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RESEARCH ARTICLE

Functional analysis of tomato *SlPP2C-A* gene subfamily, highlighting SlPP2C7's role in regulating saline-alkali stress tolerance

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Highlights

- 14 *SlPP2C-A* family genes have been isolated in tomato.
- The knockout *SlPP2C7* can enhance the saline-alkali stress tolerance of tomato.
- Integrative analysis of transcriptomics and metabolomics reveals that SlPP2C7 regulates the flavonoid biosynthetic pathway.

Abstract

The type A protein phosphatase 2C (*PP2C-A*) gene family is vital for regulating the ABA signaling pathway and plant stress responses. In this research, 14 *SlPP2C-A* genes were identified in the tomato genome, distributed across six chromosomes. Most *SlPP2C-A* genes contain *cis*-acting elements associated with growth, development, light, hormones, and stress responses. Collinearity analysis revealed high homology between the tomato and *Arabidopsis PP2C-A* gene families. Tissue-specific expression analysis indicated that *SlPP2C7* is highly expressed in flowers, leaves, and mature fruits, and is significantly induced by saline-alkali stress. Gene-edited *SlPP2C7* knockout mutants subjected to saline-alkali stress confirmed that SlPP2C7 negatively regulates saline-alkali tolerance in tomato. Combined transcriptomic and metabolomic analyses showed that under saline-alkali stress, metabolic pathways such as flavonoid biosynthesis, isoflavonoid biosynthesis, flavone and flavonol biosynthesis, phenylpropanoid biosynthesis, and phenylalanine metabolism were significantly enriched. These outcomes imply that SlPP2C7 may enhance tolerance to saline-alkali stress through modulating flavonoid biosynthesis pathways. This research reveals comprehension of the physiological and molecular mechanism responsible for saline-alkali stress tolerance mediated by SlPP2C7 in tomato.

Keywords: tomato, *SlPP2C-A* gene family, SlPP2C7, saline-alkali stress, transcriptome, metabolome, flavonoids

1. Introduction

Nowadays, soil salinization has arisen as a significant global environmental challenge that threatens agricultural production, food security, and ecosystems (Mukhopadhyay *et al.* 2021). Saline-alkali stress is generally characterized by elevated salt concentrations and high pH levels. Under such stress, excessive levels of Na^+ enter plant cells, inhibiting the uptake of K^+ and Ca^{2+}, leading to ion toxicity. This disruption modifies cellular stability, compromises membrane integrity, and diminishes root activity and photosynthetic efficiency (Raza *et al.* 2023). Additionally, the elevated external ion concentration reduces osmotic potential, resulting in physiological drought, referred to as osmotic stress (Asada 2006). Tomato (*Solanum lycopersicum*), a significant worldwide crop, experiences yield and quality declines under saline-alkali stress (Wang *et al.* 2024). Identifying key genes in tomatoes responsive to saline-alkali stress, delving into their regulatory mechanisms, then employing modern molecular research to boost plants' tolerance are of great significance.

Plant secondary metabolites are essential for resistance to abiotic stresses such as salinity and drought. They exert their functions by regulating the metabolism of plant

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hormones, the activity of kinases, and the homeostasis of reactive oxygen species (ROS) (Sachdev *et al.* 2021). Flavonoids are aromatic compounds prevalent in plants, exhibiting antioxidant and antimicrobial activities, and can delay oxidative aging in plant tissues by scavenging free radicals (Shen *et al.* 2022). Flavonoid biosynthesis primarily relies on the phenylpropanoid pathways (Clayton *et al.* 2018). The biosynthesis commences with phenylalanine, which is first transformed into cinnamic acid *via* the activity of phenylalanine ammonia-lyase (PAL), and subsequently transformed into *p*-coumaric acid by cinnamic acid 4-hydroxylase (C4H). *p*-Coumaric acid subsequently reacts with three malonyl-CoA molecules to form chalcone *via* chalcone synthase (CHS), which is then cyclized to produce the basic flavonoid skeleton (Li *et al.* 2018). Flavonoids, a diverse class of plant secondary metabolites, are classified into various subgroups according to their structural features. These subgroups encompass flavones, flavonols, isoflavones, flavanols, flavanones, anthocyanins, chalcone and dihydroflavone (Chen *et al.* 2023). Flavonoids are integral to plants' resistance against abiotic stresses, for instance drought, salinity, and ultraviolet light. Research shows that salt stress can induce flavonoid accumulation in plants, enhancing salt tolerance by scavenging excess ROS and mitigating oxidative damage (Clayton *et al.* 2018, Shen *et al.* 2022). In soybeans, overexpression of the key flavonoid-biosynthesis gene *GmF3′H* significantly improves salt-stress tolerance, enhancing antioxidant capacity and growth (Bian *et al.* 2020). In maize, exogenous application of flavonoids alleviates growth inhibition induced by salt stress, enhancing leaf photosynthetic efficiency and root activity (Song *et al.* 2022). Under salt stress conditions in tomatoes, the transcriptional activity of genes involved in flavonoid biosynthesis is significantly enhanced, leading to a notable accumulation of flavonoids (Feng *et al.* 2023). The transcription factor SlNAC12 boosts salt stress resistance by altering flavonoid biosynthesis (Chen *et al.* 2024).

PP2C, a protein phosphatase, regulates signaling pathways and various physiological and biochemical processes in plants through dephosphorylating substrate proteins. Abiotic stresses response in plants are critically dependent on this factor (Xue *et al.* 2008). It affects stress-response mechanisms by modulating metabolism and hormone levels (Fan *et al.* 2019). The *PP2C* gene family has been identified in numerous plant species, with 80, 90, 145, 102, and 178 genes encoding PP2C proteins characterized in *Arabidopsis thaliana* (Smith and Walker 1996), *Oryza sativa* (Singh *et al.* 2010), *Saccharum spontaneum* (Huang *et al.* 2023), *Zea mays* (Pang *et al.* 2024), and *Arachis hypogaea* (Wu Z *et al.* 2023), respectively. The extensive presence of PP2C proteins across the plant kingdom accentuates their crucial involvement covering a broad spectrum of growth and developmental processes. As such, the detailed characterization and analysis of the PP2C in tomatoes hold significant scientific value. In higher plants, type A PP2C proteins act as negative modulators within the ABA signaling pathway. This regulatory function leads to reduced resilience against oxidative stress and negatively impacts signaling pathways related to responses to abiotic stresses (Fan *et al.* 2019; Chen *et al.* 2020).

This research performed a genome-wide survey to identify *SlPP2C-A* family members in tomatoes using bioinformatics tools. Most *SlPP2C-A* genes are significantly induced by saline-alkali stress. We developed *SlPP2C7* knockout mutants *via* gene editing and determined that SlPP2C7 negatively regulates tomato saline-alkali stress tolerance through experiments simulating saline-alkali stress, although the potential molecular mechanism remains unclear. To elucidate the importance of SlPP2C7 in mediating saline-alkali stress tolerance, we performed a combined transcriptomic and metabolomic analysis on wild-type and *SlPP2C7* knockout mutants. Pathways related to flavonoid biosynthesis, metabolism, and photosynthesis were found to be significantly enriched. It hypothesized that SlPP2C7 may enhance plant saline-alkali stress tolerance by modulating flavonoid biosynthesis and associated metabolic pathways. Our study expected to yield vital theoretical and practical information regarding the modulation of flavonoids by SlPP2C7 under saline-alkali stress conditions, as well as contribute to the development of salt-tolerant tomato cultivars.

2. Materials and methods

2.1. Genome-wide identification of *SlPP2C-A* family genes

Utilizing type A PP2C family protein sequences from *A. thaliana*, maize, and soybean, we constructed a hidden Markov model using HMMER 3.0. Utilizing this model, a comprehensive search was conducted across the entire repertoire of tomato coding proteins to detect potential PP2C-A family sequences, ultimately resulting in the successful identification of all plausible PP2C-A family sequences within the tomato proteome. BLASTP was employed to align all tomato protein sequences of the PP2C-A family, applying an e-value threshold of 1e–10 to identify all potential PP2C-A family sequences (Altschul *et al.* 1990). Subsequently, pfamscan and the Pfam A database were utilized to annotate the domains of all candidate sequences (Finn *et al.* 2014). In conclusion, a total of 14 sequences harboring the PF00481 domain were ultimately validated as the definitive PP2C-A sequences. The physicochemical characteristics of type A PP2C family genes were analyzed *via* the ProtParam online tool. Meanwhile, the WoLF PSORT tool was used to predict the subcellular localization of PP2C-A proteins.

2.2. Phylogenetic analysis and chromosomal localization of the *SlPP2C-A* family genes

The phylogenetic tree for the PP2C-A protein family sequences identified in tomato, *A. thaliana*, maize was constructed by utilizing MAFFT for multiple sequence alignment and MEGA for tree construction (Kumar *et al.* 2008). For annotating the phylogenetic tree, iTOL v6 was used, and the chromosomal locations of the *SlPP2C-A* family genes in tomato were generated using the MG2C tool.

2.3. Analysis of gene structure and conserved motifs in *SlPP2C-A* family

The GSDS was utilized to analyze the gene structures of

SlPP2C-A. Generally, members with similar motifs indicate analogous functions. To enhance the understanding of the similarity and diversity of protein motifs, the MEME software analyzed the conserved motifs of the tomato SlPP2C-A family (Bailey *et al.* 2009).

2.4. Analysis of *cis*-elements in the promoters of the *SlPP2C-A* family

The 2-kb upstream region of the gene structure was selected, and the PlantCARE database was utilized to identify transcription factor binding sites. The locations of these sites were marked and displayed on the gene promoter physical map (Appendices A and B).

2.5. Collinearity and expression pattern analysis of the *SlPP2C-A* family

MCScanX was employed for collinearity analysis. This analysis aimed to identify segmental and tandem duplications resulting from gene duplication events in the data. In the alignment results, if a pair of aligned sequences are adjacent on a chromosome, MCScanX considers one to be a tandem duplicate of the other. If multiple aligned pairs cluster on different chromosomes, MCScanX categorizes one group as a segmental duplicate of another, indicating collinearity. As for the family-expressed genes which had gone through screening, an analysis of hierarchical clustering was implemented. This process helped to cluster genes whose expression patterns were similar. The outcomes were visualized by TBtools (Chen *et al.* 2023).

2.6. Generation of transgenic materials, treatment of experimental materials, and sample collection

In this study, the CRISPR/Cas9 system was employed to edit the *SlPP2C7* gene. The transformation method for tomatoes adhered to previous research (Hu *et al.* 2024). We utilized several plump and uniform wild-type 'Ailsa Craig' and *SlPP2C7* knockout mutant tomato seeds. Once the seeds had been soaked and had germinated, they were sown in 50-cell trays filled with growing medium. Then they were cultivated inside a light incubator (RXZ-500D, Ningbo, China). At the stage when the fourth true leaf was fully expanded, they were transplanted into 7 cm×7 cm nutrient pots. Saline-alkali treatment was administered once the fifth true leaf had completely unfurled. Each seedling received 100 mL of a saline-alkali solution (300 mmol L^{-1}, NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3=1:9:9:1, pH=8.90). The second and third true leaves (counting from the growing point downward) were collected at 0, 6, 12 and 24 h post-treatment to measure gene expression, and on the fifth day post-treatment to assess other physiological indicators. Upon collection, all leaves were swiftly frozen in liquid nitrogen and maintained at –80°C freezer. To guarantee that the results were reproducible, three independent biological replicates were conducted.

2.7. Total RNA extraction and qRT-PCR analysis

In this step, 0.1 g of leaf samples frozen in liquid nitrogen were processed into powder with a high-throughput tissue grinder. The extraction of total RNA was performed using the OmegaBio-Tek Plant RNA Kit, following which cDNA was synthesized with the PrimeScript™ RT Reagent Kit (TaKaRa Bio, Shiga, Japan). The quantification of gene expression was conducted employing the StepOne™ Real-Time PCR System. qRT-PCR amplification was carried out following the ChamQ SYBR qPCR Master Mix kit instructions. *SlACTIN7* served as the reference gene, with primers listed in Appendix C. Data analysis was executed based on the method of Livak and Schmittgen (2001).

2.8. Determination of malondialdehyde content

The extraction and determination of MDA content were executed based on the method of Hodges *et al*. (1999). Briefly, 0.2 g of tomato leaves were pulverized into powder with a high-throughput tissue grinder. A total of 1.8 mL of 5% (w/v) TCA solution was added, and the mixture was vortexed and centrifuged at 12,000×g for 20 min at room temperature. Subsequently, a mixture was made by combining 1.5 mL of the supernatant with 1.5 mL of 0.6% (w/v) TBA solution. For 30 min, the mixture was heated in boiling water and then swiftly cooled to ambient temperature. Absorbance measurements were taken at wavelengths of 450, 532, and 600 nm.

2.9. Determination of relative water content (RWC)

The RWC measurement method is based on Hu *et al.* (2015). Freshly harvested tomato leaf samples (3–8 per treatment) were initially weighed as fresh weight (FW). Following immersion in distilled water for 8 h, the leaves were air-dried to eliminate residual surface moisture and then re-measured to assess the saturated fresh weight (SFW). Subsequently, the leaves were thoroughly dried at 65°C and reweighed to determine the dry weight (DW). The RWC was computed based on equation: (FW–DW)/(SFW–DW)×100%.

2.10. Determination of relative electrical conductivity (REC)

The REC measurement technique is derived from Tan *et al.* (2017). Leaf discs with a diameter of 0.5 cm were punched out. Five to ten discs were placed in 9 mL of water and shaken for 3 h to conduct primary conductivity (R1). The discs were treated with boiling water for half an hour, cooled, and measured again to obtain R2. REC was calculated as: (R1/R2)×100%.

2.11. Transcriptomic and metabolomic analysis

In this project, total RNA was extracted from 12 samples. High-quality mRNA was utilized to construct cDNA libraries for both the wild-type (control) and *SlPP2C7* mutant (CP-7). The library's insert size was verified with a fragment analyzer to ensure quality. Qualified cDNA libraries were sequenced using the Illumina platform. Clean data were utilized for gene structure analysis, which encompasses alternative splicing, new gene discovery, and gene structure optimization (Pertea *et al.* 2015). Differential expression analysis was conducted using the criteria: $|\log_2\text{Fold Change}|\geq 1$ and FDR<0.05.

A total of 12 samples were collected for metabolomic analysis, following a procedure analogous to that of

transcriptomic analysis. The primary instruments utilized in the data collection system were ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS). Using R software's built-in Prcomp function, principal component analysis was executed. The data underwent unit variance scaling (UV) for processing. The metabolite content data underwent UV processing, followed by heatmap generation using the ComplexHeatmap package in R software to conduct hierarchical cluster analysis (HCA) on the metabolite accumulation patterns observed across various samples. The criteria for selecting differentially accumulated metabolites (DAMs) were as follows: metabolites with a variable importance in projection (VIP) value>1 were selected, indicating a significant difference in the metabolite's variation concerning sample classification. Additionally, metabolites with Fold Changes≥2 or ≤0.5 were included, indicating a significant differential content between the control and experimental groups. Following screening, KEGG functional annotation and enrichment analysis were conducted on the differential metabolites (Kanehisa *et al.* 2008).

The transcriptomic and metabolomic data were integrated and cross-validated to jointly analyze differentially expressed genes (DEGs) and differentially accumulated metabolites (DAMs) between wild-type and *SlPP2C7* mutant groups.

2.12. Data statistical analysis

Values show the mean of three individual and repeated experiments. Significant differences (*P*-values) were determined using Student's *t*-test (*, $P<0.05$) and either one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

3. Results

3.1. Genome-wide identification of the *SlPP2C-A* gene family in tomato

In our research, we identified 14 *SlPP2C-A* genes that are unevenly distributed across six chromosomes (Fig. 1). We designated these genes as *SlPP2C1* through *SlPP2C14* based on their chromosomal positions. We assessed the physicochemical properties of the 14 tomato PP2C proteins, including amino acid length, molecular weight, isoelectric point, instability index and aliphatic index. The results show that the amino acid count in these proteins spans from 142 to 955, and their MW ranges from 15,813.08 to 109,110.03 Da. All pI values are below 7. Predictions of subcellular localization suggest that *SlPP2C1*, *SlPP2C2*, *SlPP2C4*, *SlPP2C5*, and *SlPP2C10* are localized in the cytoplasm; *SlPP2C3*, *SlPP2C6* and *SlPP2C8* are localized in the nucleus; *SlPP2C7*, *SlPP2C9*, *SlPP2C13*, and *SlPP2C14* are localized in the chloroplast; and *SlPP2C11* is associated with the cytoplasmic membrane (Table 1).

3.2. Analysis of gene structure and conserved motifs of the *SlPP2C-A* family in tomato

To elucidate the diversity of *SlPP2C* gene structures in tomatoes, we analyzed the distribution of exons and introns across the 14 *SlPP2C* genes (Fig. 2-A). The exon numbers vary from 1 in *SlPP2C9* to 20 in *SlPP2C13*, indicating a relatively complex gene structure (Appendix D). Conserved motifs, critical for gene classification, functional differentiation, and protein activity, were examined in the SlPP2C-A proteins. Closely related proteins exhibit highly similar motifs (Appendix E). Each PP2C protein contains more than two motifs, with motif 1 and motif 2 prevalent in most tomato PP2C proteins, suggesting significant conservation throughout their evolution. In addition to shared motifs, specific motifs unique to particular groups were identified. For instance, motif 15 is exclusive to *SlPP2C11* and *SlPP2C13*, whereas motif 14 is unique to *SlPP2C11* and *SlPP2C14*. These distinctive motifs may regulate the functional diversity of *PP2C-A* gene family (Fig. 2-B).

3.3. Phylogenetic tree and synteny analysis of the *SlPP2C-A* family genes

We generated a phylogenetic tree using the neighbor-joining method. This tree incorporated *PP2C* genes from tomato, *A. thaliana*, and maize. The phylogenetic tree categorizes PP2C proteins into five principal groups, each exhibiting varying gene counts within subgroups. Group I has the most members (6 *PP2C* genes), followed by Group II (four genes) (Fig. 3-A). The distribution of *SlPP2C-A* and *AtPP2C-A* demonstrates substantial similarity, suggesting the conservation of *SlPP2C-A* throughout evolutionary processes. To explore the phylogenetic dynamics of the *SlPP2C-A* family genes in tomatoes, we conducted comparative genomic

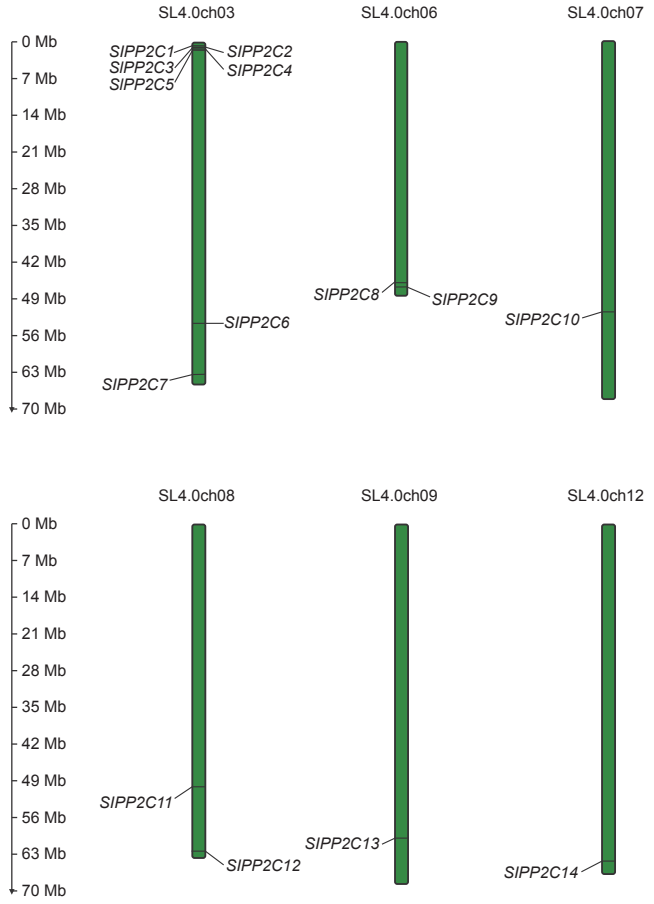


Fig. 1 Chromosomal localization of the *SlPP2C-A* gene in tomato.

Table 1 Physicochemical properties of the *SlPP2C-A* genes in tomato

Family ID	Amino acid length (aa)	CDS length (bp)	Molecular weight (Da)	Isoelectric points	Instability index	Aliphatic index	Location
SlPP2C1	331	996	36,843.85	6.09	59.95	82.78	cyto
SlPP2C2	345	1,038	37,872.93	4.95	57	85.13	cyto
SlPP2C3	347	1,044	38,049.53	6.02	53.69	84.09	nucl
SlPP2C4	304	915	33,397.94	5.7	56.99	80.16	cyto
SlPP2C5	397	1,194	43,979.53	5.72	50.8	82.92	cyto
SlPP2C6	406	1,221	44,683.73	6	52.59	76.33	nucl
SlPP2C7	480	1,443	52,169.78	5.12	42.15	100.85	chlo
SlPP2C8	410	1,233	44,859.67	5.12	46.42	79.37	nucl
SlPP2C9	142	429	15,813.08	6.09	26.13	92.68	chlo
SlPP2C10	536	1,611	59,196.43	5.38	47.81	88.88	cyto
SlPP2C11	469	1,410	51,638.63	4.89	38.97	94.95	plas
SlPP2C12	393	1,182	44,173.95	6.17	45.52	79.59	nucl
SlPP2C13	955	2,868	109,110.03	5.43	38.77	81.88	chlo
SlPP2C14	508	1,527	55,681.53	4.62	45.3	85.59	chlo

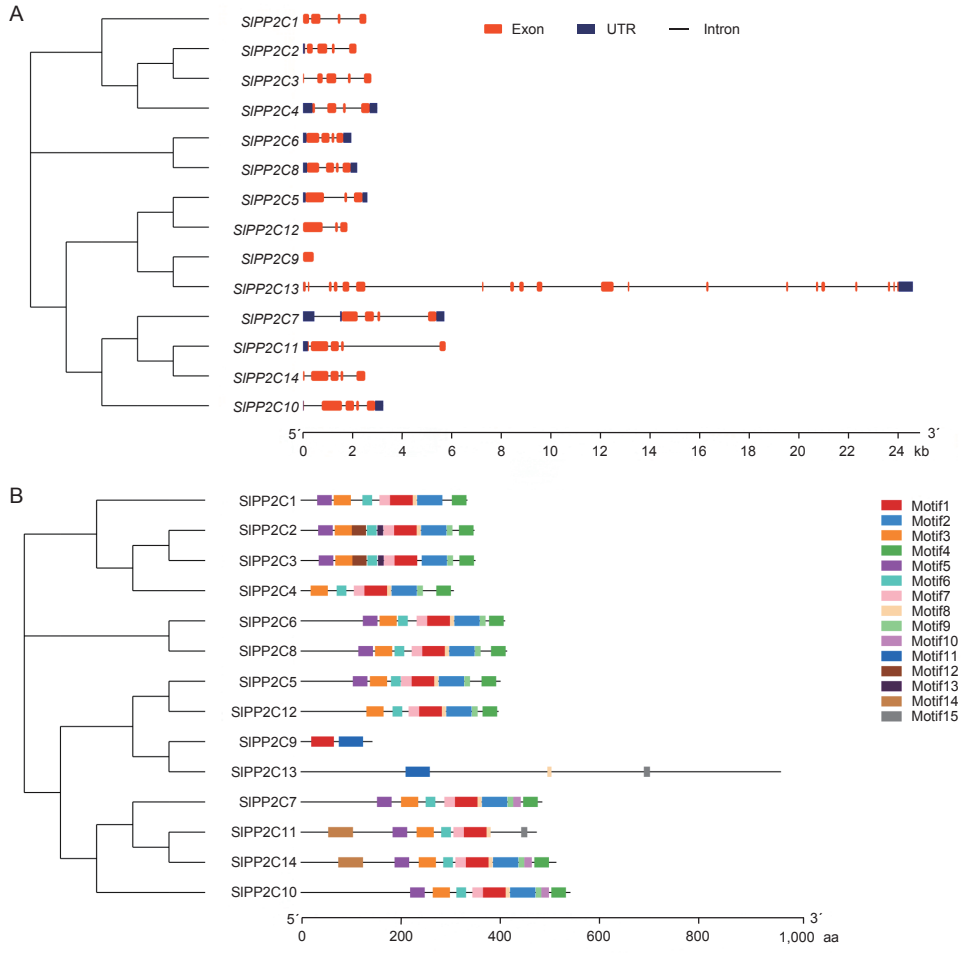


Fig. 2 Schematic representation of gene structure and protein motifs of *SlPP2C-A*. A, schematic representation of *SlPP2C* gene structures. The intron/exon structures were analyzed using GSDS. Orange boxes indicate exons, whereas lines represent introns. B, conserved motifs of *SlPP2C-A* genes. Box colors represent motifs at specific positions within each SlPP2C protein.

analyses to assess evolutionary homology among tomato, *Arabidopsis*, and maize (Fig. 3-B). Gray lines in the figure denote whole-genome synteny between species, whereas red lines indicate synteny within this gene family. The results reveal that the tomato–*Arabidopsis* collinearity map identifies nine gene pairs exhibiting collinearity, which signifies a high degree of homology between the *SlPP2C-A* and *AtPP2C-A* families.

3.4. Analysis of expression patterns of the *SlPP2C-A* family genes

To investigate the function of *SlPP2C-A*, we retrieved

RNA-seq date to examine the tissue-specific transcription of these genes. The *SlPP2C-A* genes exhibited varying expression levels across different tissues. Specifically, *SlPP2C2*, *SlPP2C3*, and *SlPP2C9* were highly expressed in buds; *SlPP2C7*, *SlPP2C8*, *SlPP2C12*, and *SlPP2C14* in flowers; *SlPP2C1* in leaves; *SlPP2C11* and *SlPP2C14* in roots; *SlPP2C5*, *SlPP2C6*, and *SlPP2C10* in immature fruits; *SlPP2C4* and *SlPP2C10* during the fruit breaker stage; and *SlPP2C13* post-fruit breaker stage (Fig. 4-A). Additional investigation of the transcriptomic data revealed that saline-alkali stress markedly elevates the transcription levels of the majority of *SlPP2C-A* genes (Fig. 4-B).

3.5. qRT-PCR analysis of *SlPP2C-A* gene response to saline-alkali stress

To elucidate the function of SlPP2C-A in tomato plants subjected to saline-alkali stress, we assessed the transcriptional profiles of 14 *SlPP2C-A* genes throughout the saline-alkali treatment period (Fig. 5). Under saline-alkali stress, *SlPP2C7* was upregulated at 6 h, *SlPP2C5*, *SlPP2C6*, *SlPP2C9*, *SlPP2C10*, and *SlPP2C13* at 12 h, and *SlPP2C8* and *SlPP2C11* at 24 h. In contrast, *SlPP2C1*, *SlPP2C4*, *SlPP2C12* and *SlPP2C14* exhibited significant downregulation.

3.6. Phenotypic analysis of *SlPP2C7* gene knockout tomato plants under saline-alkali stress

Owing to the high expression of *SlPP2C7* in tomato leaves and flowers, as well as its significant upregulation at 6 and 12 h under saline-alkali stress, Moreover, the homologous genes of the *SlPP2C7* gene in *A. thaliana* are HAB1 and HAB2. Studies have shown that HAB1 and HAB2 negatively regulate the ABA signaling pathway and abiotic stress by interacting with ABA receptor proteins PYR/PYL/RCAR (Wang *et al.* 2015). We developed transgenic tomato plants with *SlPP2C7* gene knockouts to illuminate its functional roles. In *SlPP2C7*-knockout lines, CP-5 exhibits a 1-base-pair deletion, whereas CP-7 shows an 8-base-pair deletion (Fig. 6-A). Under normal circumstances, there are no significant differences between wild-type and *SlPP2C7*-knockout plants. However, under saline-alkali stress, the transgenic plants with *SlPP2C7* knockouts demonstrated reduced wilting and leaf dehydration compared to wild-type plants (Fig. 6-B). Further physiological measurements indicated that in the *SlPP2C7* knockout lines, the MDA content experienced respective declines of 23.84% and 26.23% (Fig. 6-C), whereas the RWC in the leaves showed respective increases of 11.7% and 14.21% (Fig. 6-D). The REC decreased by approximately 15.3% (Fig. 6-E).

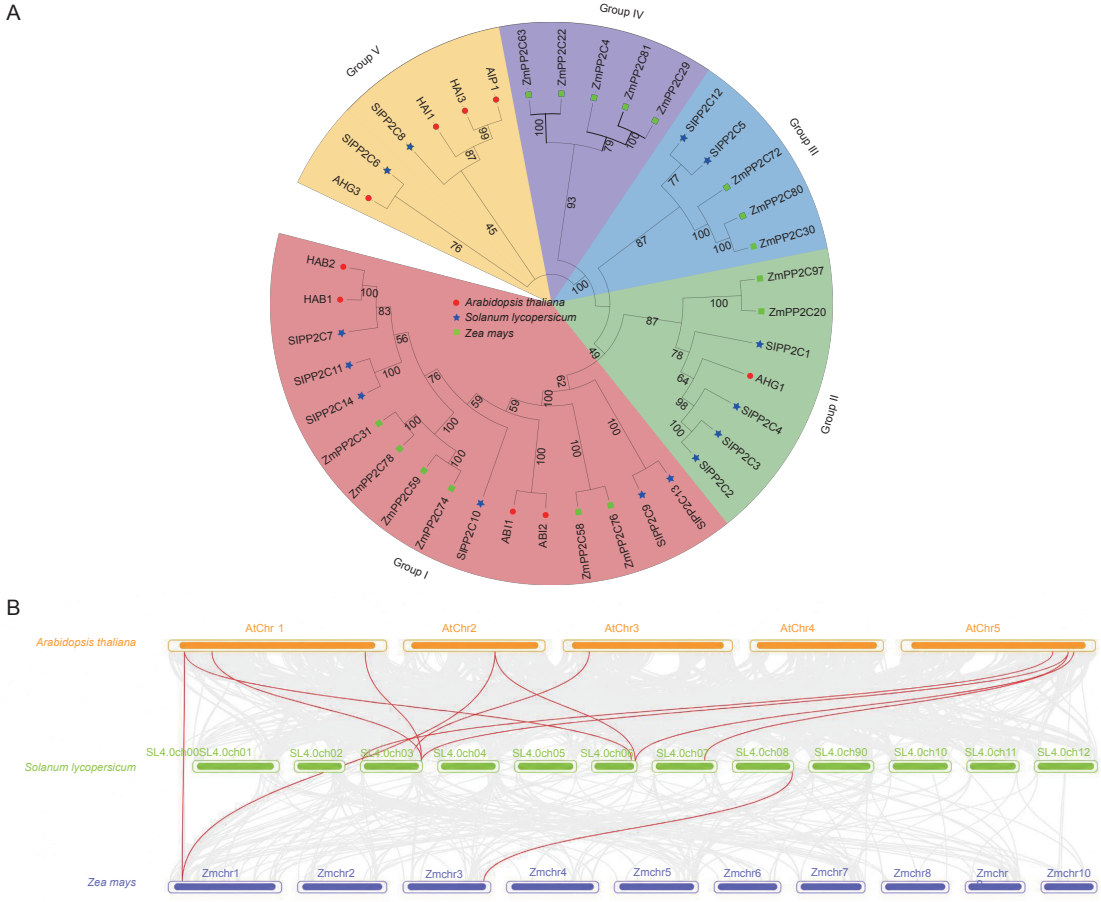


Fig. 3 Phylogenetic (A) and collinearity (B) analyses of *PP2C-A* in *Arabidopsis*, tomato, and maize. In Panel A, *SlPP2Cs* are denoted by blue stars, *AtPP2Cs* by red circles, and *ZmPP2Cs* by cyan squares. In Panel B, gray lines indicate whole-genome collinearity between species, whereas red lines show collinearity within this gene family.

3.7. Transcriptomic analysis of wild-type and *SlPP2C7*-knockout tomato plants

To illuminate the molecular mechanisms involved in *SlPP2C7*-mediated regulation of tolerance to saline-alkali stress, wild-type tomato and *SlPP2C7*-knockout plants treated with saline-alkali stress for approximately five days were utilized for transcriptomic sequencing. The samples were separated into four groups, each containing three biological replicates. We analyzed the alterations in gene expression profiles between wild-type and *SlPP2C7*-deficient plants subjected to saline-alkali stress. As depicted in Fig. 7, in total 1,571 genes exhibited significant changes, including 844 upregulated and 727 downregulated genes, after saline-alkali stress treatment of AC (with a change of two-fold or greater and $P \leq 0.05$). To explore the biological functions of DEGs, we conducted KEGG enrichment analysis. Under normal conditions, compared to wild-type AC, 307 genes exhibited significant changes in the *SlPP2C7* mutant, with 235 upregulated and 72 downregulated. Under saline-alkali stress, compared to wild-type AC, 908 genes exhibited significant changes in the *SlPP2C7* mutant, with 649 upregulated and 259

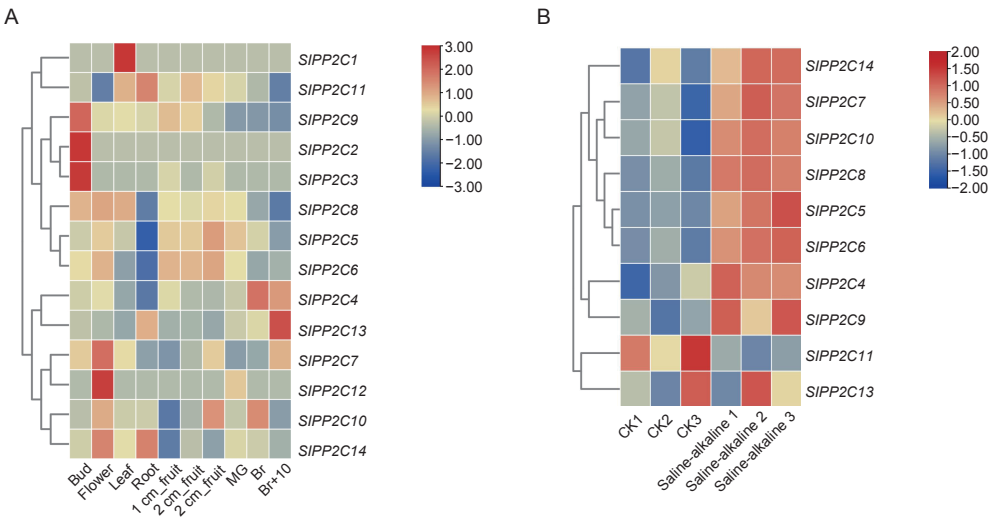


Fig. 4 Heatmap of transcription levels of tomato *SlPP2C* genes in different tissues (A) and under saline-alkali stress (B). In Panel A, RNA-seq data includes roots, buds, leaves, flowers, immature fruits, mature green fruits (MG), breaker-stage fruits (Br), and fruits 10 d post-breaker (Br+10).

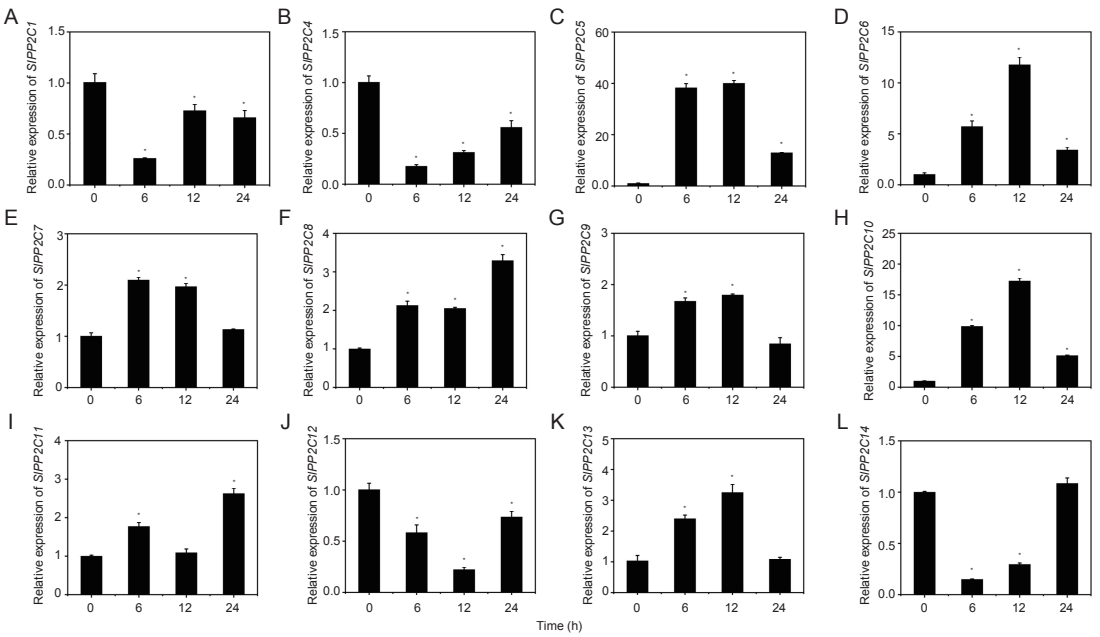


Fig. 5 Transcription levels of *SlPP2C* genes under saline-alkali stress. A, *SlPP2C1*. B, *SlPP2C4*. C, *SlPP2C5*. D, *SlPP2C6*. E, *SlPP2C7*. F, *SlPP2C8*. G, *SlPP2C9*. H, *SlPP2C10*. I, *SlPP2C11*. J, *SlPP2C12*. K, *SlPP2C13*. L, *SlPP2C14*. Asterisks mark significant differences between the treatment and control groups (*, $P<0.05$; Student's *t*-test). Data are means±SEM.

downregulated (Fig. 7-A; Appendix F). A Venn diagram revealed that under saline-alkali stress, 899 genes exhibited significant changes in both wild-type AC and *SlPP2C7*-knockout transgenic plants, with 672 unique to AC and 917 to the mutant (Fig. 7-B). KEGG analysis indicated that compared to wild-type plants, *SlPP2C7*-knockout plants under both normal and saline-alkali conditions exhibited significant enrichment in pathways such as photosynthesis, phenylalanine metabolism, flavonoid and flavonol biosynthesis, phenylpropanoid biosynthesis, and isoflavonoid biosynthesis (Fig. 7-C and D). Flavonoids, as key secondary metabolites in plants, are a significant contributor to mitigate abiotic stresses, including salinity.

3.8. Metabolomic analysis of wild-type and *SlPP2C7*-knockout tomato plants

To comprehensively analyze the metabolic alterations in AC and *SlPP2C7*-knockout plants under saline-alkali stress, we performed UPLC-MS/MS metabolomic analysis. PCA revealed significant metabolic differences between the two groups (Fig. 8-A). To further understand these changes, we extensively analyzed metabolites in samples from both groups under saline-alkali stress and identified DAMs. Compared to wild-type plants, a total of 496 DAMs were identified in *SlPP2C7*-knockout plants, comprising 327 upregulated and 169 downregulated metabolites (Fig. 8-B). Additionally, cluster heatmap analysis showed significant metabolic differences between the two groups. Biological replicates cluster together, indicating their homogeneity and the data's reliability (Fig. 8-C; Appendix G). KEGG pathway enrichment analysis was conducted to elucidate the pathways associated with these DAMs. The analysis indicated that DAMs were enriched in 83 metabolic pathways. We focused on the top 20 metabolic pathways based on P-value. Among these, nine pathways were significantly upregulated, and five were downregulated (Fig. 8-D). Notably, pathways such as biosynthesis of flavanols I and II, isoflavonoid biosynthesis, anthocyanin biosynthesis, and biosynthesis of anthocyanins II play critical roles in plant responses to abiotic stress. Flavonoids, derived from the phenylpropanoid pathway, serve as signaling molecules in pathogen defense, antioxidation, and other essential processes for plant growth and stress responses. Therefore, phenylpropanoid-flavonoid biosynthesis may represent a key metabolic pathway for tomato tolerance to saline-alkali stress. This finding aligns with the DEGs, leading us to hypothesize that SlPP2C7 may regulate tomato tolerance to saline-alkali stress through phenylpropanoid-flavonoid biosynthesis.

3.9. Integrated transcriptomic and metabolomic analysis of wild-type and *SlPP2C7*-knockout plants

In the present investigation, we performed an extensive integrated analysis of transcriptomic and metabolomic datasets to uncover the molecular pathways through which SlPP2C7 mediates saline-alkali stress tolerance in tomatoes. KEGG enrichment analysis of the metabolomic data indicated significant enrichment of "flavonoid biosynthesis" , "flavone

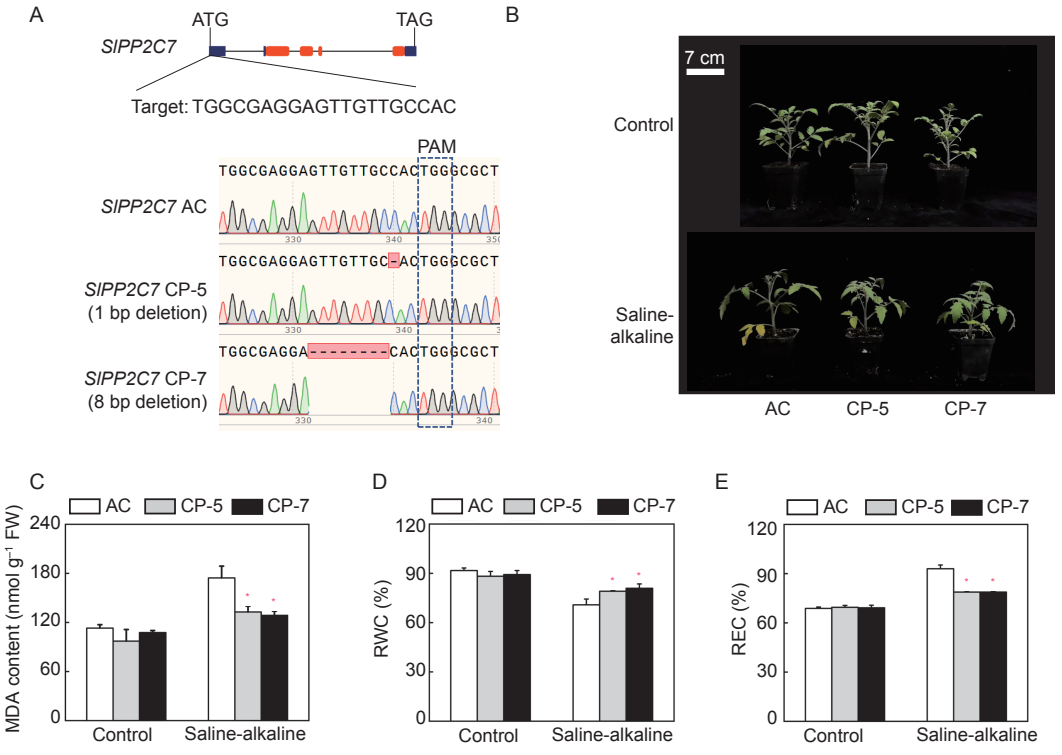


Fig. 6 Phenotype images of transgenic material constructed by knockout under saline-alkali stress. A, *SlPP2C7* knockout types. B, the phenotype of the transgenic plants under saline-alkali stress. C, malondialdehyde (MDA) content. D, relative water content (RWC). E, relative electrical conductivity (REC). Asterisks mark significant differences between the treatment and control groups (*, $P<0.05$; Student's *t*-test). Data are means±SEM.

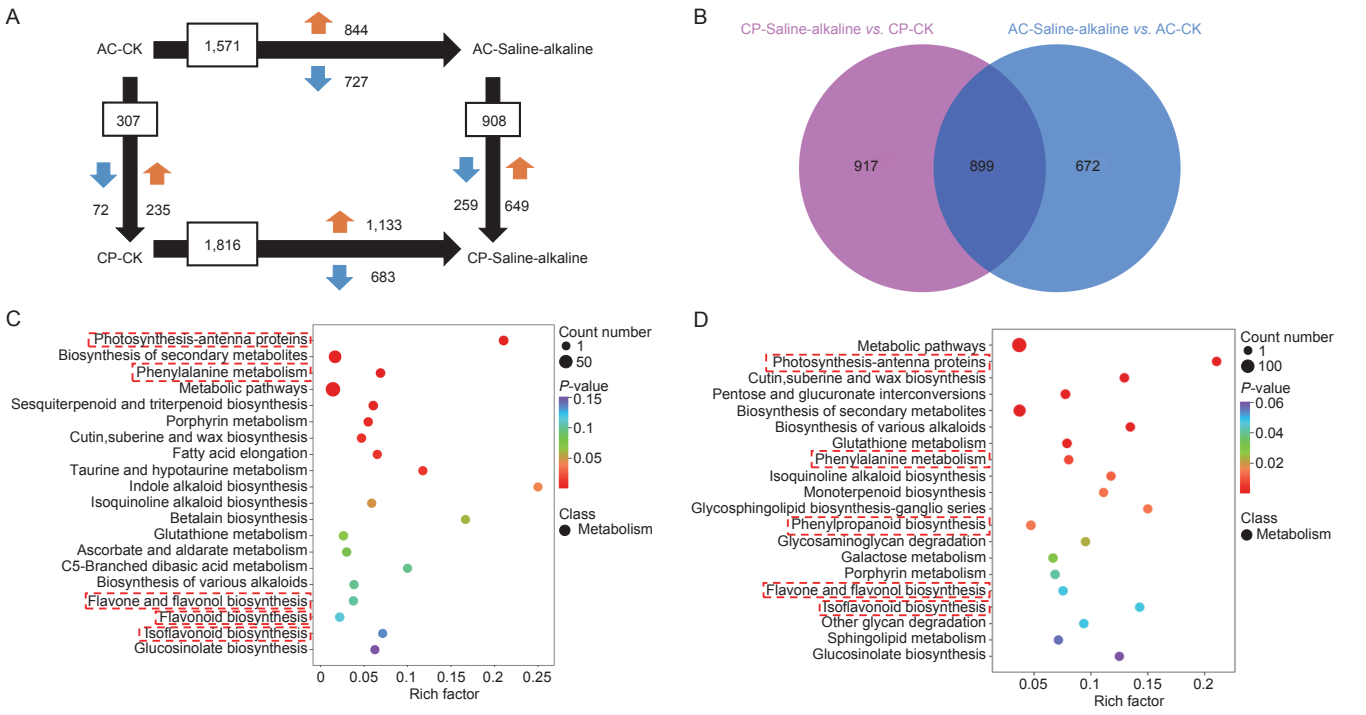


Fig. 7 Transcriptomic analysis between wild-type and *SlPP2C7*-knockout plants. A, differentially expressed genes (DEGs) in wild-type and *SlPP2C-CP-7* plants before and after saline-alkali stress. B, Venn diagram of DEGs in wild-type and *SlPP2C-CP-7* plants under saline-alkali stress. C and D, KEGG enrichment in wild-type and *SlPP2C-CP-7* plants under normal conditions (C) and saline-alkali stress (D).

and flavonol biosynthesis" , "isoflavonoid biosynthesis" , and "anthocyanin biosynthesis" pathways in both wild-type and *SlPP2C7*-knockout plants. This finding highlights the critical role of these pathways in the tomato's reaction to saline-alkali stress. Heatmaps of genes and metabolites in these pathways were also created (Fig. 9). The genes related to the flavonoids biosynthetic pathway included *PALs*, *C4Hs*, *4CLs*, *CHS*, *CHI*, *F3H*, *FLS*, *F3′H*, *F3′5′H*, *DFR*, and *ANS*, all of which were upregulated in the *SlPP2C7*-knockout plants (Fig. 7; Appendix H). The identified metabolites included flavanones, isoflavonoids, flavones, flavonols, flavanols, and anthocyanins (Figs. 8 and 9). The integrated analysis of transcriptomic and metabolomic profiles indicates that SlPP2C7 modulates saline-alkali stress tolerance in tomatoes *via* the flavonoid biosynthetic pathways.

4. Discussion

4.1. Genome-wide identification and analysis of the *SlPP2C-A* gene family in tomato

Researchers have identified PP2C protein genes in a variety of plant species, such as *A. thaliana*, *O. sativa*, *S. spontaneum*, *Z. mays* and *A. hypogaea*. (Smith and Walker 1996; Singh *et al.* 2010; Huang *et al.* 2023; Wu Z *et al.* 2023; Pang *et al.* 2024). In *A. thaliana*, 80 members of the PP2C genes were classified into 12 subfamilies (A to L) (Fuchs *et al.* 2013). Among these PP2Cs, subfamily A members such as ABI1, ABI2, AHG1, HAB1, and HAB2 exert a negative regulatory effect on ABA signaling. They achieve this by interacting with ABA receptor proteins PYR/PYL/RCAR (Wang *et al.* 2015). Many research studies have shown that group A of the PP2C family has an important regulatory function in how plants respond to stress, primarily through the dephosphorylation of substrate proteins, which is particularly evident in ABA-dependent responses. For example, the regulatory role of clade A PP2C is crucial during plant responses to abiotic stress (Ma 2009; Krzywińska *et al.* 2016). In this research, we carried out an extensive analysis of the tomato *SlPP2C-A* gene family. This analysis included genome-wide identification, phylogenetic analysis, gene structure, conserved motifs, chromosomal location, collinearity, *cis*-elements, and expression patterns. Using homology-based methods, we identified 14 *SlPP2C* genes (Fig. 1) and predicted their physicochemical properties and subcellular localization, with most found in the nucleus, chloroplasts, and cytoplasm (Table 1; Appendix D). We speculate that these genes may be closely associated with critical physiological processes such as photosynthesis and cell growth. Furthermore, phylogenetic and collinearity analyses revealed nine pairs of collinear genes between tomato and the *Arabidopsis* type A *PP2C* families, indicating high homology (Fig. 3). Additionally, the variation in exon/intron structure, position, and conserved motifs significantly influences gene family function (Zhang *et al.* 2022). In *Arabidopsis* and maize, salt-tolerant *PP2C* genes contain numerous salt-tolerance-related *cis*-elements (Bhaskara *et al.* 2019; He *et al.* 2019). A comprehensive investigation of the *SlPP2C-A* gene structure, motifs, and *cis*-elements indicates that *SlPP2C7* resembles *PP2C* genes in *Arabidopsis* and maize (Fig. 2; Appendices A, B and E). RT-qPCR results demonstrate that most *SlPP2C* genes are significantly upregulated under

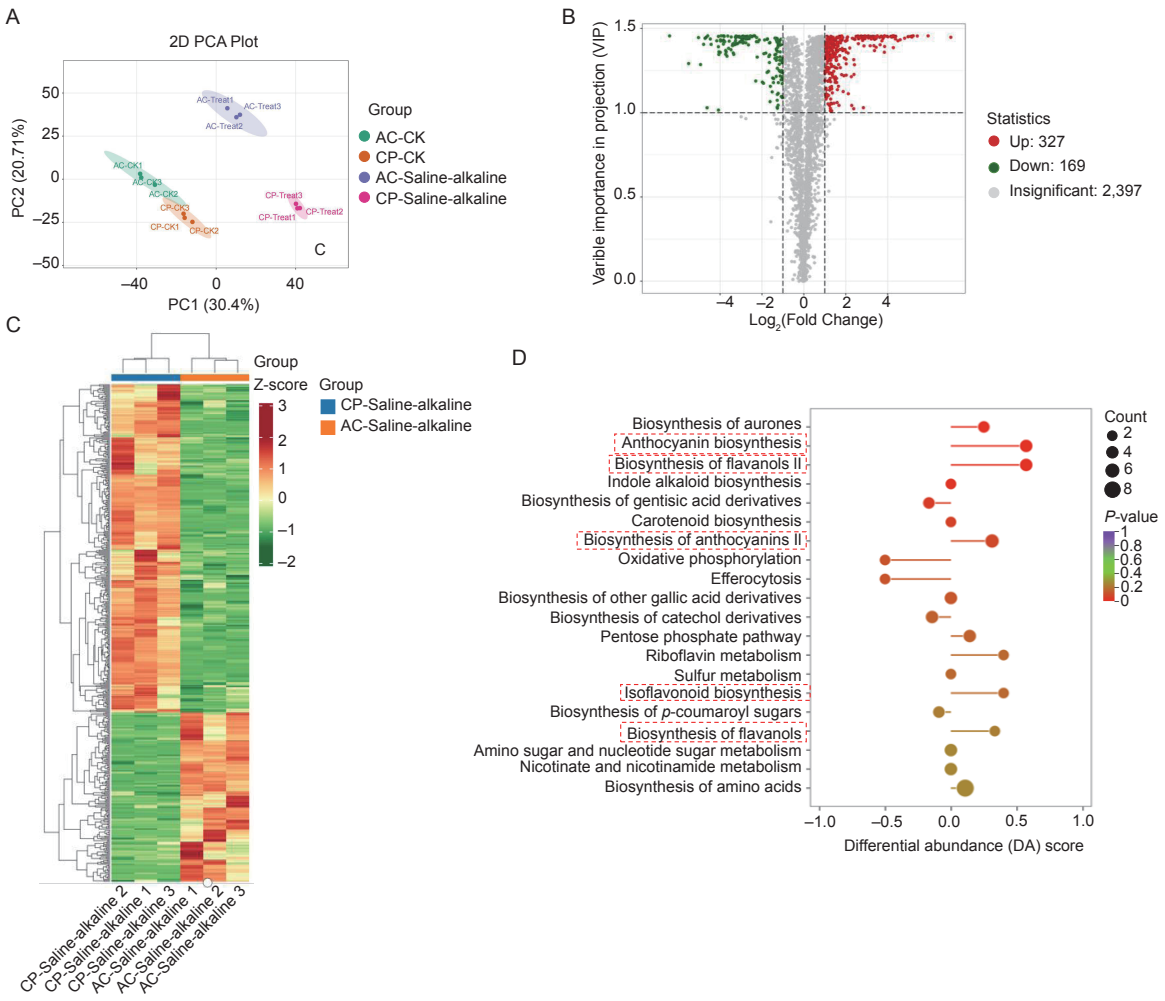


Fig. 8 Metabolomic analysis between wild-type and *SlPP2C7*-knockout plants under saline-alkali stress. A, PCA of metabolomes. B, volcano plot of differentially accumulated metabolites (DAMs). C, cluster analysis of DAMs. D, KEGG analysis of top 20 pathways.

saline-alkali stress (Fig. 5). Studies have indicated that high pH during saline-alkali stress can impair root vitality and photosynthetic ability in plants (Mukhopadhyay *et al.* 2021). Notably, *SlPP2C7* is localized in chloroplasts. Subsequent phenotypic analyses of saline-alkali stress revealed that *SlPP2C7*-knockout plants exhibited reduced leaf yellowing and enhanced saline-alkali stress tolerance compared to wild-type plants (Fig. 6-B). In transcriptomic KEGG enrichment analysis under saline-alkali conditions, transcriptional of photosystem antenna proteins related genes in *SlPP2C7*-knockout plants were remarkably higher than in wild-type plants (Appendix I). It has been reported that light quality, particularly blue light, markedly upregulates anthocyanin biosynthesis related genes, strongly promoting anthocyanin accumulation (Zeng *et al.* 2023). Similarly, ABA plays a role in light-mediated chloroplast movement, influencing plant photosynthesis (Rojas-Pierce *et al.* 2014). This observation aligns with our integrated transcriptomic analysis (Fig. 9). Thus, SlPP2C7 regulates tomato saline-alkali stress tolerance through mechanisms involving photosynthesis and anthocyanin synthesis.

4.2. SlPP2C7 confers saline-alkali stress tolerance in tomatoes by regulating flavonoid biosynthesis

Soil salinization, a global challenge, poses a significant threat to agricultural production (Mukhopadhyay *et al.* 2021). It not only directly impedes plant growth, development, and productivity but also disrupts internal physiological, biochemical, and molecular processes, hindering normal plant life activities (Raza *et al.* 2023). Numerous studies indicate that PP2C modulates plant physiological responses to abiotic stress by negatively regulating the ABA signaling pathway (Meyer *et al.* 1994). For instance, in *A. thaliana*, PP2C proteins (e.g., ABI1) inhibit SnRK2 activity, thereby negatively regulating ABA signaling (Merlot *et al.* 2001). Our findings demonstrate that the majority of *SlPP2C* genes are significantly induced by saline-alkali stress. *SlPP2C7*, an A1 subfamily gene, is homologous to *HAB1* and *HAB2* in *A. thaliana*. *SlPP2C7* is predominantly expressed in floral and leaf tissues, and its expression can be significantly upregulated upon exposure to saline-alkali stress within 6 h of treatment (Fig. 5). We developed transgenic tomato plants with *SlPP2C7* knockouts. Under saline-alkali stress, these knockout plants exhibited enhanced tolerance (Fig. 6-B). In

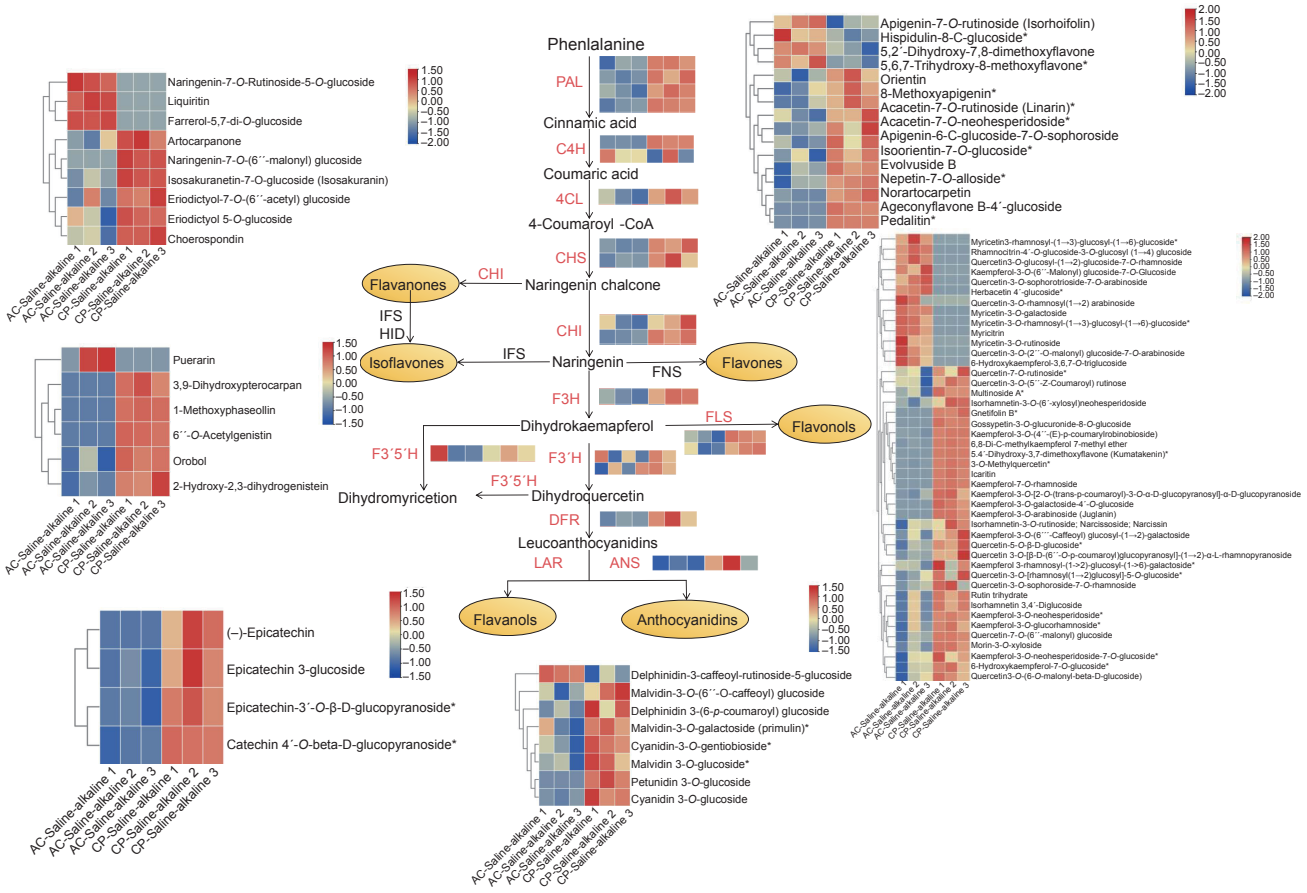


Fig. 9 Integrated transcriptomic and metabolomic analysis of wild-type and *SlPP2C-CP-7* plants under saline-alkali stress.

comparison to wild-type plants, the MDA content and REC in knockout lines CP-5 and CP-7 decreased, whereas relative water content in CP-5 and CP-7 leaves increased (Fig. 6-C–E). These findings indicate that *SlPP2C7* knockouts enhance tomato saline-alkali stress tolerance.

Flavonoids are regulated by plant development and environmental factors such as drought, UV light, and salinity (Wu J *et al.* 2023). As antioxidants, flavonoids scavenge ROS and mitigate cellular damage (Speisky *et al.* 2022). Flavonoids include flavones, flavonols, flavanones, isoflavonoids, flavanols, anthocyanins, and proanthocyanidins. Under salt stress, overexpression of *GSA1* in rice increases flavonoid glycoside accumulation, enhancing salt tolerance (Dong *et al.* 2020). Rice SQD2.1 catalyzes flavonoid glycosylation; its mutant exhibits reduced tolerance to abiotic stresses such as salt and drought, whereas overexpression increases tolerance (Zhan *et al.* 2019). In wild soybean, HSFB2b promotes salt tolerance by enhancing flavonoid accumulation (Bian *et al.* 2020). Metabolomic analysis of *Camellia japonica* reveals DEGs and DAMs enriched in phenylpropanoid, flavonoid, and flavonol biosynthesis pathways, which improve abiotic stress resistance (Wu *et al.* 2021). In alfalfa, integrative transcriptomic and metabolomic analysis under salt stress indicates that flavonoids positively regulate root salt tolerance (Wang *et al.* 2023). ABA, a stress-associated phytohormone, serves as a key factor in modulating how plants react to multiple abiotic stress conditions such as drought, high salinity, and low temperature (Deng *et al.* 2025). Under saline-alkali stress, ABA upregulates *MsFLS13* in alfalfa, promoting flavonol accumulation, reducing ROS, and enhancing photosynthetic efficiency to stabilize the antioxidant system and photosynthesis (Zhang *et al.* 2023). The ABA-signaling transcription factor SlAREB promotes flavonoid accumulation by regulating anthocyanin-related genes *SlDFR* and *Sl3′5′H* (Xu *et al.* 2022). *SlPP2C7*, a type A *PP2C* gene, negatively regulates the ABA signaling pathway. The regulatory mechanism of SlPP2C7 in tomato plants' saline-alkali stress tolerance is still unclear. To explore this mechanism, we conducted transcriptomic and metabolomic analyses on wild-type and *SlPP2C7*-knockout plants. Our transcriptomic results indicated significant enrichment in pathways such as photosynthesis, phenylalanine metabolism, phenylpropanoid biosynthesis, flavonoid and flavonol biosynthesis, and isoflavonoid biosynthesis under both normal and salt-stress conditions (Fig. 7). Additionally, we used RT-qPCR to test the expression of genes in the flavonoid biosynthesis pathway and found that they were significantly up-regulated in *SlPP2C7*-knockout plants under saline-alkali stress (Appendix H). We also performed extensive UPLC-MS/MS metabolomic analyses on samples from both wild-type and *SlPP2C7*-knockout plants to identify DAMs . We analyzed the top 20 metabolic pathways based on *P*-value and found that 9 were significantly upregulated

and 5 downregulated. In the *SlPP2C7*-knockout lines, pathways such as biosynthesis of flavanols I, II, isoflavonoid biosynthesis, anthocyanin biosynthesis, and biosynthesis of anthocyanins II were significantly upregulated (Fig. 8). The integrated analysis of transcriptomic and metabolomic profiles indicates that SlPP2C7 modulates saline-alkali stress tolerance in tomatoes *via* the flavonoid biosynthetic pathways (Fig. 9). We speculate that the *SlPP2C7*-knockout lines may enhance tomato saline-alkali stress tolerance by promoting flavonoid accumulation.

5. Conclusion

In summary, we identified 14 members of the type A *SlPP2C* gene family in tomato. Based on their homology, these members are categorized into five subgroups. Analysis of gene structure, motifs, and *cis*-elements suggests that *SlPP2Cs* may participate in plant growth, development, abiotic stress responses, hormone signaling, and light-responsive pathways. Saline-alkali stress markedly upregulates *SlPP2C7* transcription levels. To elucidate its function in tomato saline-alkali stress tolerance, we generated *SlPP2C7* knockout lines. Results indicate that *SlPP2C7* knockout plants exhibit a significant enhancement in saline-alkali stress tolerance. Transcriptomic and metabolomic analyses reveal that, under saline-alkali stress, genes and metabolites associated with phenylpropanoid and flavonoid biosynthesis pathways are notably enriched in *SlPP2C7* knockout plants compared to wild-type plants. The increase in flavonoids likely accounts for the enhanced saline-alkali stress tolerance observed in *SlPP2C7* knockout plants (Fig. 10). The findings presented here supply a robust theoretical underpinning for future investigations into the functions of type A PP2C family members in tomatoes.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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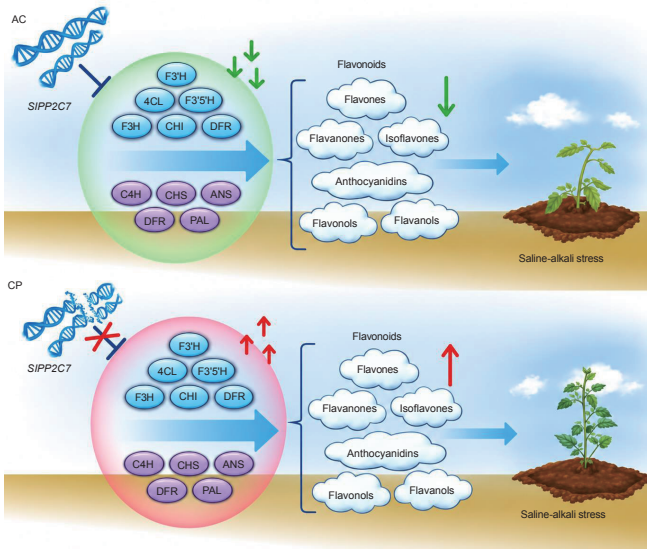


Fig. 10 The proposed module of SlPP2C7 in regulating saline-alkali stress tolerance. Under normal conditions, *SlPP2C7* inhibits flavonoid synthesis, negatively regulating tomato saline-alkali stress tolerance. When *SlPP2C7* gene is knocked out, the inhibition of flavonoid metabolic pathways is weakened. The upregulation of flavonoids biosynthesis genes and the accumulation of flavonoids enhance the saline-alkali stress resistance of tomato plants.

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