BMC Plant Biology

RESEARCH Open Access

Ribo-seq and RNA-seq analyses enrich the regulatory network of tomato fruit cracking

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Abstract

Tomato (*Solanum lycopersicum* L.), one of the most widely grown vegetable crops in the world, faces cracking problems before and after harvest. Fruit cracking reduces the commercial value and seriously affects the economic performance of the fruits by affecting the appearance and quality of the fruit. Clarifying the molecular mechanism underlying tomato fruit cracking is of great importance for selecting and breeding cracking-resistant varieties. At present, research on the molecular mechanism of tomato fruit cracking has made progress, but few studies have been conducted to explore the genes related to fruit cracking regulation using combined multi-omics analysis. We applied Ribo-seq (ribosome analysis sequencing) and RNA-seq (RNA-sequencing) techniques to uncover potential fruit cracking regulatory genes and improve the regulatory network of fruit cracking using extremely cracking-resistant (CR) and cracking-susceptible (CS) tomato genotypes. Combining these two sets of histological data and translation efficiency, 41 genes were identified to be associated with fruit cracking. The genes played functions on hormone synthesis (e.g. *Solyc09g089580.4*, *Solyc07g049530.3*), reactive oxygen species regulation (e.g. *Solyc08g080940.3*), cell wall metabolism (e.g. *Solyc04g071070.2*, *Solyc03g123630.4*), aquaporins activity (e.g. *Solyc03g096290.3*, *Solyc10g083880.2*), cuticle and wax composition, as well as mineral elements transport (e.g. *Solyc10g006660.3*, *Solyc01g057770.3*), while 10 of them were transcription factors (TF) (e.g. *Solyc05g015850.4*, *Solyc08g078190.2*). Based on the investigation of the interaction relationship between these genes, the synergistic regulation of multi-gene tomato fruit cracking was predicted. This study suggests that the synergistic action of transcription and translation is an important molecular mechanism in regulating tomato fruit cracking.

Highlights

- We report a comprehensive analysis of tomato cracking based on the RNA-seq (RNA-sequencing) and Ribo-seq (ribosome profiling sequencing) using extremely cracking-resistant and -susceptible tomatoes.
- The 41 possible genes regulating tomato fruit cracking were identified.
- Two regulation networks, including ethylene-cell wall metabolic pathway and transcription factor-water channel-mineral element were found to be involved in regulating tomato fruit cracking.

Keywords Tomato, Cracking, RNA-sequencing, Ribosome profiling sequencing, Translation efficiency

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Introduction

Fruit cracking is a common physiological disease which happens in various fruits, including tomatoes, sweet cherries, grapes, watermelons, blueberries, and nectarines [1, 2]. Fruit cracking affects fruit's appearance and quality and reduces its commercial value, thereby leading to great economic losses [3–5]. Many factors affect fruit cracking, such as genetic background, fruit morphology, and unsuitable environmental conditions [6]. Previous research found that tomato fruit cracking is a quantitative trait controlled by multiple genes [7], but major genes associated with tomato fruit cracking have not yet been reported.

In recent years, transcriptomes and other omics have been widely used in plant sciences, especially in fruit cracking. Research on the molecular mechanism of fruit cracking has made great progress [8]. Transcriptome sequencing of cracking-resistant and susceptible date genotypes revealed 61 differentially expressed genes related to calcium, cell wall, cuticle structure, hormone metabolism, starch/sucrose metabolism, TFs, and water transport [8]. In litchi, a highly economical subtropical fruit, by transcriptome sequencing and weighted gene co-expression network analysis, seven co-expression module genes (1733 candidate genes) were found to be associated with fruit cracking [9]. Among them, high expression levels of genes, specifically phytohormones (growth hormone and abscisic acid), starch/sucrose metabolism, and sugar/water transport (e.g. *Lc.0.190*, *Lc.0.938*, and *Lc.0. 806*) may increase aril expansion pressure, which can prevent the fruit from cracking. Simultaneously, low expression level genes, specifically phytohormones (growth hormone, gibberellin, and ethylene), TFs (WRKY, calcium transport and signaling), and wax synthesis genes (*Lc.12.1668*, *Lc.7.1507*, and *Lc.8.658*) may reduce the mechanical strength of the dehiscent litchi pericarp, which can cause fruit crack [9]. Transcriptome studies of watermelon genotypes with different crack levels revealed that xylan endotransferase genes such as *Cla002042* and *Cla010096* were significantly differentially expressed, which may contribute to the occurrence of watermelon cracking [10]. In addition, plants such as grape and tomato have also undergone transcriptome sequencing of interspecific cracking-resistant (CR) and cracking-susceptible (CS) genotypes to identify the genes associated with fruit cracking [11, 12]. Analysis of transcriptome sequencing data from the grapevine cracking-prone variety 'Xiangfei' suggested that genes related to cell wall metabolism and cuticle biosynthesis played an important role in regulating berry cracking [12]. By mRNA and lncRNA sequencing, we found that hormone metabolic processes (e.g. *Solyc09g008720.2*), cell wall organization (e.g. *Solyc08g077910.3*) and oxidoreductase activity (e.g. *Solyc02g080530.3*) coordinately regulated tomato fruit cracking [11].

Ribosome imprinting sequencing (Ribo-seq) is a transformative technique for enriching and sequencing mRNA fragments bound to ribosomes that are in the process of translation (enabling global analysis of translational and co-translational events in vivo) [13, 14]. It is also called ribosomal footprints with sequencing fragments approximately 30 nt in length in most species [13]. Ribo-seq is a beneficial complement for transcriptional process, which was first applied in budding yeast and has started to be gradually applied in plant science [14, 15]. In-depth studies on translation regulation in species such as Arabidopsis and tomato have highlighted the ability of Ribo-seq for crop improvement by regulating the mRNA translation process [15, 16]. Ribo-seq studies on tomato have identified a large number of unannotated ORFs (open reading frame), while their combination with proteomic studies have revealed that some ORFs can produce stable proteins [16]. Analysis of ribosome profiles of maize seedlings revealed a highly dynamic translational mechanism in response to drought stress in plants, acting in concert with transcriptional mechanisms [17]. RNA-seq, Ribo-seq, and ATAC-seq analyses revealed that tea tree may suppress overall gene expression by enhancing the translation of uORF under low temperature stress [18]. Xiong's study revealed the translational regulatory pattern of N application after drought-flood alternation stress [19]. The results also suggested that gene translation efficiency (TE) was influenced by sequence characteristics, such as coding sequence length, GC content, as well as normalized minimal free energy [19]. The coordination of transcriptional and translational regulation may help to improve the translation efficiency of structural genes in the flavonoid biosynthesis pathway during tea development [20]. Ribo-seq and RNA-seq joint analysis in tomato fruit cracking, however, has not been reported so far.

To study the mechanism of tomato fruit cracking at both transcriptional and translational levels, fruits from extremely cracking-resistant and -susceptible genotypes before and after induced irrigation were collected and analyzed using Ribo-seq and RNA-seq techniques. This study will gain our knowledge concerning the key genes regulating tomato fruit cracking and predict the regulatory network of tomato fruit cracking at transcription and translation levels.

Materials and methods

Plant materials and treatment

An extremely cracking-resistant tomato genotype (CR / 'Tomato cherry' from the USA) and the cracking-susceptible tomato genotype (CS / 'LA2661' from Tomato Genetics Resource Center) were selected as plant

materials (Fig. 1). The plant materials were self-fertilized for six consecutive generations with stable cracking traits [21]. The experiment was conducted at Nanjing Agricultural University (Nanjing, Jiangsu province, China) in March 2019. The seeds were firstly soaked in warm water for 6 h, then sterilized and washed. Afterwards, the seeds were put on wet filter paper to promote germination. Germinated seeds were sown in 72-hole cavity trays. Seedlings with five to six true leaves were planted in the plastic greenhouse of Jiangsu Agricultural Expo Park. Plants spacing followed a 40×50 cm pattern and single vine pruning were set up. When fruits ripen, cracking was induced using saturated irrigation [22]. Fruits at the red ripening stage before and 30 h after irrigation treatment were collected for RNA-seq and ribosome analysis with three biological replicates [23] (Fig. 1).

Preparation and analysis of ribosomal footprints

Ribo-seq, a technique for detecting RNA translation at the genome-wide level based on high-throughput sequencing, is currently the mainstream method for translationomics. Specifically, tissue samples were snap frozen in liquid nitrogen, ground and added to lysis buffer. The samples were mixed and centrifuged, and the supernatant was then added to RNase I and DNase I. Subsequently, DNA and RNA were removed, ribosomes were separated using a MicroSpin S-400 Column, and RFs were isolated using the RNA Cleaner and Concentrator-25 kit (Zymo Research; R1017), whereas rRNA (ribosomal RNA) was removed using the method reported previously [24]. Finally, RFs were further purified using magnetic beads (Vazyme, Nanjing, Jiangsu, China). These RNA fragments that were protected by ribosomes and

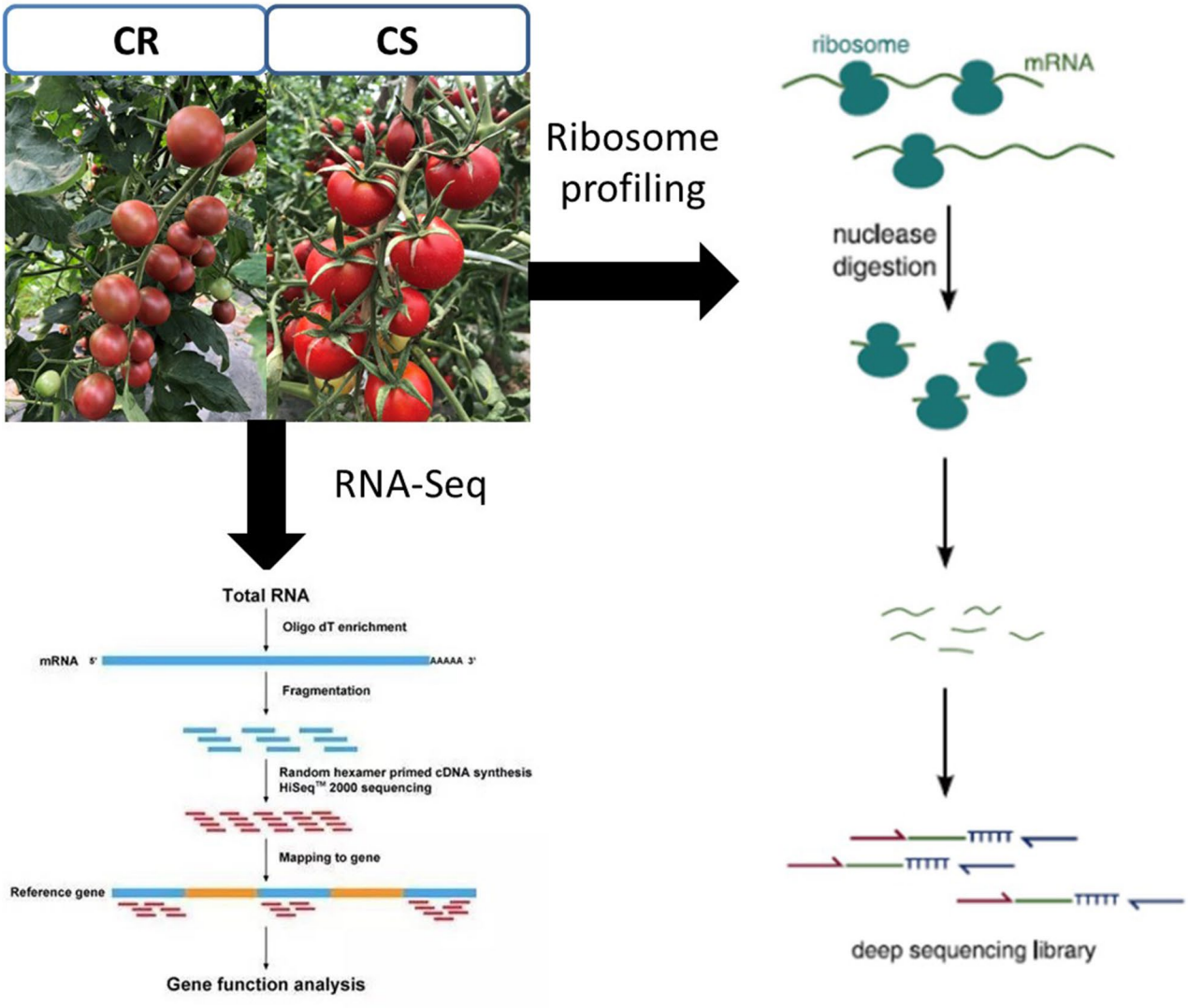

Fig. 1 Experimental design using CR and CS tomato genotype for transcriptome and translatome sequencing. Red ripening tomato pericarp were selected for RNA extraction, library construction and sequencing, in which only RNA fragments bound to ribosomes were selected for sequencing library of Ribo-seq

later extracted for sequencing were also known as ribosome footprints (RFs).

The Illumina HiSeq 4000 sequencing platform was used for sequencing, and a large number of high-quality reads or raw data were obtained. The higher accuracy of base sequencing means that there was less probability of the base being sequenced incorrectly. The quality of this sequencing data should reach Q20 or even Q30, which means the data was reliable and could be further analyzed.

The number of RFs located around the coding gene CDS start codon and stop codon was counted according to the comparative position of RFs 5' end in the genome. Since the ribosome stops every 3 bases (1 codon, a peptide extension of an amino acid is completed) to produce mature mRNA coding for protein. We grouped the RFs that were compared to the CDS region into three categories (codon 1 to 3 bases) according to the codon position corresponding to the 5' end of the RFs comparison position.

RNA-seq data processing and analysis

To ensure data quality, filtering was carried out on the raw data before information analysis to reduce the interference in the analysis coming from invalid data. Firstly, we performed quality control on the raw reads from the downstream machine using fastp (https://github.com/OpenGene/fastp) to filter low-quality data and obtain clean reads. The steps of read filtering were as follows: (1) Remove reads containing adapters; (2) remove reads containing an N proportion greater than 10%; (3) remove reads with all A bases; (4) remove low-quality reads (the bases with quality value $Q \leq 20$).

Based on the HISAT2 comparison results, we reconstructed the transcripts using String Tie and calculated the expression of all genes in each sample. The expression values were expressed as fragments per kilobase per million values (FPKM). The data were then analyzed using DESeq2 software. Based on the results of the analysis, genes with FDR < 0.05 and $|\log_2 FC| > 1$ were screened as significant DEGs (Differentially Expressed Genes). GO and KEGG enrichment analyses of DEGs were further performed using ClusterProfiler89. For visualization of the results, data were published on the OmicShare website (http://www.OmicShare.com/tools).

Ribo-Seq data processing and analysis

In order to ensure data quality, quality control and filtering of off-board data were carried out before bioinformatic analysis. The raw reads obtained after the initial filtering. The reads containing connectors. All A bases and with more than 10% N and low-quality reads ($Q20 \leq 50\%$) were removed. These filtered data were clean reads for subsequent bioinformatic analysis.

Ribosomal RNA removal: To eliminate the effects of contamination of ribosomes for subsequent analysis, the short reads matching tool bowtie2 (2.2.8) were adopted to match clean reads to the ribosome database (no mismatch allowed). The reads that did not match the ribosomes were removed and the retained data were used for subsequent analysis.

Remove transit RNA: The presence of tRNA (transit RNA) may affect the speed of translation and the type of protein synthesized. To identify and remove tRNAs from the samples, the reads of ribosomal RNA removal were compared to GenBank and Rfam databases.

Compare and remove small nucleolar RNA (snoRNA), small nuclear RNA (snRNA) and microRNA (miRNA): After removing rRNA and tRNA, the snoRNA (small nucleolar RNA) and snRNA (small nuclear RNA) were found and removed from the samples by blasting the GenBank and Rfam databases. The miRNA sequences identified in miRBase were also removed from the data. The filtered reads were compared to the tomato reference genome ITAG 4.0, and the reads that matched the expected length of the reference genome were called ribosome footprints (hereafter referred to as RFs).

The number of reads at the Ribo-seq level in the ORF region of the coding gene was calculated using Rsem software and converted to FPKM values to obtain the expression of the gene at the translational level. The FPKM method was used to calculate the gene expression, and the FPKM values could be directly used to compare the gene expression differences between samples. Differentially translated genes were screened using FDR and $\log_2 FC$ using edgeR software, and the screening conditions were FDR < 0.05 and $|\log_2 FC| > 1$.

Two-omics joint data analysis

Two-omics correlation analysis

Pearson correlation coefficients were calculated between translational gene expression and transcript abundance within the groups, and scatter plots were drawn to analyze the correlation between the two omics of the translational and transcriptional groups.

All DEGs association analysis

The genes were classified into 5 categories according to their variation patterns in both omics, which corresponded to: (1) Transcription: genes with significant differences in the transcriptome; (2) Translation: genes with significant differences in translation only; (3) Homodirection: genes with significant differences in both omics and with the same up- and down-regulation directions; (4) Opposites: genes with significant differences in both omics and with opposite up- and down-regulation directions; (5) Unchanged: genes with no significant differences in both omics [25, 26].

Crack-related DEGs screening

The screened DEGs were classified according to GO functional analysis and KEGG pathway analysis, and those with high expression and significant differences were used as candidate genes for regulating fruit cracking. The DEGs with high and significant expression were further classified according to GO functional analysis and KEGG pathway analysis.

Interaction network prediction of proteins

To understand the interactions between proteins encoded by differentially expressed genes, we predicted the interactions of these proteins using the online websites (https://cn.string-db.org/ and https://www.chiplot.online/) to map the protein interaction network. Network construction is based on the proteins with confirmed interactions, or gene neighborhood, gene fusions, and gene co-occurrence.

Translation efficiency analysis

Translational efficiency (TE) represents the total number of RNA molecules (usually mRNA) from a gene in a sample that binds to the ribosome and is translated. Translation efficiency was calculated by the following formula:

$$TE = FPKM \text{ in Ribo-seq} / FPKM \text{ in } RNA\text{-seq}$$

Subsequently, Ribodiff [27] was used to analyze the differences in gene translation efficiency between groups, whereas FDR and $\log_2$FC were adopted to screen for DTEG (differential translation efficiency genes) with the screening conditions of FDR < 0.05 and $|\log_2 FC| > 1$.

qRT-PCR analysis

To validate the RNA-seq data, we randomly selected 10 DEGs for qRT-PCR (real-time fluorescent quantitative PCR) analysis with the same samples and performed correlation analysis of qRT-PCR and RNA-seq data. RNA extraction kit Buffer RL was purchased from Huapu Biotechnology (Changchun, China); reverse transcription kit PrimeScriptTM RT reagent Kit was purchased from abm (Vancouver, Canada); and qRT-PCR kit TORO Green qPCR Master Mix was purchased from Toroivd (Virgin Islands, UK). qRT-PCR reaction system was 10 μL and *Solyc04g011500.2* gene was used as control, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression. The genes and associated primers involved in the qRT-PCR experiments are provided in Table S1.

Results

Data generation and ribosome analysis

From CR0, CS0, CR30, and CS30, 1775.8 million, 2105.3 million, 1958.9 million, and 1930.5 million bases were obtained from Ribo-seq, while 6253.5 million, 6220.9 million, 6582.7 million, and 6704.1 million were obtained from RNA-seq respectively.

More than 97% of the fragments were distributed in the CDS region, with a smaller number in the UTR and Intron regions (Fig. 2.A, S1.A). Gene coverage analysis (i.e., the percentage of each gene covered by reads) was performed (Fig. 2.B, S1.B). The codon positions were compared by the identified RFs (Fig. 2.C, S1.C). The bar graph showed that the ribosome stayed at the first base of the codon for the longest time (Fig. 2.C, S1.C). Analysis of the RFs length revealed that they were mainly distributed around 29 to 32 bp (Fig. 2.C, S1.C). The results showed that the translation abundance was extremely low upstream of the start codon or downstream of the stop codon (Fig. 2.D, S1.D). The RFs signal gradually decayed with increasing distance from the start codon and the stop codon, probably because of the accumulation of RFs at the start codon, where the 60 S large subunit and 40 S small subunit assembly and the termination codon undergo ribosome dissociation, resulting in RFs stagnation (Fig. 2.D, S1.D).

Correlation analysis between the two omics

The Pearson correlation coefficients were calculated as $R^2 = 0.7113$ for CR0 and $R^2 = 0.7099$ for CS0 before irrigation, and $R^2 = 0.845$ for CR30 and $R^2 = 0.7145$ for CS30 after irrigation (Fig. 3.A). Saturated irrigation treatment resulted in excessive water uptake by the fruit, causing adverse stress to the fruit and promoting fruit cracking [23]. By comparing the Pearson coefficients between the two omics before and after irrigation, we found that the correlation between the two omics increased after irrigation treatment for both CR and CS. This correlation showed the consistency in the number of transcripts with the abundance of mRNA-bound ribosomes, suggesting that adversity promotes a positive synergistic response between transcription and translation and that there is a correlation between transcription and translation, which is consistent with previous findings [17].

Validation by qRT-PCR showed that the relative expression of the test genes had the same trend and difference as the RNA-seq data. The correlation analysis of the data from qRT-PCR and RNA-seq yielded $R^2 = 0.807$, and $R^2 > 0.8$ proved the reliability of the RNA-seq data (Fig. S2).

Analysis of DEGs

Compared with CS0 and CR0, 1753 up-regulated genes and 1010 down-regulated genes at the transcriptional level, and 1447 up-regulated genes and 679 down-regulated genes were detected at the translational level (Fig. 3.B). While compared with CS30 and CR30, 2933 up-regulated genes and 747 down-regulated genes were detected at the transcriptional level (Fig. 3.B). Altogether,

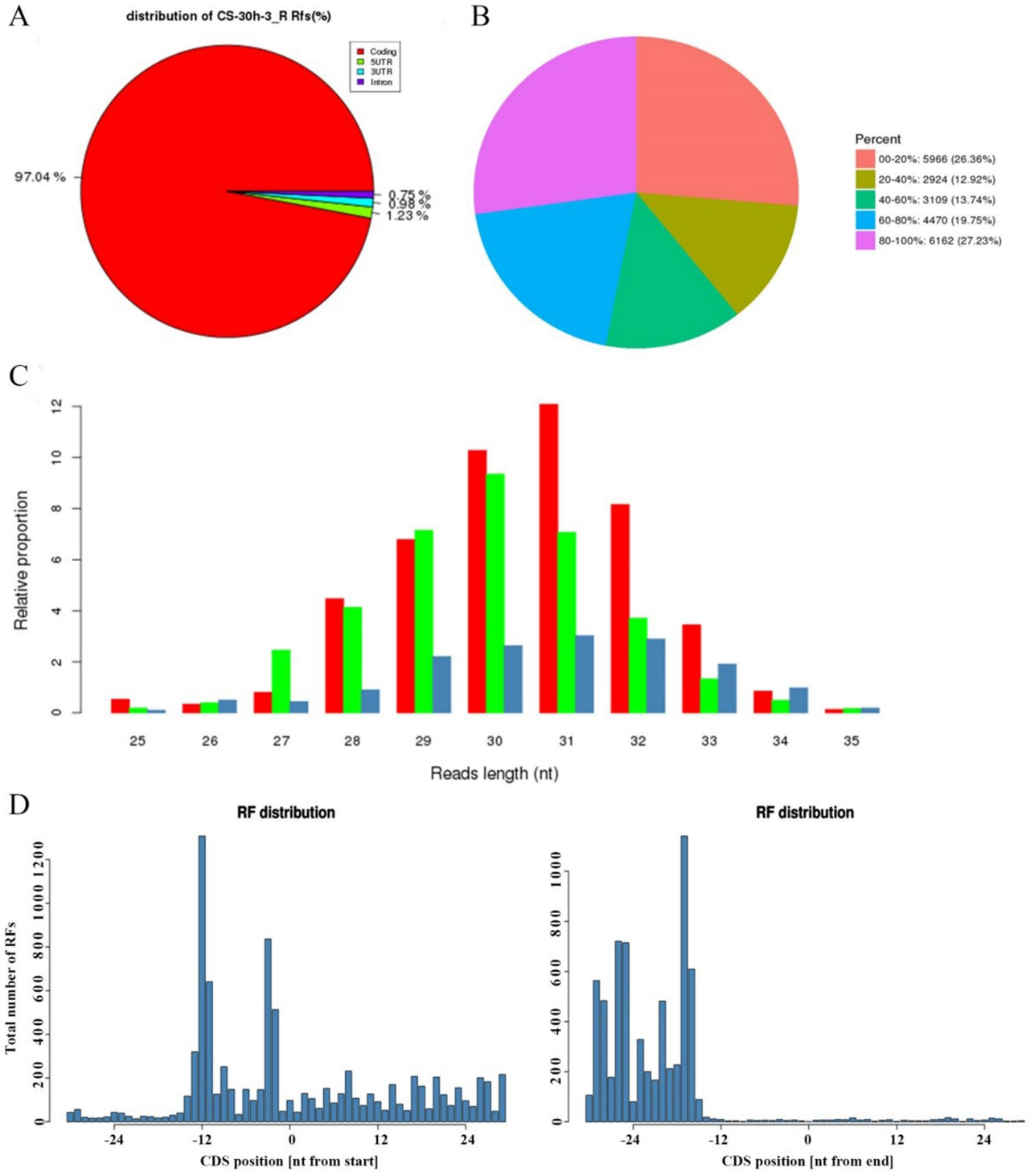

Fig. 2 The Data of the Ribo-seq Feature: (**A**) Distribution of RFs in Coding Genes, (**B**) Gene Coverage Statistics, (**C**) Distribution of RFs around codons (Red represents the first base on the codon, green represents the second base on the codon, and blue represents the third base on the codon), (**D**) RFs alignment codon positions

we detected 2853 up-regulated genes and 656 down-regulated genes at the transcriptional level (Fig. 3.B).

Compared with CR30 and CR0, 359 up-regulated genes and 1,106 down-regulated genes were detected at the transcriptional level, and 210 up-regulated genes and 733 down-regulated genes were detected at the translational level. Compared with CS30 and CS0, 153 up-regulated genes and 278 down-regulated genes were detected at

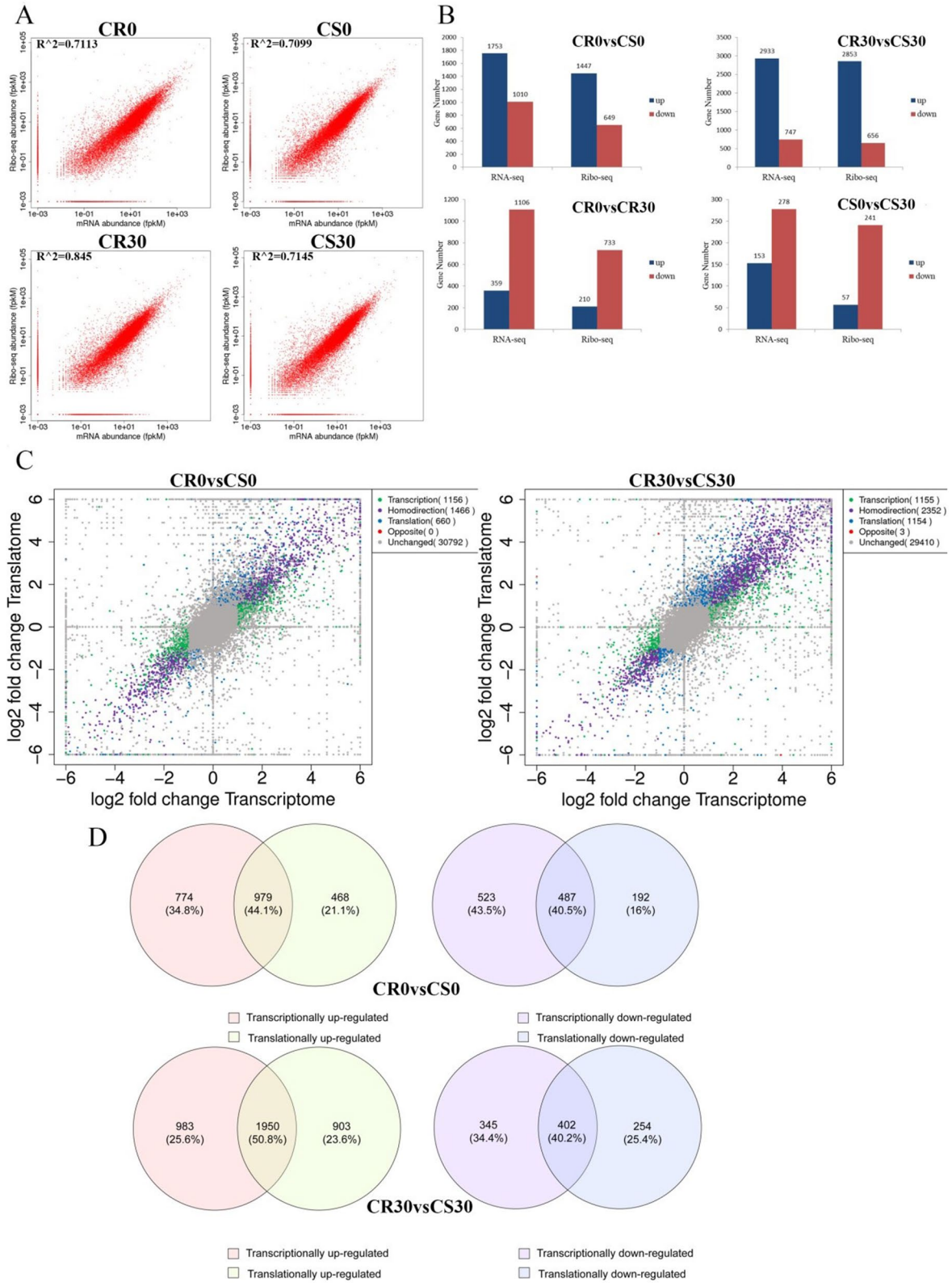

Fig. 3 (**A**) Correlation analysis between the two omics of the four treatments, (**B**) Analysis of DEGs, (**C**) Comparison of translational and transcriptional differences, (**D**) Consistency analysis of DEGs at the transcriptional and translational levels

the transcriptional level; 57 up-regulated genes and 241 down-regulated genes were detected at the translational level (Fig. 3.B). The number of down-regulated genes was higher than the number of up-regulated genes after irrigation treatment in both CR and CS fruits. Comparison of the two tomato genotypes, it was found that the CR fruits showed more DEGs after saturated irrigation at both transcriptional and translational levels.

Comparison of translation differences and transcriptional differences

Before irrigation treatment, 1156 genes were differentially expressed at the transcriptional level only, accounting for 35.22% of all differentially expressed genes. 660 genes were differentially expressed at the translational level only, accounting for 20.11%. A total of 1466 genes (44.67%) showed significant differences in both transcription and translation and were up- and down-regulated in the same direction. After irrigation treatment, among all differentially expressed genes, 1155 genes were differentially expressed only at the transcriptional level, accounting for 24.76% of all differentially expressed genes; 1154 genes were differentially expressed only at the translational level, accounting for 24.74%; a total of 2352 genes, accounting for 50.43%, were significantly differentially expressed at both the transcriptional and translational levels with the same up- and down-regulation direction. Finally, only 3 genes (0.06%) were significantly different at both transcriptional and translational levels, with the opposite direction of up- and down-regulation (Fig. 3.C). Analyzing all DEGs before and after irrigation, we found that only about half of the genes were up- or down-regulated at both transcriptional and translational levels, both before and after irrigation treatment (Fig. 3.D). At the same time, the proportion of genes that were significantly different at both transcriptional and translational levels and up- and down-regulated in the same direction increased after irrigation treatment, indicating that the plant body responds to stress at both transcriptional and translational levels after being subjected to external stress (Fig. 3.D).

After obtaining the DEGs, we classified the DEGs into GO-term statistics. As shown in Fig. S3, the horizontal coordinates represent the three GO ontologies: molecular function, cellular component, and biological process. At the transcriptional level, the DEGs of CR and CS were mainly enriched in metabolic processes, cellular processes, single organism processes, cells, cellular components, catalytic activity, and integration, and the number of up-regulated genes in each function was slightly higher than the number of down-regulated genes. The main enriched functions of DEGs in CR and CS did not change after irrigation treatment, but the number and percentage of up-regulated genes increased substantially. At the translational level, the functional enrichment before and after irrigation treatment was consistent with the transcriptional level, and the number and proportion of up-regulated genes in CR and CS increased substantially after irrigation treatment (Fig. S3). Combining RNA-seq and Ribo-seq data, we performed GO analysis of genes that were significantly different in both omics and were up- and down-regulated in the same direction, and found that the main enriched functions were consistent with either single histology (Fig. S3). KEGG pathway analysis revealed that DEGs were mainly enriched in metabolic and biosynthetic pathways of secondary metabolites at the transcriptional and translational levels. The KEGG pathway analysis revealed that DEGs were mainly enriched in metabolic and biosynthetic pathways of secondary metabolites at the transcriptional and translational levels, and the analysis combined with both omics revealed significant differences between both omics. Genes with the same up- and down-regulation direction were mainly enriched in metabolic and biosynthetic pathways of secondary metabolites, consistent with the results of the single histology (Fig. S4).

Fruit cracking-related DEGs screening

To obtain genes regulating tomato fruit cracking, we combined RNA-seq and Ribo-seq data to screen DEGs of CR and CS with FDR < 0.05 and $|\log_2 FC| > 1$.

Previous studies suggested that fruit cracking may be related to cell wall modification and degradation, phytohormones, wax synthesis and degradation, mineral elements such as calcium, and water channel proteins [8, 9, 28, 29]. Therefore, we screened the DEGs of these pathways and found that a total of 11 DEGs, such as *Solyc03g111690.4* (*PL*), *Solyc03g123630 0.4* (*PMEU1*), *Solyc07g064180.2* (*PME2.1*) and *Solyc06g051800.2* (*EXP1*) were associated with cell wall modification and degradation, mainly involving the modification and degradation of major cell wall components such as pectin, mannans, xyloglucan, and extension proteins. There are 8 DEGs such as *Solyc09g089580.4* (*ACO3*), *Solyc07g049530.3* (*ACO1*), *Solyc04g071580.3*(*ASR1*), *Solyc08g080630.4* (*TIMPA*), *Solyc02g077880.3* (*DRMH1*), *Solyc12g089300.2* (*GASA1*), *Solyc07g056670.3* (*GA2OX1*), *Solyc03g113910*.3 (*GASA1*). related to plant hormones, mainly involved in the synthesis and regulation of growth hormone, ethylene, gibberellin, and abscisic acid. 4 DEGs were associated with cuticular waxes, such as *Solyc07g006890.1* (*CYP94A1*), *Solyc01g088400.4* (*CER1 like*), *Solyc12g100270.2*(*CER1 like*), and *Solyc03g065250.4*(*CER1 like*).

The DEG before and after induced irrigation were mainly concentrated in mineral elements and water channel proteins. Five minerals related DEGs were mainly associated with calcium and boron, specifically

Solyc10g006660.3(*KRP1*), *Solyc04g009800.3*(*CPK1*), *Solyc10g079420.1*(*CML36*), *Solyc03g118810.1*(*CML41*) and *Solyc01g057770.3*(*BOR1*). Three DEGs, *Solyc03g096290.3* (*PIP1-1*), *Solyc10g083880.2*(*TIP1-1*) and *Solyc08g081190.3* were found to be associated with water channel proteins. And *PIP1-1* was highly expressed in CS, especially in RNA-CS30(Fig. 4.A). *Solyc08g081190.3* showed very high expression and presented slowly decrease in CR30 while sharply decrease in CS30 fruits compared with their 0 h control group.

TFs are a class of proteins involved in gene transcription, and in a previous study, a TF (*Ethylene response factor 4*, *ERF4*) in the ethylene signaling pathway was found to regulate watermelon fruit cracking [30]. By analyzing the data from both omics, we identified a total of 10 differentially expressed TFs. The differentially expressed TFs mainly cover the WRKY (e.g. *Solyc05g015850.4* and *Solyc01g079260.4*) and ERF/AP2 families (e.g. *Solyc05g052040.1* and *Solyc08g078190.2*) (Fig. 4.B). Detailed information on all 41 DEGs is provided in Table S2.

Among the differentially expressed genes, we found that three genes for cell wall metabolism function (*PL*, *PMEU1*, *PME2.1*) were enriched in the same KEGG Pathway (ko00040//Pentose and glucuronate interconversions). Searching for the KEGG official website (https://www.kegg.jp/), the results revealed that the three differentially expressed genes were located upstream of the pathway and encoded proteins involved in the pectin degradation pathway. For gene expression analysis, those highly expressed in CS (*PL*, *PMEU1*) were marked in red and those highly expressed in CR (*PME2.1*) were marked in green (Fig. 4.C).

Prediction of the interaction network of genes associated with fruit cracking

We predicted the function of the screened DEGs and the interaction network of the encoded proteins. (Fig. 5.A). Two potential pathways that may regulate tomato fruit cracking were obtained from macroprotein regulatory networks, the hormone-cell wall metabolic pathway and transcription factor-water channel-mineral element pathway, respectively (Fig. 5.B, C).

Among the protein interaction networks in hormone and cell wall metabolism, we found that the functions of the covered proteins include ethylene synthesis key enzyme 1-aminocyclopropane-1-carboxylate oxidase 1 and 3(ACO1 and ACO3), pectin lyase during pectin metabolism (PL), pectin esterase (PMEU1, PME2.1) and an expansion (EXP1) (Fig. 5.B).

In the transcription factor-water channel protein-mineral element uptake and transport protein interaction network, we found that the proteins covered include a WRKY family (WRKY23, Solyc01g079260.2.1), two ethylene TFs (Solyc08g078190.1.1) (DDTFR/10A, Solyc05g052040.1.1), three proteins encoded by water channel protein family genes (LOC544086, Solyc08g081190.2.1), (Solyc03g096290.2.1), (Solyc10g083880.1.1) and a boron transporter protein (Solyc01g057770.2.1) (Fig. 5.C).

Translation efficiency (TE) analysis between two cultivars

Translation efficiency (TE) is an important indicator of the translation process [31, 32]. We calculated TE for genes of the two species before and after irrigation using the formula (Fig. S5.A). We found that there were no more differential translation efficiency genes (DTEGs) before and after a single irrigation, while there were a large number of DTEGs between the CR genotype and the CS genotype before irrigation. There were 68 up-regulated DTEGs and 13 down-regulated DTEGs in the CS genotype compared with the CR genotype before irrigation, and 286 up-regulated DTEGs and 61 down-regulated DTEGs in the CS genotype compared with the CR genotype after irrigation treatment (Fig. 6.A). After obtaining DTEGs, we compared TE differences with transcriptional differences and found that there were 10 genes with significantly different TE levels and transcriptional levels in the same direction and 16 genes in the opposite direction before irrigation. After irrigation, there were 38 genes with significant differences between TE and transcript levels in the same direction and 54 genes in the opposite direction. (Fig. 6.B)

We then performed functional enrichment analysis on the screened DTEGs. By GO terms analysis, we found that DTEGs were mainly enriched in functions consistent with the previous single-omics analysis and two-omics association analysis (Fig. S5.B). KEGG pathway analysis revealed that DETGs were mainly enriched in ribosomal, shear body, and RNA transport pathways (Fig. S5.C).

Discussion

Currently, studies on tomato fruit cracking using BSA-seq, BSR-seq, QTL localization, and GWAS have been reported, but combining transcriptomic and translational studies on tomato fruit cracking has not been reported. In this study, two tomato genotypes with significantly different fruit cracking rates were used to investigate tomato fruit cracking at both transcriptional and translational levels. In addition, we constructed the regulatory network based on this study and previous lncRNA research [11], which will help to enrich the molecular mechanism of tomato fruit cracking.

Transcription and translation synergistically regulate tomato fruit cracking

Ribo-seq is currently the mainstream method for translation omics research, and analyzing transcription and

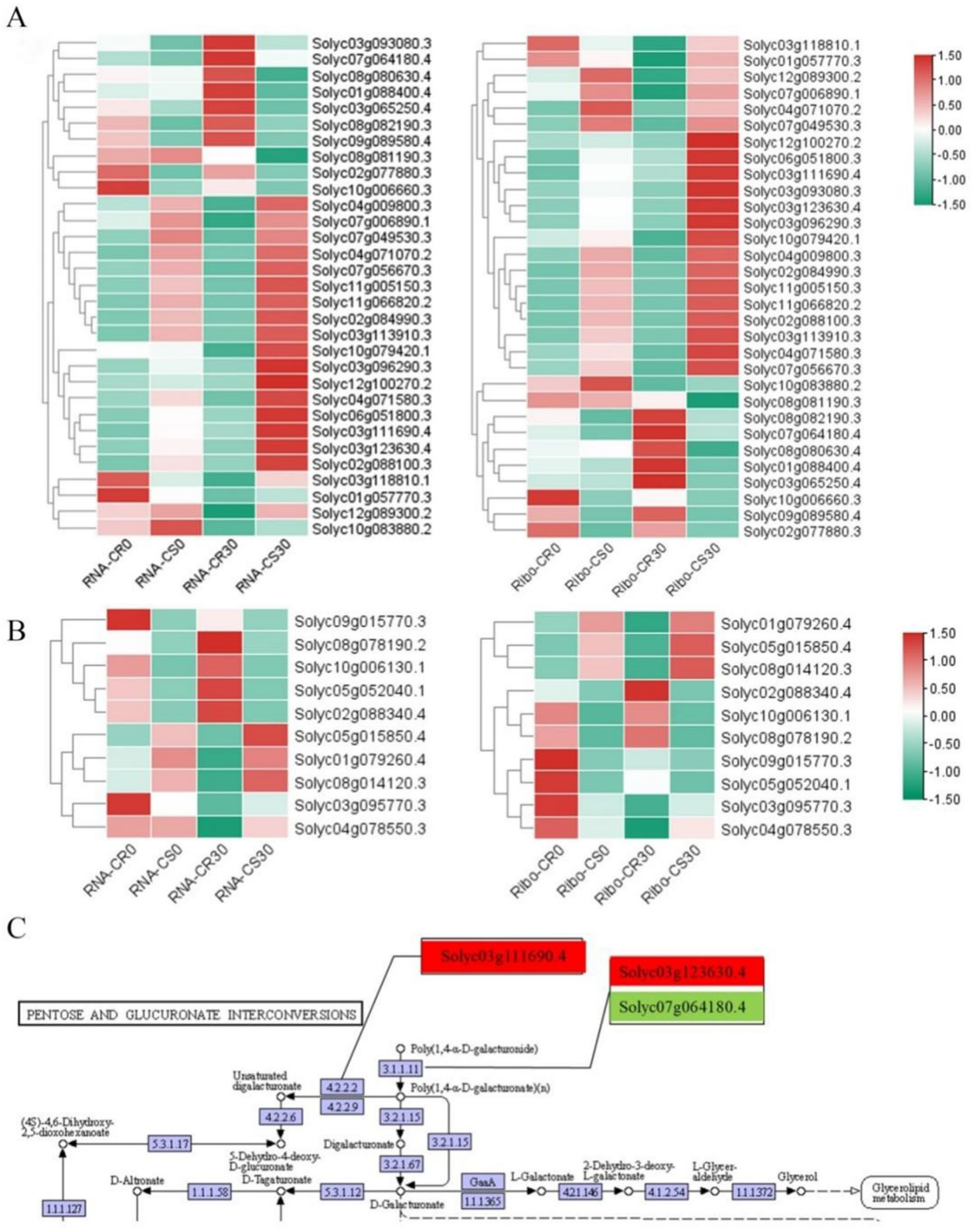

Fig. 4 Screening of differential genes related to fruit cracking. (**A**) Differentially expressed genes in tomato fruit cracking in RNA-seq and Ribo-seq, (**B**) Differentially expressed transcription factors in tomato fruit cracking in RNA-seq and Ribo-seq, (**C**) Location and differential expression of pectin-degrading enzyme class-related genes in the KEGG pathway

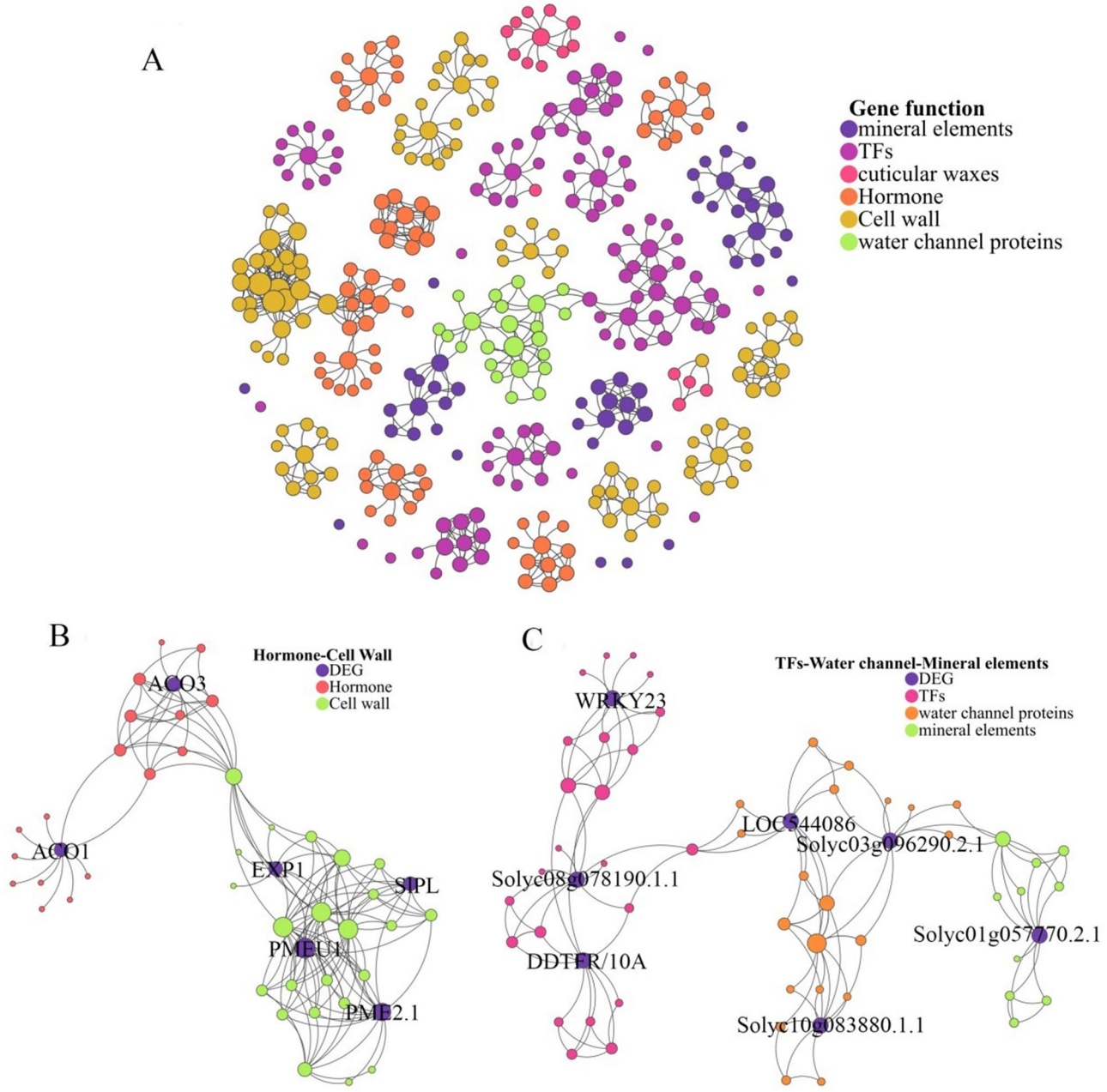

Fig. 5 Construction of protein interactions network encoded by differentially expressed genes, (**A**) Network of proteins encoded by all differentially expressed genes that may be associated with fruit cracking, (**B**) Hormone-cell wall metabolism protein regulatory network, (**C**) Transcription factor-water channel protein-mineral element uptake and transport protein regulatory network

translation synergy can boost a more comprehensive understanding of gene expression and function [33]. Analysis of the reliability of Ribo-seq data revealed that the location, coverage and length of ribosomal footprints on mRNA were consistent with existing studies [16]. After calculating the Pearson correlation coefficients between translated gene expression and transcript abundance, we found that the Pearson correlation coefficients between the two omics of both genotypes before and after irrigation were >0.7, indicating the synergistic effect of transcription and translation (Fig. 3.A). This was later confirmed by the analysis of DEGs, where the genes that showed differences at both transcriptional and translational levels and were up- and down-regulated in the same direction between CR and CS before irrigation treatment accounted for a total of 44.67% of all DEGs. Genes that differed at both transcriptional and translational levels and were up- and down-regulated in the

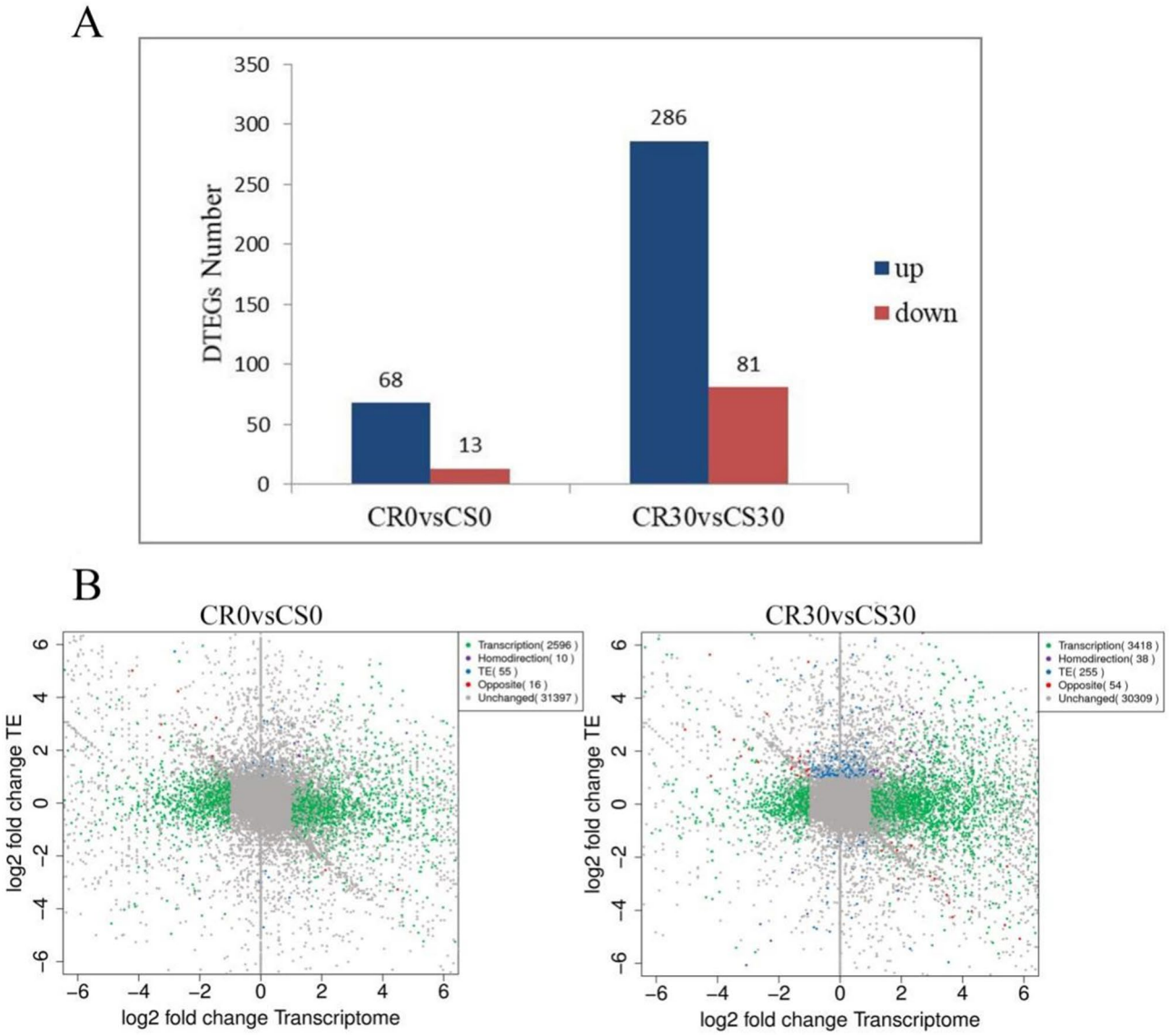

Fig. 6 (**A**) DTEGs up and down between CR and CS under 0 h and 30 h saturated irrigation, (**B**) Comparison of CR and CS genome-wide transcriptional levels with TE at 0 h and 30 h of saturated irrigation

same direction after irrigation treatment accounted for 50.43% of all DEGs. Nearly half of the DEGs were able to show concordance at the transcriptional and translational levels. Both before and after irrigation, the number of up-regulated genes was greater than the number of down-regulated genes at transcriptional and translational levels in CS compared with in CR, indicating that CS fruits were recruiting more candidate genes in response to stress.

About 50% of the DEGs showed single histological differences between the two species, e.g., *PIP1-3*, *PME2.1*; even in the opposite direction, e.g., *XTH23*. The presence of these genes indicates that transcription and translation are independent, which aligns with previous findings [34, 35]. Before and after irrigation treatment, 660 and 1154 genes differed between CR and CS only at the translational level, respectively. This indicated that the translation level was translated on the original mRNA without requiring the production of new mRNAs, which makes it a faster and more rapid form of regulation [33].

Hormone-cell wall and transcription factor-water channel-mineral elements network synergistically regulate tomato fruit cracking

DEGs from CR and CS fruits identified through two omics were mainly involved in phytohormone synthesis, hormone, ROS, cell wall metabolism, mineral element, wax and cuticle metabolism, water uptake and transport, as well as some TFs. These DEGs may play important roles in regulating tomato fruit cracking. In this study,

two potential pathways, specifically the hormone-cell wall metabolic pathway and transcription factor-water channel-mineral element pathway were obtained from macroprotein regulatory networks. Based on the networks and previous studies, we further enriched fruit cracking regulatory networks.

Ethylene is one of the most important hormones. Ethylene metabolic pathways include ethylene synthesis related gene *ACO*, as well as ethylene signaling factors such as *ERFs*. *ERFs* have been reported to regulate fruit ripening in response to ethylene signaling [36–38]. Ethylene plays an important role in fruit ripening and softening, which may decrease pericarp strength and increase the possibility of fruit cracking [38]. In this study, *ACO1* and *ACO3*, as well as four differentially expressed *ERFs* (*Solyc10g006130.1*, *Solyc05g052040.1*, *Solyc08g078190.2*, *Solyc09g075420.3*) were differentially expressed in CR and CS fruits. In our previous study, we also found *ERF4* (*Solyc11g042560.2*) and *Ethylene receptor* (*Solyc09g008720.2*), as well as some long non-coding RNA (lncRNA) such as *XLOC_033909* and *XLOC_012774* were involved in tomato fruit cracking [11]. The ethylene-responsive factor *ERF4* in the ethylene signaling pathway was found to regulate watermelon fruit dehiscence [30]. We hypothesized that *ERFs* and *ACOs* screened out from this study also have some effects on tomato fruit cracking. In addition, cell wall related genes, such as *SLPL*, *PMEU1*, *PME2.1* and *EXP1* were also differentially expressed in this study. Xue et al. (2020) found xyloglucan endotransglucosylase/ hydrolase (*XTH*) such as *Solyc07g055990.3* (*XTH7*), *Solyc12g011030*.3 (*XTH 9*), as well as *expansin-like* Solyc08g077910.3, *Solyc09g075330*.3 (*pectinase*), *Solyc10g080210*.2 (*PG2a*), *XLOC_041113*, *XLOC_023442*, *XLOC_005571*, *XLOC_023442*, *XLOC_033910* associated with fruit cracking [11]. Previous researches have also indicated that cell wall metabolism and its related enzymes such as pectin methylesterase (PME), β-galactosidase (β-gal) and cellulase (Cx) could regulate the softening and cracking process of fruits [39, 40]. Jiang et al. (2019) found that silencing the *PG* and *EXP* significantly reduced tomato fruit cracking rate [22]. Our previous research also found that the fruits of *SlPL* overexpression (*OEPL*) were more susceptible to cracking than that of the wild type and showed lower fruit firmness. *OEPL* fruit had significantly lower pro-pectin content and higher pectin cleavage enzyme activity. The *OEPL* fruit also showed higher expression of cell wall metabolism-related genes *PG2*, *PME2.1*, *Cel2*, *GH9C5* and ethylene synthesis pathway-related genes *ACS4* and *ACO1* [41]. These researches suggested that cell wall-degrading enzymes and genes could influence fruit softening, and could further affect fruit cracking with high potential to cooperate with ethylene. Furthermore, ethylene-responsive factors can regulate the expression of cell wall-related genes [36]. We also found that ethylene responsive elements were presented in the promoter region of *PL*, *PMEU1*, *PME2.1* and *EXP1*, indicating that ethylene and cell wall related genes might coordinately regulate tomato fruit cracking. Treatment of apples with ethylene inhibitor 1-MCP significantly reduces the expression of *PG* in the pericarp, reduces fruit cracking rate and extends shelf life [42]. It proved that ethylene can regulate the expression of cell wall related genes such as *PG*. Through the analysis of protein interaction networks, reciprocal relationships were obtained between ethylene-related genes and cell wall-degrading genes. To sum up, we established an ethylene-cell wall model that can regulate tomato fruit cracking (Fig. 7).

In addition, ROS may regulate fruit cell wall modification, which in turn influence fruit cracking. In our study, two significantly different and highly expressed reactive oxygen species-related genes were identified (*Solyc08g080940.3*, *Gpxle1*; *Solyc06g072840.3*, *Bn15D1B*). Previous research has proved that ROS related *Solyc02g080530.3* and *XLOC_020819* could influence fruit cracking [11]. Peroxidase in the cell wall results in cell wall sclerosis by giving rise to cross-linking of cell wall components, thereby inhibiting cell elongation and influencing fruit cracking, which will lead to fruit cracking when water absorption swelling occurs [43–45]. ROS are also involved in cell wall expansion [46, 47]. Therefore, ROS may be involved in fruit cracking by influencing fruit cell wall or other ways.

Except for ethylene, other hormones related genes such as auxin (*DRMH1*), ABA (*ASR1*) and GA (*GASA1*, *GA2OX1*) also showed different expressions in CR and CS fruits in our study. *IAA3* has been shown to be a molecular bridge between the auxin and ethylene signaling pathways in tomato [48]; whereas *IAA17* plays a critical role in controlling fruit quality, and *IAA17*-silenced lines showed thicker pericarp compared with wild-type lines [49]. Furthermore, ROS can be induced by auxin, and H_2O_2 disassembles polymers by producing ·OH at the cell wall [50]. Programmed cell death results in a decrease or loss of plasma membrane permeability, which in turn will affect cell activity, water absorption and fruit cracking [51]. Meanwhile, increasing auxin may accelerate the accumulation of H_2O_2 and promote cell elongation [52]. Rayle and Cleland (1970) [53] raised the acid growth theory, suggesting that hydrogen ions may cause a purely physical or chemical reaction, for instance, they may cleave acid-labile bonds on the wall, or activate normal enzymatic to play a role in wall loosening. The interaction between ethylene and abscisic acid also affects fruit ripening [54], and abscisic acid promotes fruit cracking by increasing water influx into the fruit [22]. Applications of GA_3 can decrease the activities

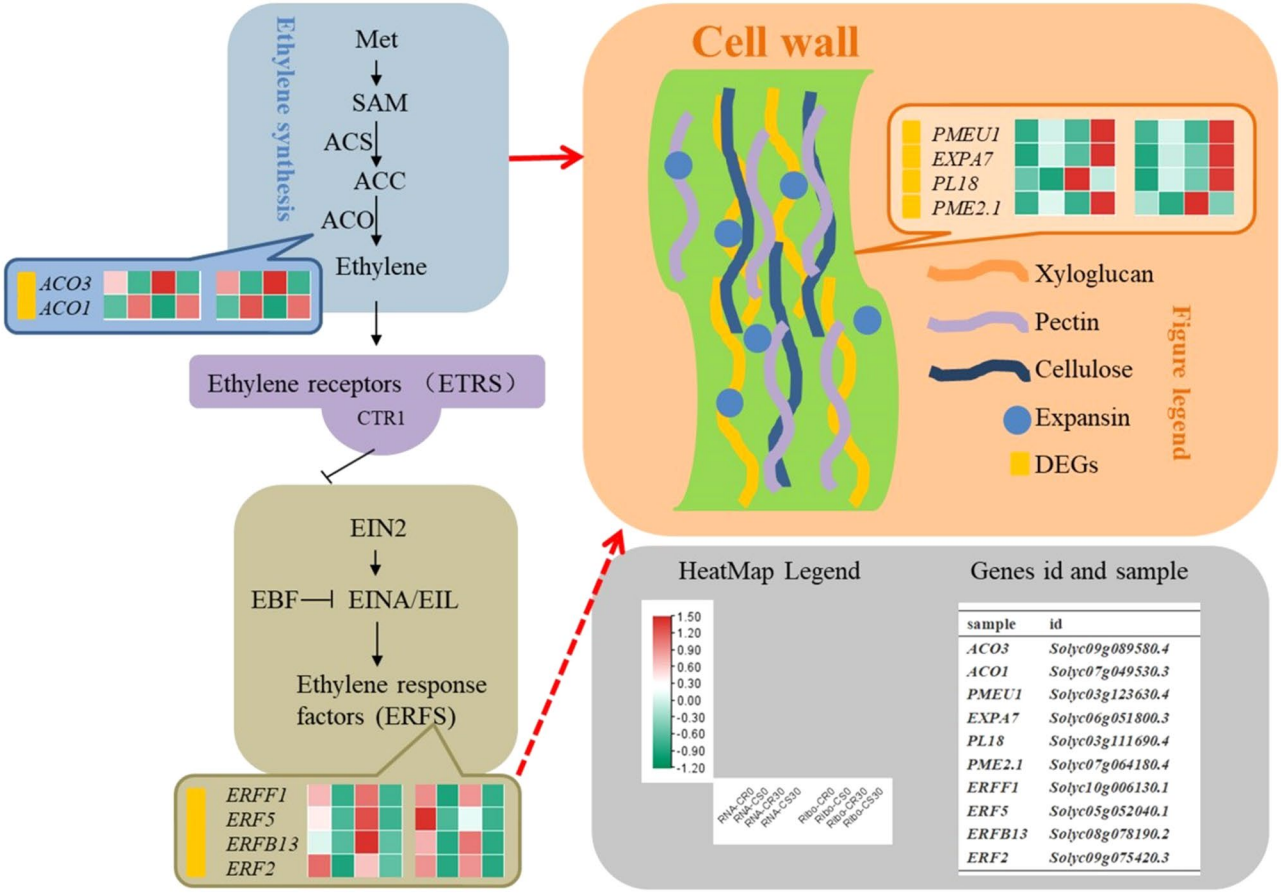

Fig. 7 Prediction of the ethylene-cell wall model regulating tomato fruit cracking. The solid arrows indicate regulatory pathways, the red arrows indicate regulatory pathways found in this study, and the dashed arrows indicate predicted regulatory pathways

of *PG* and *PME* and increase fruit firmness, thereby preventing fruit cracking [55]. We used the online website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to analyze the promoter cis-acting elements of the screened DEGs related to cell wall metabolism, cuticle metabolism and mineral elements and found that the promoter of DEGs contained ethylene, abscisic acid, growth hormone, and gibberellin response elements.

TFs play very important roles in growth, development and aging. In our research, several TFs such as *WRKY*s and *BHLH*s were screened out. The *WRKY*s can function up- and downstream of hormones, which are involved in the antagonistic functions of ethylene (ET), and manage developmental processes via auxins. In addition, some genes with potential regulation of tomato fruit cracking have been discovered, whose functions involve plant hormones, keratin wax synthesis, mineral element absorption, and cell water absorption. *WRKYs* are also identified in transcriptional regulation of ABA-responsive element binding factors [56, 57]. Hence, hormones such as ethylene, auxin, ABA and GA, cooperative with TFs may influence fruit cracking.

Through prediction from macroprotein regulatory networks, we also obtained a transcription factor-water channel protein-mineral element interaction network. Water is an important factor influencing fruit cracking, and fruit uptake of large amounts of water through the root system leads to cell swelling and elevated swelling pressure, which causes cell and tissue rupture [58, 59]. In this experiment, water channel proteins (*PIP1-1*, *TIP1-1*, *PIP1-3*) located on cell membranes were screened out through irrigation treatment, which may be critical for massive water uptake by cells and influence fruit cracking. Mineral elements may affect fruit cracking, and pre-harvest spraying with mineral salt solutions can be effective in avoiding fruit cracking [60]. Calcium was found to play an important role in maintaining cell wall structure and strength and in regulating cellular osmotic pressure, reducing fruit cracking [60, 61]. Application of elements such as boron and iron prior to kernel hardening reduced the rate of sweet cherry cracking [62]. In our study, five DEGs, including *Solyc10g006660.3*, *Solyc01g057770.3*, *Solyc04g009800.3*, *Solyc10g079420.1*, *Solyc03g118810.1* were screened, which played roles in boron transport and calcium binding.

Besides, the cuticle is attached around the plant epidermal cells, consists of keratin, waxes and other components, and its structure and function have an important influence on the mechanism of fruit cracking [63, 64]. It has been shown that epidermal waxes are effective in preventing water loss from plant tissues, and the change of osmotic pressure caused by fruit water absorption is also one of the factors causing tomato fruit cracking [65]. In our study, a total of four DEGs related to keratin wax synthesis were identified.

Previous findings suggest that fruit cracking is the result of synergistic regulation of multiple genes [22]. We proposed a hypothesis of tomato fruit cracking network based on DEGs, protein interaction analysis and previous researches, involving hormone-cell wall-transcription factor-water channel-osmotic pressure network (Fig. 8, supplementary Table 1). Specifically, water enters cells through roots, leaves or pericarp, and potentially causing pressure on cell wall and pericarp. When cell wall and pericarp were not strong enough, it would cause fruit cracking. Aquaporins (*PIP1-1*, *TIP1-1*), cuticle and wax (*CER1*, *CYP*) related genes as well as lncRNA will influence water absorption. Hormones (e.g. *ACOs*, *ERFs*, *DRMH1*, *GASA1*), ROS (e.g. *Gpxle1*), mineral element (e.g. *BOR1*) and cell wall associated genes (e.g. *PL*, *EXP1*, *PG2a*, *XTHs*) may affect cell wall strength. Transcription factors can influence aquaporins or hormones. All or several of these factors work together may cause tomato fruit cracking.

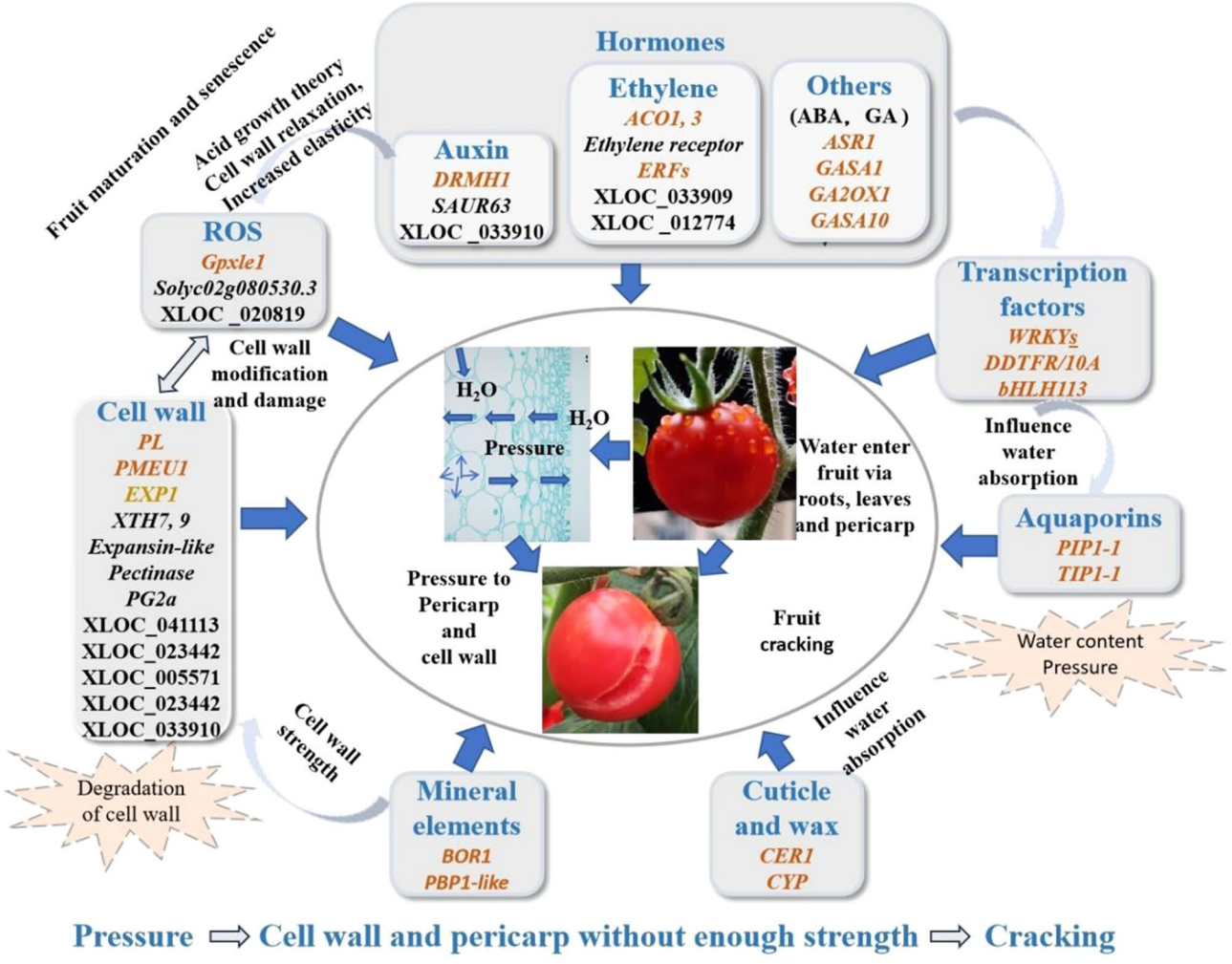

Fig. 8 Regulatory network for tomato fruit cracking, genes in orange were identified in this research, and genes in black were identified in our previous research [11]. *PL*, *Solyc03g111690.4*; *PMEU1*, *Solyc03g123630 0.4*; *EXP1*, *Solyc06g051800.2.1*; *ACO3*, *Solyc09g089580.4*; *ACO1*,*Solyc07g049530.3*; *ASR1*, *Solyc04g071580.3*; *TIMPA*, *Solyc08g080630.4*; *DRMH1*, *Solyc02g077880.3*; *GASA1*, *Solyc12g089300.2*; *GA2OX1*, *Solyc07g056670.3*; *GASA1*, *Solyc03g113910.3*; *PIP1-1*, *Solyc03g096290.3*; *TIP1-1*, *Solyc10g083880.2*; *CML41*, *Solyc03g118810.1*; *BOR1*, *Solyc01g057770.3*; *CYP*, *Solyc07g006890.1*; *CER1*, *Solyc01g088400.4*; *WRKY*, *Solyc05g015850.4*; *ERF*, *Solyc05g052040*

Conclusion

By RNA-seq and Ribo-seq techniques and bioinformatics analysis using two tomato genotypes with significant differential traits in fruit cracking, we established two potential regulatory networks associated with fruit cracking, including ethylene-cell wall and TFs-water channel protein-boron transporter protein. In addition, a comprehensive regulatory network was set up to explain the mechanism of fruit cracking including hormones, ROS, cell wall, mineral element, TFs, water channel protein, cuticular and wax related genes, as well as long non-coding RNA. All or several of these factors work together may cause tomato fruit cracking. This research enriched the molecular mechanism of fruit cracking; however, the major genes regulating fruit cracking need further investigation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05937-1.

Supplementary Material 1

Acknowledgements
We appreciated the help from Guangzhou Genedenovo Biotechnology Co., Ltd for assisting in sequencing analysis.

Author contributions
Fangling Jiang led and coordinated the project. Zhaojiang Zhong wrote the main manuscript text, Zhen Wu and Rong Zhou provided writing comments on the paper, Xiaowei Yu , Yuanyuan Zhou, Haowei Lin and Yinghao Zhai drawing the figures in the manuscript. Fangling Jiang is the corresponding author and is responsible for all contact and correspondence. All authors reviewed the manuscript.

Funding
This project was funded by National Natural Science Foundation of China (32072581), National Agricultural science and technology major project (NK20220904), The earmarked fund for CARS (CARS-23), Jiangsu Seed Industry Revitalization Project (JBGS(2021)015) and The Fundamental Research Funds for the Central Universities(KYZZ2022004).

Data availability
The datasets generated during and analyzed during the current study are included in this published article (and its supplementary information files). All RNA sequencing data from this study are available in the NCBI sequence read archive (SRA) under accession numbers: CS30-1-RNAseq, CR30-3-RNAseq, CR30-2-RNAseq, CR30-1-RNAseq, CS0-3-RNAseq, CS0-2-RNAseq, CS0-1-RNAseq, CS30-3-Riboseq, CS30-2-Riboseq, CS30-1-Riboseq, CR30-3-Riboseq, CR0-3-RNAseq, CR30-2-Riboseq, CR30-1-Riboseq, CS0-3-Riboseq, CS0-2-Riboseq, CS0-1-Riboseq, CR0-3-Riboseq, CR0-3-Riboseq, CR0-2-Riboseq, CR0-1-Riboseq, CS30-3-RNAseq, CS30-2-RNAseq, CR0-2-RNAseq, CR0-1-RNAseq (https://www.ncbi.nlm.nih.gov/sra/PRJNA967039).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Statement
Tomato material 'Tomato cherry' was introduced from the USA and 'LA2661' was introduced from Tomato Genetics Resource Center. The seeds were purchased from a domestic agent and were self-fertilized for more than six generations by the Vegetable Physiology and Ecology Laboratory of Nanjing Agricultural University. This study complies with the relevant national and institutional regulations.

Competing interests
The authors declare no competing interests.

Received: 18 May 2023 / Accepted: 5 December 2024
Published online: 19 December 2024

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