan *et al.* (2020) reported that BEL1-LIKE HOMEODOMAIN4 (BLH4) participates in cell wall metabolism during tomato fruit ripening and is a crucial regulator of firmness. Additionally, *SlBES1* has been found to promote tomato fruit softening by transcriptionally inhibiting pectin methylesterase ubiquitously 1 (*PMEU1*) (Liu *et al.*, 2021). Fruit firmness is closely linked to fruit ripening, and previous studies have reported the impact of ethylene receptors on the ethylene regulation pathway, influencing fruit development and ripening (Anas *et al.*, 2022; Chang *et al.*, 1993; Kamiyoshihara *et al.*, 2022; Shi *et al.*, 2022). In our study, through GWAS, we successfully excavated a strong SNP peak related to fruit firmness. Specifically, we focused on an ethylene receptor, *EIN4*, which is predominantly expressed in fruits and contains a non-synonymous SNP mutation, as indicated by linkage analysis. The SNP (A → G) of *SlEIN4* was found in various materials, and intriguingly, haplotype AA was predominantly found in low-firmness materials. In contrast, haplotype GG was mainly detected in high-firmness materials, strongly suggesting *SlEIN4* as a candidate gene associated with fruit firmness. Considering evolutionary perspectives, this variation is likely one of the factors contributing to the variation in tomato fruit firmness. To further validate the role of *SlEIN4* (AA) in regulating tomato firmness, we employed MT tomato, which possesses the haplotype AA of the *SlEIN4* gene, as the genetic background material. Gene editing of *SlEIN4* accelerated tomato fruit ripening, whereas overexpression of this gene delayed fruit ripening compared with the control. Additionally, the ethylene release of CR was earlier than that of WT, while this of OE was later than that of WT. These observations provided the basis for earlier fruit ripening in gene-edited lines and delayed ripening in overexpression lines. Based on previous research, *CTR1*, *EIN2*, *EIN3* and *ETR5* are known as essential regulatory genes in the ethylene pathway (Alonso *et al.*, 1999; Chao *et al.*, 1997; Kieber *et al.*, 1993; Klee and Tieman, 2002). In this study, we also found that the expression levels of these genes were significantly altered in the fruit at the MG stage of the transgenic lines. The ethylene receptor is the receiver of the ethylene signal, located downstream of ethylene, so it cannot directly regulate ethylene production. It was speculated that the ethylene receptor regulates ethylene production by influencing the expression of downstream genes with feedback pathways.

According to a previous study, it was reported that ethylene-responsive factor 4 (ERF4) binds to the promoter of *ERF3*, promoting ethylene synthesis and resulting in a decrease in fruit firmness (Hu *et al.*, 2020). While a few genes in the ethylene regulation pathway have been reported to regulate firmness, whether ethylene receptors directly regulate fruit firmness remains largely unknown. In this research, we found that gene-edited *SlEIN4* enhanced the fruit firmness of tomatoes at the YR and RR stages, prolonging the shelf life of the fruits. Instead, overexpression of this gene reduced fruit firmness,
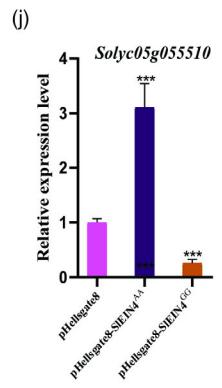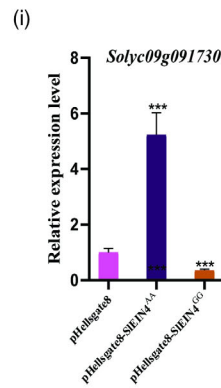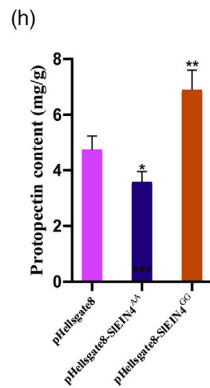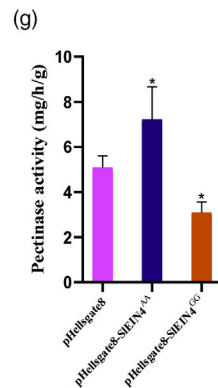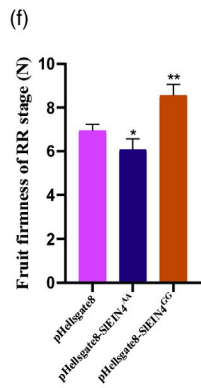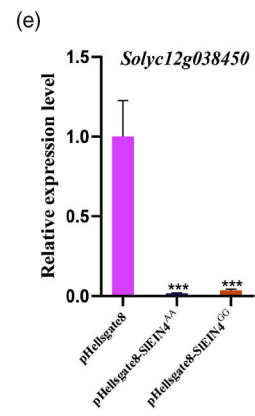
Figure 6 Phenotype, physiological data and related gene expression of pHellsgate8, phellsgate8-SlEIN4^{AA} and phellsgate8-SlEIN4^{GG} fruits. (a) Fruits phenotype from Day 1 to 14 after transient transformation. (b) The expression levels of *SlEIN4^{AA}* in phellsgate8-SlEIN4^{AA} fruits at Day 14 after transient transformation. (c) The expression levels of *SlEIN4^{GG}* in phellsgate8-SlEIN4^{GG} fruits at Day 14 after transient transformation. (d-e) 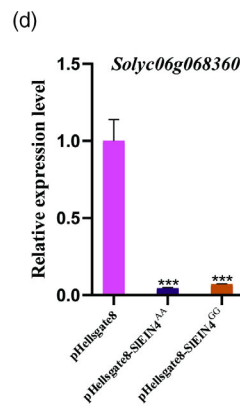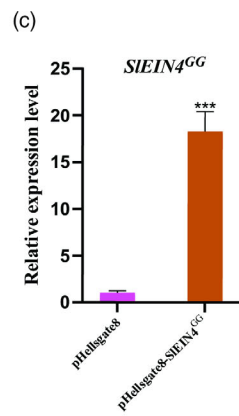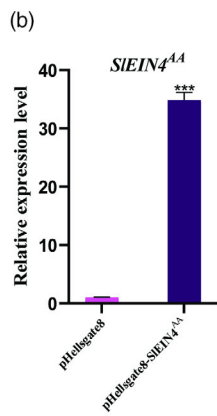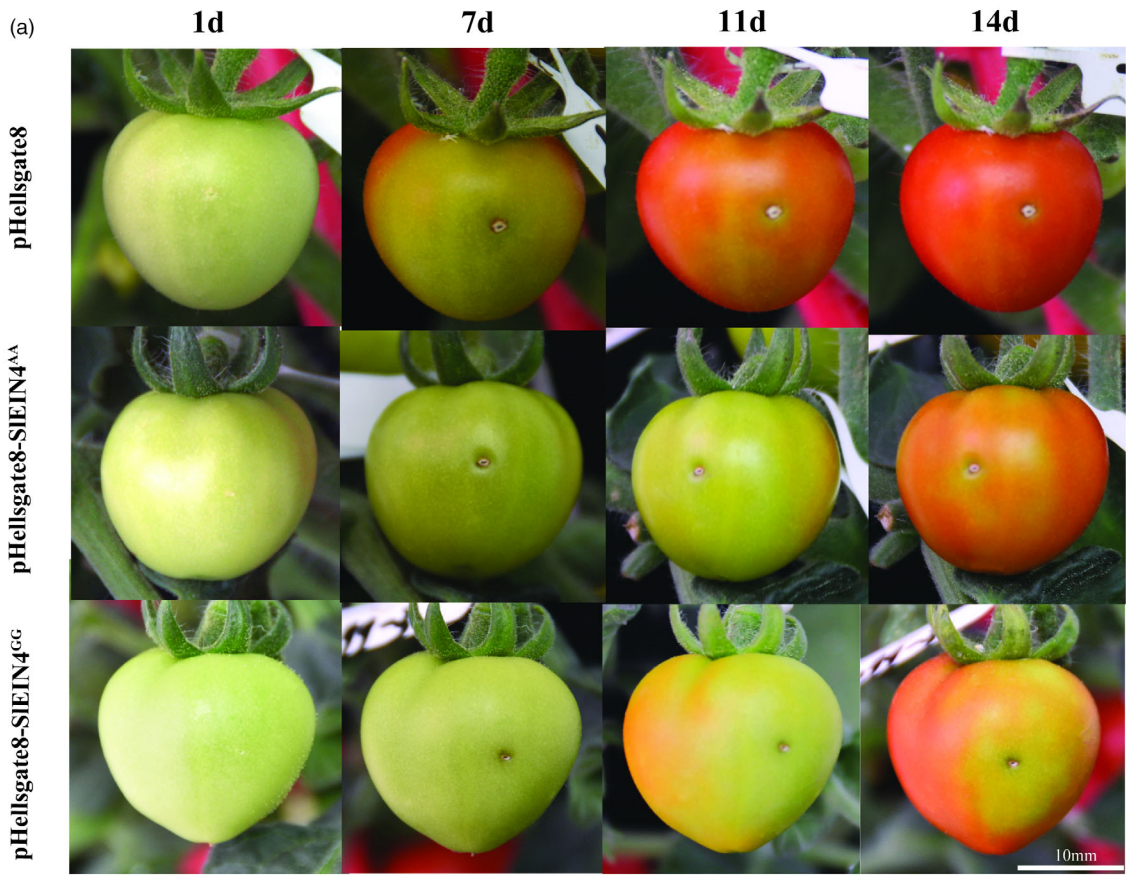The relative expression levels of ERF genes in pHellsgate8, phellsgate8-SlEIN4^{AA},\ and phellsgate8-SlEIN4^{GG} fruits at Day 14 after transient transformation. (f-h) Fruit firmness, pectinase activity and protopectin content of pHellsgate8, phellsgate8-SlEIN4^{AA} and phellsgate8-SlEIN4^{GG} fruits at the RR stage. (i-j) The relative expression level of pectinase genes in pHellsgate8, phellsgate8-SlEIN4^{AA} and phellsgate8-SlEIN4^{GG} fruits at the RR stage. Values are presented as means ± SD (*n* = 10). Asterisks indicate the significant difference between wild-type and transgenic plants revealed by *t*-test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
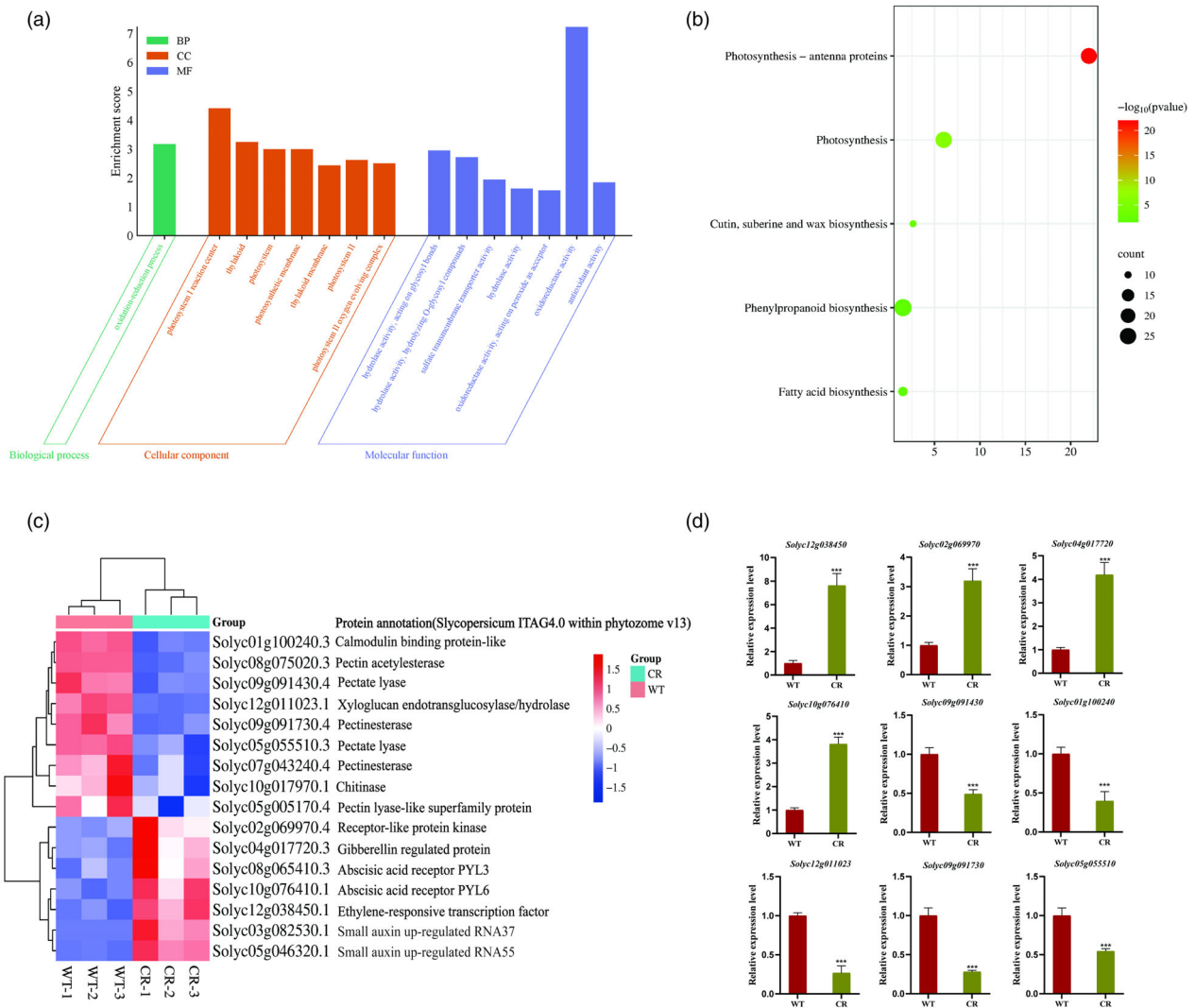

Figure 7 Transcriptome sequencing analysis of wild-type (WT) and CR fruits at 39dpa. (a) Gene Ontology (GO) pathway annotation classification of differentially expressed genes (DEGs). The x-coordinate represents the GO Term, and the y-coordinate shows the GO Term enriched −log10 (*P*-value), DEGs were selected with a value of | log2 (FoldChange) | >1 and $P_{adj} < 0.05$, as indicated below. (b) Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment of differentially expressed genes. The horizontal coordinate represents the rich factor, and the vertical coordinate represents the pathway. The size of the dots in the figure corresponds to the number of differentially annotated genes in the corresponding pathway, and the depth of colours represents the significance level. (c) Clustering heatmap of DEGs related to pectinase and ethylene regulatory pathways. Genes are represented horizontally, with one sample for each column. Red indicates high expression, and blue indicates low expression. (d) The expression levels of pectinases genes, ethylene and GA regulatory pathway-related genes in WT and CR lines. Values are presented as means ± SD (*n* = 3). Asterisks indicate the significant difference between wild-type and transgenic plants revealed by *t*-test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

indicating that *SlEIN4* negatively regulated fruit firmness. To validate the SNP (A → G) effect on SlEIN4-mediated firmness, we also overexpressed haplotype AA and GG *SlEIN4* in MT tomato. The firmness of pHellsgate8-EIN4^{AA} fruits was significantly decreased while it was significantly increased in *SlEIN4^{GG}* over-expression fruit compared with control, suggesting the SNP (A → G) on *SlEIN4* causes different genetic effects. In the 266 natural population, haplotype AA exists primarily in low-firmness

fruits, haplotype GG mainly exists in high-firmness fruits, and heterozygous AG mainly in low-firmness fruits. It could be inferred that haplotype AA is a dominant gene, and haplotype GG is a recessive gene. When the dominant gene was over-expressed, the firmness was reduced, and the firmness was increased when the gene was edited.

Fruit firmness is an intricate trait influenced by various characteristics of fruit cells, such as thickness, size and arrangement (Cao *et al.*, 2012; Kendra and James, 2015; Lahaye *et al.*, 2018). To further explore the mechanism of *SlEIN4* in regulating fruit firmness, we investigated the fruit pericarp microstructure of WT and transgenic fruits. The pericarp cell size of CR lines was significantly decreased, while in the OE lines, it was noticeably increased compared with the control. It has been reported that larger pericarp cells are associated with lower fruit firmness (Cao *et al.*, 2012; Vrebalov *et al.*, 2002). Additionally, the cell density of CR lines was dramatically increased, while that of OE lines was decreased compared with the control. These data suggest that *SlEIN4* likely regulates fruit firmness by simultaneously affecting pericarp cell size and cell density. Flesh firmness in tomatoes is closely linked to cell wall degradation. Enzymes such as PE, pectate lyase, PG, cellulase and chitinase participate in cell wall degradation, leading to the breakdown of fruit pectin, softening of fruit and a decrease in firmness (Su *et al.*, 2022). RNA-Seq analysis demonstrated that the transcription of most of the genes associated with cell wall degradation was down-regulated in CR lines compared with the control, proving that cell wall degradation was hindered in CR lines. $SlEIN4^{AA}$ positively regulated pectinase activity and pectinase gene expression while negatively regulating firmness and protopectin content. The genetic effects of $SlEIN4^{GG}$ were the opposite.

Furthermore, several genes related to ethylene signal transduction and other hormonal pathways, such as ethylene-responsive transcription factor, receptor-like protein kinase, abscisic acid receptor and gibberellin-regulated protein mainly were up-regulated in the CR lines compared with WT, resulting in an advancement in fruit maturity in CR lines. The ethylene regulatory network is complex, and other hormones related to development and maturation, such as GA and ABA, also play a role and can cooperate with ethylene regulation (Li *et al.*, 2022; Wu *et al.*, 2023). Our results suggested that *SlEIN4* does influence fruit firmness to some extent. However, the exact mechanism of how *SlEIN4* regulates fruit firmness needs further investigation.

In conclusion, this study represents the first report demonstrating that an ethylene receptor gene can regulate fruit firmness by affecting cell wall degradation and fruit pericarp microstructure. Our findings offered a valuable genetic understanding of the natural variability of tomato fruit firmness and maturity, which can further facilitate the effective use of these traits in tomato improvement.

Experimental procedures

Plant materials and sequencing

GWAS was conducted using a diverse collection of 266 tomato resources, comprising 149 large tomatoes (*S. l. lycopersicum*, BIG), 103 cherry tomatoes (var cerasiforme, CER) and 14 currant tomatoes (*Solanum pimpinellifolium*, PIM), which were carefully selected from a larger pool of 360 natural population resources reported in a previous study and sourced from various global regions (Lin *et al.*, 2014). The experimental population material was cultivated at two distinct locations in Wuhan, China, in 2013:

the HZ and ZD locations. During GWAS, we harvested at least six fruits from at least six plants per row at the RR stage to test for fruit firmness.

Genome read mapping and variant calling

We obtained the reads dataset of 266 tomato accessions, which had been previously reported, from the Sol Genomics Network (https://solgenomics.net/). Using the burrows-wheeler aligner (BWA) (v0.7.12) with default parameters, the reads were cleaned and aligned to the 'Heinz 1706' tomato genome version SL2.4 (https://solgenomics.net/). Subsequently, duplicated read pairs were identified using Picard (v2.0.1) with default parameters. For variant calling, we utilized the HaplotypeCaller function of the genome analysis toolkit (GATK) (McKenna *et al.*, 2010) (version 20 171 018) to generate genome variant call format (GVCF) files for each accession. Then, we performed population variant calling with the GenotypeGVCFs function of GATK, employing the default parameters. Finally, we applied hard filtering to the raw variant set using GATK, with parameters 'QD<2.0 ‖ FS> 60.0‖ MQ< 40.0 ‖ MQRankSum<−12.5 ‖ ReadPosRankSum<−8.0' applied to SNPs, and 'QD < 2.0 ‖ FS > 200.0 ‖ ReadPosRankSum<−20.0' applied to small indels.

Phylogenetic and population analyses

For population genomic analyses, we retained bi-allelic SNPs with a missing data rate of <15% and a minor allele frequency (MAF) > 0.05. PCA was conducted using Plink (Purcell *et al.*, 2007) (v1.9) with the complete set of SNPs (5 485 483 SNPs). We employed STRUCTURE (Falush *et al.*, 2003) (v2.3.4) to assess population structure. The optimal number of clusters (*K*) was determined, showing the most probable quantity of population clusters. Using 8000 randomly selected SNPs at fourfold degenerated sites, STRUCTURE analyses were run 20 times for each *K* value ranging from 2 to 20. The optimal *K* was determined as 3 by following the Δ*K* method (Evanno *et al.*, 2005) implemented in STRUCTURE. The PCA plots and population structure graph (for each *K* = 2–4) were drawn with R packages. The Balding–Nichols kinship matrix (BN matrix) was constructed in emmax-kin and the proportion of SNPs was randomly selected with the default parameter (emmax-kin -v -h -d 10). The heatmap of the kinship matrix among 266 tomato access was drawn by the pheatmap package (https://cran.r-project.org/web/packages/pheatmap/) in R.

Linkage disequilibrium analysis

Haploview 4.2 software was used to analyse LD (Barrett *et al.*, 2005). Specifically, we evaluated the average linkage decay along each 0.5-Mb region of the entire genome, parameters set as: -maxdistance 500 -minMAF 0.05 -hwcutoff 0. To identify the physical locations of the SNPs, we referred to the tomato genomic sequence version SL2.40 (http://solgenomics.net/).

Genome-wide association study

A total of 5 485 483 SNPs consist in the GWAS analysis, with a minor allele frequency of 0.01 or higher and a lost data rate of 15% or less across the total population. The GWAS was conducted using a mixed linear model (MLM) fitted in the GEMMA algorithm (Zhou and Stephens, 2012). The MLM considers kinship (BN matrix calculated by emmax-kin) and population structure (Q matrix) to control for potential confounding factors. The *P*-value was counted for each SNP, and statistical significance was determined using a uniform threshold of

$\leq 1.8 \times 10^{-7}$ ($P = 1/n$; n = total number of markers used) as the cut-off for identifying significant associations with the firmness traits (Wen *et al*., 2014). An R package qqman (https://cran.r-project.org/web/packages/qqman/) was used to create (quantile-quantile) Q-Q and Manhattan plots from GWAS results.

RNA isolation and gene expression analysis

Tomato plant RNA was extracted and purified following the Hua Yue Yang kit's instructions. Genomic DNA was removed using Monad's reverse transcription reagent, and the remaining RNA was reverse transcribed to generate cDNA. For qRT-PCR, we used Primer Premier 5.0 design primers. The experiments used 2 × HQ SYBR qPCR Mix (ZOMANBIO, China) and the CFX96 real-time PCR system (Bio-Rad, USA). The reference gene used was Solyc11g005330. Each qRT-PCR experiment was performed in triplicate as three biological replicates. The relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ approach. The primer sequences used for qRT-PCR are found in Table S6.

Subcellular localization

PCR was utilized to amplify the coding sequence (CDS) fragment of *SlEIN4*, ensuring the absence of a stop codon. Subsequently, the ClonExpress II One Step Cloning Kit was employed to insert the amplified fragment into the pCAMBIA1302 vector (Vazyme, China). The pCAMBIA1302-*SlEIN4*-GFP with cell membrane marker Cd-1007-mCherry (Yang *et al*., 2018) and the control vector (pCAMBIA1302-GFP) with Cd-1007-mCherry were subsequently infiltrated into approximately 4-week-old *Nicotiana benthamiana* leaves by introduced into *Agrobacterium tumefaciens* GV3101 (Batistic *et al*., 2010). After 48 h of infiltration, protoplasts were prepared from infiltrated tobacco leaves using a protoplast extraction kit (Coolaber, China). Green fluorescent protein (GFP) and mCherry fluorescence were visualized using a laser confocal microscope (Nikon, Japan).

Amino acid sequence alignment and phylogenetic analysis

The full-length amino acid sequences of EIN4 and SlETR1-SlETR7 homologous genes from tomatoes were obtained from the NCBI public database (https://www.ncbi.nlm.nih.gov/). DNAMAN software was used to compare the amino acid sequences obtained. Molecular evolutionary genetics analysis (MEGA) 7 software was used to construct the evolutionary tree, and the result was obtained by further calculation.

Plant transformation

The full-length *SlEIN4* open reading frame (ORF) was amplified from tomato MT cDNA and integrated into the pHELLSGATE8 vector through homologous recombination (ClonExpress II One Step Cloning Kit, Vazyme). Two specific targets (sgRNA1 and sgRNA2) were manually chosen for the gene editing process for *SlEIN4*. The CP098 recombinant vector was designed to incorporate two specific sgRNAs targeting the *SlEIN4* CDS, resulting in a defined deletion of the Cas9 endonuclease gene (Ye *et al*., 2017). All primers utilized for vector construction are listed in Table S4. The plasmid was transferred into the tomato genome via cotyledon explants by introducing it into *Agrobacterium tumefaciens* strain GV3101. Micro Tom was the chosen plant for the transformation process. Positive T0 plants were certified by PCR using CRISPR/Cas9 detection primers (Table S5). CRISPR/Cas9-induced mutations were further characterized using PCR sequencing employing *CR-SlEIN4*-specific primers (Table S5).

Overexpression plants were verified through PCR using CaMV35S promoter forward primers and *OE-SlENI4*-specific reverse primers (Table S5). Both OE and CR transgenic lines used in this research were homozygous lines. The *SlEIN4* promotor sequence from MT was inserted into a pMV2 vector harbouring the β-glucosidase (GUS) gene, resulting in the *ProSlEIN4*::GUS vector. This construct was then transformed into MT.

Tomato transient transformation

The full-length Haplotype GG of *SlEIN4* ORF was amplified out of tomato M82 (TS-3) cDNA and integrated into the pHELLSGATE8 vector (Figure S11). The recombinant vector was named pHellsgate8-SlEIN4^{GG}. pHellsgate8-SlEIN4^{AA} was the same vector transformed into OE. Recombinant vectors and pHellsgate8 empty vector were transformed into *Agrobacterium tumefaciens* strain GV3101. pHellsgate8 empty vector was used as a control. MT fruits at the MG stage (34dpa) were used in the transient expression experiments following the method of Zhang and Duan (Duan *et al*., 2022; Zhang *et al*., 2015). A single fruit was considered a biological replicate, and at least six replicates were taken.

Determination of ethylene production, fruit firmness and other fruit quality traits

To assess ethylene production and ACC content in fruit, we collected tomato fruits from WT, OE and CR lines at various ripening stages, including 31 dpa, 34 dpa, 37 dpa, 39 dpa and 41 dpa. The ethylene measurement method followed the protocol described by Gao (Gao *et al*., 2018). The method for determination of ACC content in fruit followed the protocol described by Kazmierczak and Petritis (Kazmierczak and Kazmierczak, 2007; Petritis *et al*., 2003). For firmness measurement, we used a texture analyser TA.XTPlus (Godalming, UK) to determine the fruit firmness in Newtons (N).

The ripening time of the fruits was determined by counting the number of days from flowering to the RR stage. The fruit shape index was defined as the longitudinal diameter divided by the transverse diameter. Pericarp thickness was measured using digital vernier callipers (Guang Lu, China) from the central cavity to the outer peel. Fruit weight was assessed using an electronic balance (YOKE, China). For chemical analysis, the titratable acidity of the fruits was estimated following the method supplied by Vijayakumar *et al*. (2021), and soluble sugar content was measured as per the procedure outlined by Hu *et al*. (2015). For the microscopic examination of pericarp tissue, paraffin sections were prepared using the method described by Li *et al*. (2019). The protopectin content was determined using the protopectin assay kit (Michy Biomedical Technology, China), and the fruit pectinase activity was detected using a pectinase assay kit (Michy Biomedical Technology, China). A single fruit was considered a biological replicate, and at least 10 replicates were taken.

RNA-Seq analysis

The samples of MT and *SlEIN4*-CR fruits at 39 days post-flowering were taken for transcriptomic analysis, respectively; each line has three replicates. The purity, concentration and integrity of total RNA were checked, and the libraries were produced, sequenced and analysed by Shanghai Personal Biotechnology Co. Ltd. Heatmap of DEGs using TBtools software. The sequencing data were of good quality and satisfied the subsequent analysis conditions (Tables S7 and S8). The conditions for screening differentially expressed genes were as follows: expression difference multiple |log2FoldChange| > 1, with adjusted *P*-value

$(P_{adj}) < 0.05$ (*P*-value was calculated by negative binomial distribution method). topGO was used for GO enrichment analysis, and the *P*-value was calculated by the hypergeometric distribution method (the standard for significant enrichment was $P_{adj} < 0.05$) to determine the GO term of significant enrichment of differential genes to assess the main biological functions of differential genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using clusterProfiler (3.4.4) software, focusing on significant enrichment pathways with $P_{adj} < 0.05$. Finally, an online mapping website (https://www.bioinformatics.com.cn/) was used for drawing.

Statistical analysis

All experiments proceeded independently and were repeated in triplicate to ensure reproducibility. Data analysis was executed using SPSS (SPSS Corporation, Chicago, USA) with analysis of variance procedures. Significant differences among the means were assessed using Student's *t*-test at the $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels. The reported values represent the mean $\pm$ standard deviation.

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Author contributions

S.Z., W.W. and Z.Y. designed the research. S.Z., S.W., B.F. and X.M. performed experiments. Z.J. and Y.L. provided the background material. S.Z., J.Z. and P.W. analysed the GWAS data. S.Z., S.W. and Y.G. analysed the experimental data. S.Z. and W.W. wrote and revised the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The data used to support the findings of this study are included in the article and the supporting information file.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Population structure analysis of 266 tomato natural population GWAS panels based on 5 485 483 SNPs. (a) A phylogenetic matrix diagram displays the relationships between populations. The X-axis and the right Y-axis show individual plants, while the left Y-axis represents a tree plot illustrating phylogenetic relationships derived from SNP data. (b) Population structure of tomato germplasm with varying cluster numbers (K = 2, 3 and 4). The x-axis denotes different groups and their respective positions. (c–d) PCA plot of the tomato accessions, with the percentage of variance explained by each PC indicated in parenthesis. PC1, the first principal component; PC2, the second principal component; PC3, the third principal component.

Figure S2 The conserved domain in SlEIN4. One histidine kinase domain is predicted using the InterPro database.

Figure S3 *SlEIN4* gene sequence in wild-type tomato.

Figure S4 Phylogenetic tree analysis of SlEIN4 and SlETR1-SlETR7 proteins in tomato. The scale bar presents the substitution per amino acid based on the bootstrap method (B = 1000 replications).

Figure S5 Amino acid sequence alignment of SlEIN4 and SlETR1-SlETR7 in tomato. Black shade indicates amino acid residues 100% conserved, pink shade indicates amino acids that are >80% conserved, and blue shade indicates amino acids that are >50% conserved.

Figure S6 PCR genotyping of two T0 generation *CR-SlEIN4* mutants, PCR amplicon of *Cas9*, is a positive control.

Figure S7 Sequencing analysis of *CR-SlEIN4* alleles sequences from T2 homozygous lines of CR-11 and CR-13.

Figure S8 Fruit ripening phenotypes of WT, OE-18 and CR-11 plants. Scale bar, 2 cm.

Figure S9 Transverse section and quality of WT and transgenic fruits. (a) Transverse section of WT, OE-18 and CR-11 fruits. Scale bar, 10 mm. (b-d) Fruit weight, sugar-acid ratio and total soluble solids of WT and transgenic fruits at red ripe stage. Values are presented as means ± SD (n = 10). Asterisks indicate the significant difference between wild-type and transgenic plants revealed by *t*-test: *P < 0.05, **P < 0.01, ***P < 0.001.

Figure S10 Volcano map and clustering heatmap of differentially expressed genes (DEGs) between WT and CR lines. (a) Volcano map. The x-coordinate represents the log2 (FoldChange), and the y-coordinate represents the negative log value of 10 at the significance level. The two dotted lines in the figure represent the threshold values of the difference multiples, the dotted line is the threshold of significance level. The colour indicates whether the gene is up-regulated, down-regulated or insignificantly differentially expressed (DEGs were selected with a value of | log2 (FoldChange) | >1 and P_{adj} < 0.05, as indicated below). (b) Clustering heatmap. Genes are represented horizontally, with one sample for each column. Red indicates high expression, and blue indicates low expression.

Figure S11 Sequencing of *SlEIN4* haplotype in tomato varieties MT and M82.

Table S1 The geographic distribution of SNP (ch04_55126611) in 266 tomato accessions.

Table S2 List of 50 SNPs significantly associated with tomato fruit firmness.

Table S3 Genes included in the location of 55 127 814–55 157 228.

Table S4 Primers used for vector constructions.

Table S5 Primers used for positive and gene sequencing detection of transgenic plants.

Table S6 Primers used for qRT-PCR.

Table S7 Reads quality test.

Table S8 Clean Reads comparison of reference genomes.

Table S9 Expression analysis of DEGs related to pectinase, ethylene or other hormone regulatory pathways.