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Characterization of ATP-dependent phosphofructokinase genes during ripening and their modulation by phytohormones during postharvest storage of citrus fruits (*Citrus reticulata* Blanco.)

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ABSTRACT

The level of sweetness in citrus fruit is crucial for consumer appeal and market competitiveness, determined mainly by soluble sugars and organic acids. ATP-dependent 6-phosphofructokinase is central to regulating sugar metabolism, yet its role in citrus fruit ripening and postharvest storage remains underexplored. We characterized phosphofructokinase genes in citrus, identifying eight genes classified into pyrophosphate-dependent phosphofructokinase (PFP) and ATP-dependent 6-phosphofructokinase (PFK) subgroups using phylogenetic analysis, genomic architectures, and protein motifs. Comparative genomic analysis with other plants highlighted significant protein homology among CitPFKs. The motif analysis indicated conserved phosphofructokinase domains in CitPFK sequences, with upstream promoter regions containing diverse cis-regulatory elements, most notably light-responsive (LREs). The gene expression profiling throughout fruit development and ripening revealed differential patterns, with responses to gibberellic acid and salicylic acid phytohormones during postharvest indicating their roles in regulating CitPFK genes. The analysis of the transcriptome showed high expression of ATP-dependent 6-phosphofructokinase 3 (*CitPFK3*) during fruit development, indicating a positive role in fruit maturation. Consequently, silencing *CitPFK3* through virus-induced gene silencing (VIGS) increased hexose sugar content, suggesting its function in sugar accumulation. These findings improve our understanding of PFKs in citrus, particularly *CitPFK3*'s pivotal role in regulating hexose sugar dynamics and their modulation by exogenous phytohormones after harvest. This study provides a foundation for optimizing soluble sugar regulation to enhance fruit quality and postharvest handling in citrus production.

1. Introduction

Mandarin (*Citrus reticulata* Blanco.) is the second leading category of citrus fruits globally, after sweet oranges. It is renowned for its nutritional benefits, ease of peeling, and pleasant flavor (Goldenberg et al., 2018). The crop is extensively grown in tropical and subtropical regions across numerous countries and is of significant economic and cultural importance (Tietel et al., 2011). Over the past five decades, one of the foremost breeding objectives has been the selection of cultivars that mature both early and late to extend harvest seasons and satisfy consumer demands. Despite efforts to improve fruit quality, challenges remain with early-ripening citrus varieties and their sweetness. These varieties often exhibit a lower sugar-to-acid ratio, significantly impacting consumer satisfaction (Chen et al., 2023). In response, research efforts have surged, particularly in countries such as China, Italy, Brazil, and Spain, focused on developing new mandarin varieties with improved quality traits, with fruit taste emerging as a primary focus (Raveh et al., 2020).

Fruit ripening, the third phase of fruit development, involves several biochemical alterations, such as fruit softness, carotenoid buildup,

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organic acid degradation, and sugar accumulation (Zhang et al., 2014). Central to these processes are sugars and organic acids, which serve as energy sources and essential components for the biosynthesis of amino acids, vitamins, and volatile compounds crucial for fruit aroma and flavor (Plaxton, 1996). In citrus fruits, sucrose and hexose sugars (fructose and glucose) gradually accumulate during development and ripening, contributing to the sweet taste and nutritional profile (Komatsu et al., 2002). However, sugar content typically stabilizes or declines during postharvest storage, influenced by various physiological and environmental factors (Yao et al., 2018). Despite these changes, sugars are crucial in determining fruit quality and shelf-life after harvest across various horticultural products (Zhao et al., 2023).

Soluble sugars determine the fruit's taste and organoleptic quality at harvest, making it a pivotal breeding goal and a significant factor influencing customer preference for a variety of fruit (Wang et al., 2023). Fructose, the sweetest hexose sugar, is mainly predominant in acidless and low-acid citrus cultivars, where it stands out as the only sugar showing significant differences in comparative studies with acidic varieties. Despite numerous molecular research on the fruit growth and maturation processes in citrus (Chen et al., 2012; Feng et al., 2021; Komatsu et al., 2002; Liu et al., 2009; Qiao et al., 2017), the molecular mechanisms related to fruit sweetness in citrus is not clear. Understanding the dynamics of sugar metabolism during fruit development and after harvest is crucial for optimizing fruit quality and enhancing consumer satisfaction. Improving fruit quality may depend primarily on manipulating the pathways involved in carbohydrate metabolism, such as glycolysis, which influences hexose levels.

In plants, the regulation of the glycolytic/gluconeogenetic metabolic flow mainly occurs through the biological process by which fructose-6-phosphate (Fru-6-P) is phosphorylated to form fructose-1,6-bisphosphate (Fru-1,6-P2). Two enzymes facilitate the reaction: ATP-dependent 6-phosphofructokinase (PFK, EC:2.7.1.11) and pyrophosphate-dependent phosphofructokinase (PFP, EC:2.7.1.90). PFP catalyzes a reversible reaction with pyrophosphate (PPi), whereas PFK irreversibly catalyzes the reaction using ATP as the phosphoryl donor (Mustroph et al., 2013). Despite their functional overlap, PFK typically exhibits equal or lower activity than PFP (Huber, 1986; Mustroph et al., 2013). PFK has garnered attention for its involvement in sugar accumulation and fruit ripening processes (Katz et al., 2011). Research has demonstrated that PFK is strongly expressed during the progression from the second stage (cell expansion) to the third stage (maturation) in various fruits, including citrus, pears, peaches, and blueberries, correlating with increased hexose accumulation as fruits mature (Jiang et al., 2023; Katz et al., 2011).

ATP-dependent 6-phosphofructokinase has historically received less attention, primarily attributed to challenges in its purification process (Turner and Plaxton, 2003). Recent decades have witnessed significant advancements in sequencing technologies, enabling the comprehensive identification of the phosphofructokinase gene family across diverse plant species. They include notable studies in Arabidopsis (Mustroph et al., 2007), bananas (Turner and Plaxton, 2003), spinach (Kelly and Latzko, 1977), cassava (Wang et al., 2021), sugarcane (Zhu L. et al., 2013), Cotton (Mehari et al., 2022), Chinese white pear (Lü et al., 2019), Northern red oak (Kim et al., 2023) and rice (Mustroph et al., 2013). Functional studies involving PFK in plants have primarily focused on model organisms like Arabidopsis, where knockout studies have provided insights into their physiological roles (Hess et al., 2021; Perby et al., 2022). However, functional investigations of PFK in citrus remain unexplored.

In this work, we characterized citrus's phosphofructokinase gene family during fruit ripening and postharvest storage. We used the *Citrus clementina* v1.0 genome to identify and analyze 8 PFK genes. We investigated phylogenetic relationships, physiochemical characterization, chromosome localization, identification of conserved protein motifs, analysis of gene structures, and examination of cis-regulatory elements in promoters. We evaluated their expression patterns in citrus throughout fruit ripening and in response to gibberellic acid and salicylic acid phytohormones during postharvest storage. Furthermore, virus-induced gene silencing (VIGS) was employed to characterize ATP-dependent 6-phosphofructokinase 3 (*CitPFK3)*, elucidating its functional role in hexose sugar metabolism during fruit maturation. Our findings highlight the regulatory mechanisms of phytohormones on CitPFK genes and the specific contributions of *CitPFK3* to fruit sugar metabolism.

2. Materials and methods

2.1. Plant materials

'Dafen 4' (*Citrus reticulata* cv. Dafen 4) and 'Shatangju' mandarin (*Citrus reticulata* Blanco cv. Shatangju) fruit samples were obtained from Yuanjiang, Hunan Province, China. Dafen 4 fruit sampling duration was from August to October 2023. The 'Dafen 4' fruit sampling was done at three ripening points, 122, 136, and 150 days after flowering (DAF); each sample had three biological replicates. The fruit's flesh was frozen in liquid nitrogen and stored at −80^{o} C for further analysis. 'Shatangju' fruits were harvested at the mature stage based on the TSS (exceeding 10°Brix) and uniformity in their appearance. This cultivar was used for postharvest experiments.

2.2. Identification and phylogenetic tree analysis of CitPFK

For data retrieval, the *Citrus clementina* v1.0 protein data and *Citrus sinensis* v1.0 protein data were obtained from the Citrus Pangenome to Breeding Database website (http://citrus.hzau.edu.cn/). We retrieved Arabidopsis protein data from the TAIR website (http://www.arabidopsis.org), while rice and cassava sequences were downloaded from the annotation database Phytozome v13 (https://phytozome-next.jgi.doe.gov/info/). Using HMMER (version 3.2.1, HHMI, Chevy Chase, MD, USA) with an E-value set at $1e^{-5}$, the phosphofructokinase domain (PF00365) from the Pfam database (http://pfam.xfam.org/) was applied as a query to search against the local *Citrus clementina* v1.0 protein database and the other four species protein databases. The corresponding sequences were then verified using BLASTP from two more citrus genome databases: the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) and Phytozome v13 (http://phytozome.jgi.doe.gov/). Members of the CitPFK family whose amino acid sequences could be inferred were compared to those of other plant species whose PFKs were previously identified. The MUSCLE program in MEGA11 software was used to conduct multiple alignments of PFK proteins to create the evolutionary tree using the Neighbor-Joining (N-J) approach and test with 1000 bootstrap repeats.

2.3. Analysis of chromosome location and physiochemical characteristics CitPFKs

To determine the chromosomal positions of CitPFK genes, the GFF3 file of *Citrus clementina* was obtained from the Citrus Pangenome to Breeding Database website (http://citrus.hzau.edu.cn/). Additionally, chromosomal mapping was created using this data and displayed using TBtools (Chen et al., 2020). The CitPFK genes' physical properties, including their IDs, protein lengths, gene lengths, molecular weights (MWs), isoelectric points, and grand average of hydropathicity (GRAVY) values, were calculated by Expasy ProtParam (https://web.expasy.org/protparam/). The subcellular localization of the PFK genes was determined using the WoLF PSORT online program (https://wolfpsort.hgc.jp).

2.4. Gene structure, conserved protein motifs, and promoter analysis of CitPFKs

The MEME online tool (http://meme-suite.org) was used to examine conserved protein motifs using the following settings: anticipate that

motif sites will be spaced out in sequences: maximum motifs = 10; minimum motif widths = 6; maximum motif widths = 50; and maximum sites = 300 (Bailey et al., 2006). Annotation of the individual motifs was confirmed using SMART (https://smart.embl.de/) and InterPro (https://www.ebi.ac.uk/interpro/) tools. For exon-intron gene structure analysis, the GFF3 annotation file of *Citrus clementina* was obtained from the Citrus Pangenome to Breeding Database website (http://citrus.hzau.edu.cn/). TBtools was used to visualize *CitPFK* gene structures and the protein motifs (Chen et al., 2020).

Potential cis-regulatory motifs were investigated in the CitPFK genes' promoter regions up to 2000-bp in the 5′upstream region using the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) online tool. The data were acquired from the Phytozome database (https://phytozome-next.jgi.doe.gov/info/) (Lescot, 2002). The cis-regulatory components were visualized using the TBtools program (Chen et al., 2020).

2.5. CitPFK gene expression analysis during fruit development and ripening in citrus

Transcriptome data of fruit endocarp of the 'Dafen 4' cultivar at different development stages (66, 80, 94, 108, 122, and 136DAF) from our previous study (Chen et al., 2023) was used. Expression data of PFK genes were retrieved from this transcriptome data and processed as log_2Foldchange values of pairwise comparisons. Expression heatmap was drawn using TBtools (Chen et al., 2020). RT-qPCR was conducted at three ripening stages (122, 136, and 150 DAF), focusing on PFK genes to validate the transcriptome data. Table S1 lists the precise primer sequences used in constructing PCR primers using the Primer3 Plus tool (http://www.primer3plus.com).

2.6. Expression analysis of CitPFK genes in response to exogenous gibberellic acid and salicylic acid phytohormones during postharvest storage

Mature 'Shatangju' mandarin (*Citrus reticulata* Blanco cv. Shatangju) fruits were collected at the mature ripe stage, immersed in distilled water (control), 200 μmol/L Melatonin (MT) (Solarbio, China), and 0.05 g/L gibberellic acid (GA) (Macklin Biochemical Co., LTD., Shanghai, China) and air-dried at room temperature, then stored at room temperature 25 °C and 85–90% relative humidity. The fruit samples were collected from three biological replicates at 0, 8, and 20 days under storage. After being collected, samples were immersed in liquid nitrogen and stored at −80 °C for RNA extraction and RT-qPCR analysis.

2.7. VIGS vector construction and agrobacterium infiltration

According to the manufacturer's protocol, genomic DNA was extracted from citrus fruit endocarp using the SteadyPure Genomic DNA Extraction Universal Plant Kit (Accurate Biotechnology, China). The *CitPFK3* genomic sequence was obtained from the NCBI genome database. The efficient DNA sequences were predicted for silencing, and PCR amplified the 5′ UTR of the *CitPFK3* sequence. The specific PCR primers (Supplementary Table S1) were designed using Primer Premier 5.0 (Lalitha, 2000). The PCR products were first cloned into p-TOPO vectors (Aidlab, China). Subsequently, the PCR amplicons were double digested with *Xba*I and *Sma*I enzymes and ligated with T4 DNA ligase into the tobacco rattle virus (pTRV2) vector. The pTRV2 plasmid was created using the freeze-thaw method and prepared for silencing experiments.

Agrobacterium tumefaciens (strain GV3101) cells were transformed with pTRV1 and pTRV2, or pTRV2-*CitPFK3*, using methods described previously (Li et al., 2016). The cell cultures were cultivated in liquid LB medium supplemented with 50 mg/L kanamycin and 25 mg/L rifampicin for a whole night at 28 °C or until they reached an OD_{600} of 0.6–1.0. The *A. tumefaciens* cells were subsequently collected by centrifugation (4000 g, 10 min) and suspended in an inoculation buffer (10 mM $MgCl_2$, 10 mM MES (pH 5.7), and 200 μM acetosyringone) to attain an OD_{600} of 1.0. After a 3-h incubation period at room temperature, a 1:1 ratio was established between the *A. tumefaciens* culture harboring pTRV1 and the culture containing either *pTRV2* or pTRV2-*CitPFK3*. This bacterial mixture was then injected into 'Dafen 4' fruits using a 2-mL syringe. Fruits were injected with pTRV1, and pTRV2 alone was used as a control. Injected fruits were subsequently bagged and left on the tree for eight days before sampling. Five biological replicates were used in the experiment. To confirm the silencing of the PFK gene in citrus fruits injected with the TRV2-VIGS vector, we initially screened for the presence of TRV2-derived transcripts in all infiltrated fruits. We then assessed the transcript levels of *CitPFK3* in fruits displaying clear silenced phenotypes and in control fruits. For each sample, three biological replicates were analyzed using RT-qPCR. Gene-specific primers are listed in Supplementary Table S1.

2.8. Quantification of soluble sugars by HPLC

Fructose, glucose, and sucrose were quantified using high-performance liquid chromatography (HPLC) with slight modifications to a previously described method (Wang et al., 2023). Citrus flesh samples (3 g) were pulverized into a powder using liquid nitrogen and put into a 10 mL plastic centrifuge tube. After adding 5 mL of 80% (v/v) ethanol, the homogenate was incubated for 20 min at 35 °C in a water bath. Following a 10-min centrifugation at 10,000 rpm, the supernatant was transferred into a 25 mL volumetric flask. After three extraction cycles, the supernatants were pooled and diluted to 25 mL (v/v) of ethanol to ensure homogeneity. Before being injected into the HPLC system, 1 mL of the combined supernatant was first dissolved in 1 mL of deionized water, evaporated at 60 °C, and filtered through a 0.45-μm membrane filter.

The HPLC analysis was carried out using a Shimadzu Corporation system (Tokyo, Japan) equipped with a 4.6 × 250 mm InertSustain NH_2 5 μm column (GL Sciences, China) and a refractive index detector (RID-20A, Shimadzu Corporation). The column and reference cell temperatures were kept at 85 °C and 35 °C, respectively. Acetonitrile and water (80:20) were used as the mobile phase, and the flow rate was kept constant at 1 mL/min. Sugars were identified and quantified by comparing the retention times and integrated peak areas with those of external standards for sugars (Solarbio, China).

2.9. Enzyme activity assay

ATP-PFK (ATP-phosphofructokinase) and PFP (phosphofructokinase) activities were determined following the manufacturer's guidelines using commercial assay kits (Pointe Bioengineering Co., Ltd., Hubei, China). A UV–visible spectrophotometer (UV-2700, Shimadzu Corporation, Tokyo, Japan) with absorbance at 340 nm was used to evaluate the enzymes' activity. The essays were conducted in triplicate for each treatment.

2.10. RNA extraction, cDNA synthesis and RT-qPCR

According to the manufacturer's instructions, total RNA was extracted from the flesh of citrus fruits individually using the FastPure Universal Plant Total RNA Isolation Kit (Nanjing Vazyme Biotech Co., Ltd, China). High-quality DNA-free RNA was reverse-transcribed using the TrueScript RT Master Mix with gDNAwiper (Aidlab Biotechnologies, China). The generated cDNA samples were used for the RT-qPCR investigation. Gene expression analysis was performed on the CFX96 real-time system (Bio-Rad, USA) using the SYBR Green Premix Pro Taq HS qPCR kit from Accurate Biotechnology Co. Ltd. (Hunan, China). The thermal cycling and the PCR conditions were based on the manufacturer's instructions. The Ct value corresponding to the *Citrus sinensis* actin gene (XM_006464503) was used as an internal reference for normalization (Li et al., 2017). All experiments were conducted in three

replicates. The relative transcript levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All gene-specific primers used for RT-qPCR are listed in Table S1.

2.11. Statistical analysis

Statistical analysis was done using GraphPad Prism version 10.1.2 software. Student's t-test and one-way ANOVA were used to analyze the data, with a significance threshold of $P < 0.05$. Each measurement's data is the mean ± standard error (SE) based on at least three biological replicates.

3. Results

3.1. Identification and phylogenetic analysis of phosphofructokinase proteins in citrus

Eight genes were identified from the *Citrus clementina* genome, comprising five PFK genes and three PFP genes. The identified CitPFK genes exhibited varying mRNA lengths, ranging from 2075 bp to 2344 bp. These genes encoded proteins with varying lengths (490–615) of amino acids at the protein level. They had molecular weights (MWs) between 54,188 and 68,497 Da. The CitPFK proteins were predicted to be hydrophilic based on the grand average of hydropathicity (GRAVY) values (Table 1).

A Neighbor-Joining phylogenetic tree was constructed per multiple sequence alignments of CitPFK amino acid sequences alongside *Citrus sinensis, Arabidopsis thaliana, Oryza sativa, and Manihot esculenta.* The eight CitPFK proteins are classified into two main subfamilies, PFK and PFP, according to the results of the phylogenetic study (Fig. 1). The PFK subfamily proteins were further divided into three subgroups, PFK-A, PFK-B, and PFK-C. In contrast, the PFP subfamily consisted of two subgroups: PFP-α and PFP-β.

3.2. Subcellular localization and chromosomal mapping of CitPFK genes

The subcellular localization of CitPFK genes was predicted by the Wolf PSORT tool. The findings revealed distinct distributions among cellular compartments. Specifically, four CitPFK genes (*CitPFK3*, *CitPFK5*, *CitPFK6,* and *CitPFPB1*) were identified in the chloroplast, while three genes (*CitPFK2, CitPFPA1* and *CitPFPB2*) were localized in the cytoplasm. One gene (*CitPFK4*) was localized in the mitochondria (Table 1).

The genomic distribution analysis further characterized the spatial arrangement of CitPFK genes across the *C. clementina* genome. The distribution of these genes was uneven over five chromosomes (Supplementary Fig. S1). Scaffold_2 harbored three genes, namely *CitPFK2*, *CitPFKB1*, and *CitPFKB2*. In contrast, scaffold_7 contained two genes, *CitPFK5* and CitPFK6. The remaining three genes, *CitPFK3, CitPFKA*, and *CitPFK4*, were mapped to chromosomes on scaffold_1, scaffold_5, and scaffold_9, respectively. The results highlight a dispersed genomic distribution across different chromosomes.

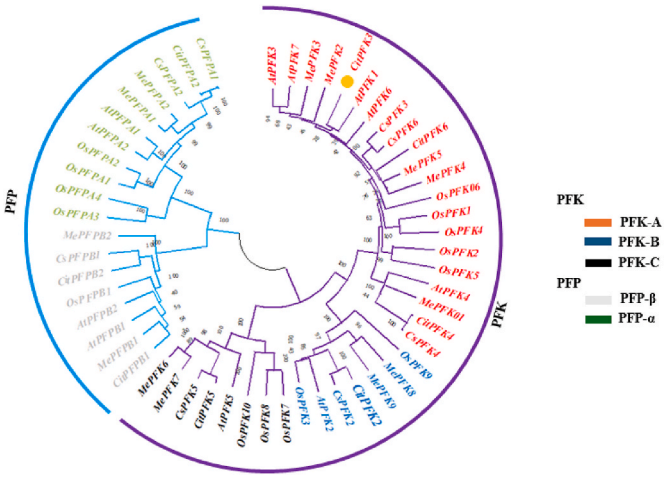

Fig. 1. Phylogenetic tree analysis of PFK genes in *C. clementina*, *C. sinensis*, *A. thaliana*, *O. sativa*, and *M. esculenta*. The Neighbor-Joining tree was constructed with PFK proteins using the Muscle program of MEGA11 software with 1000 bootstraps. PFK protein was classified into two groups and five subgroups. The five subgroups are indicated with different color ranges.

3.3. Insights into CitPFK protein motifs and genes architecture

We conducted a detailed comparison of the exon-intron organization in the coding sequences (CDSs) of CitPFK genes to deepen our knowledge of the structural diversity of these genes. The analysis revealed a close genetic relationship among the CitPFK genes, as evidenced by their similar exon-intron structures (Fig. 2A). Most CitPFK genes contain more than ten exons, except for *CitPFK2*, which belongs to the PFK-B subgroup and contains only two exons.

We analyzed the motif composition of all CitPFK protein sequences. Ten motifs were identified within the citrus PFK gene families, which were highly conserved across the genes (Fig. 2B and C). Motifs 1, 7, and 8 were common across all CitPFKs, while motif 10 was exclusive to the PFP subfamily. Motifs 4, 5, and 6 were specific to the PFK subfamily. It's important to note that motif 9 was absent in the PFP subgroup and *CitPFK5*.These findings indicate that while the PFK and PFP subfamilies share some common functional domains, they also possess specialized motifs that likely correspond to their distinct functional roles.

Further, SMART and InterPro programs were used to annotate the individual motifs identified by MEME. We discovered that only motifs 1 and motif 5 contained the functionally characterized domain of the phosphofructokinase (PF00365); the tools were unable to predict the biological importance or functional roles of the remaining eight potential motifs (2, 3, 4, 6, 7, 8 and 10) (Supplementary Table S2).

3.4. Analysis of cis-acting elements in the promoter region of CitPFK

Plant gene promoter regions function as critical nodes for modulating gene expression and are necessary for modifying gene transcription. We analyzed the CitPFK genes' promoter regions and identified

Table 1
Characteristics of the *CitPFK* family.

Gene name	Locus ID	Gene lengths	Length(aa)	PFK domain position (aa)	MW(Da)	Predicted subcellular localization	GRAVY
CitPFK2	CICLE_v10015031mg	2101	490	126–435	54,188	cytoplasm	−0.205
CitPFK3	CICLE_v10007886mg	2264	561	155–460	61,744	Chloroplast	−0.315
CitPFK4	CICLE_v10004702mg	2058	559	148–453	61760	Mitochondria	−0.268
CitPFK5	CICLE_v10025337mg	2176	532	177–479	58,310	Chloroplast	−0.119
CitPFK6	CICLE_v10025255mg	2289	535	149–454	63,770	Chloroplast	−0.273
CitPFPA1	CICLE_v10000595mg	2344	615	96–461	68,497	cytoplasm	−0.135
CitPFPB1	CICLE_v10014760mg	2307	566	96–461	61,583	Chloroplast	−0.145
CitPFPB2	CICLE_v10014750mg	2075	570	92–339	62,763	cytoplasm	−0.261

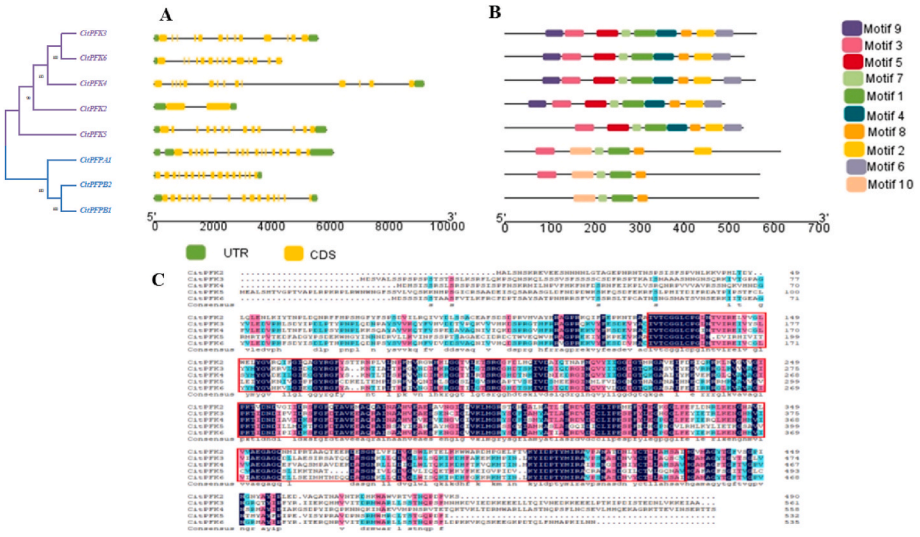

Fig. 2. Protein conserved motifs, gene structure analysis, and multi-sequence alignment of conserved regions of the five CitPFK protein sequences. A, Gene structure of *CitPFK* genes. Yellow boxes represent CDS, green boxes represent UTR, and grey lines represent introns. **B** is Conserved motifs in the CitPFK proteins identified by the MEME online tool. A different color represents each putative conserved motif. **C.** The multiple sequence alignment was performed with DNAMAN software. Black, pink, and light-blue shading represent amino acids with 100, >75, and 50% similarity of amino acids, respectively. The red rectangles represent the phosphofructokinase domain.

distinct types of cis-regulatory elements (Fig. 3 and Supplementary Table S3). The eight general types of these components were identified: elements associated with development, promoters, light- and hormone-responsiveness, environmental stress, site-binding, and other elements. Among these, the light-responsive category was highly enriched, demonstrating that light significantly impacts the regulation of CitPFK genes.

Additionally, all CitPFK gene promoters had multiple hormone-responsive elements, including Auxin-responsive element (AuxRE), Abscisic acid-responsive element (ABRE), Gibberellin-responsive element (GARE), Ethylene-responsive element (ERE), Salicylic acid-responsive element (SARE) and MeJA-responsive element (MeJARE). These results suggested that different plant hormones might influence the expression of the CitPFK genes. Moreover, anaerobic induction elements (AREs) and other stress-related components, including drought-responsive elements (DIRE), defense- and stress-responsive elements (DSRE), wound pathogen-responsive components (WPRE), low-temperature-responsive elements (LTRE) were also widely distributed in the upstream of all CitPFK genes. The findings suggested that various environmental stimuli modulate CitPFK genes.

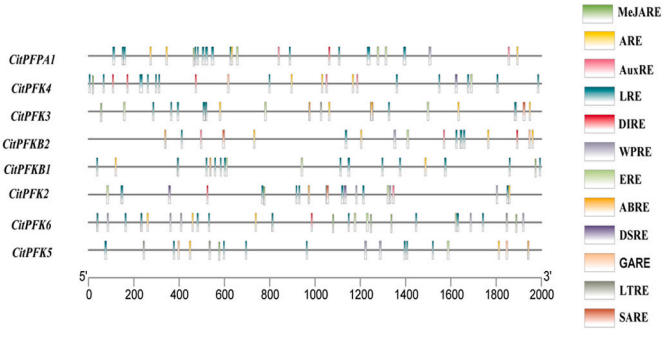

Fig. 3. Hormone response and environmental stress-related elements in the promoter regions of *CitPFK* genes. MeJARE (MeJA-responsive elements) ARE (anaerobic induction elements), AuxRE (auxin responsive elements), LRE (Light-regulated elements), DIRE (drought-inducibility elements), WPRE (wound responsive elements), ERE (Ethylene-responsive elements), ABRE (ABA-responsive elements), DSRE (Defense and stress elements), GARE (Gibberellin-responsive element), LTRE (low-temperature responsiveness)and SARE (Salicylic acid responsive elements) in the 2000-bp promoter upstream regions of *CitPFK* genes were predicted by Plant CARE. The lines denote promoter sequences. Different colors on the lines represent the elements.

3.5. *Patterns of CitPFK gene expression during citrus fruit development and ripening*

We utilized transcriptome data from citrus fruit development to analyze the expression profiles of CitPFK genes at various growth and ripening stages. This analysis aimed to improve our understanding of the roles of CitPFK genes in citrus fruit development and ripening. The results revealed that all five CitPFK genes had distinct expression patterns (Fig. 4A*). CitPFK3*'s expression level progressively increased across the various developmental stages, suggesting an increasing role during fruit maturation. *CitPFK2* showed a heterogeneous expression pattern, suggesting context-dependent activity.

During the early stages, *CitPFK6* showed notably increased levels (66 vs. 80 DAF) and downregulation in the later stages, indicating varied expressions. *CitPFK5* consistently showed a slight increase in expression in the majority of stages. *CitPFK4* primarily exhibited downregulation, suggesting a role that diminishes with development. *CitPFK6* and *CitPFK5* were grouped in the same cluster by hierarchical clustering, while *CitPFK4, CitPFK2,* and *CitPFK3* clustered closely, showing similar expression profiles. These findings suggest possible shared regulatory processes or roles among these groups. Overall, the CitPFK gene family shows dynamic regulation during development. Specific genes display distinct patterns of upregulation and downregulation, demonstrating diverse roles in fruit development.

The expression levels of the five ATP-PFK genes demonstrated distinct patterns over the ripening stages (122, 136, and 150 DAF) (Fig. 4B). The expression of *CitPFK2* and *CitPFK3 significantly* decreased at 136 DAF and then increased at 150 DAF. *CitPFK4* displayed consistent transcript levels across the three stages. C*itPFK5* showed a gradual increase in expression. Conversely, transcript levels for *CitPFK6* showed a declining tendency, declining somewhat at 136 DAF and dramatically at 150 DAF. These results emphasize the differential regulation of the CitPFK gene family, indicating their distinct functions in the developmental timeline of citrus fruit.

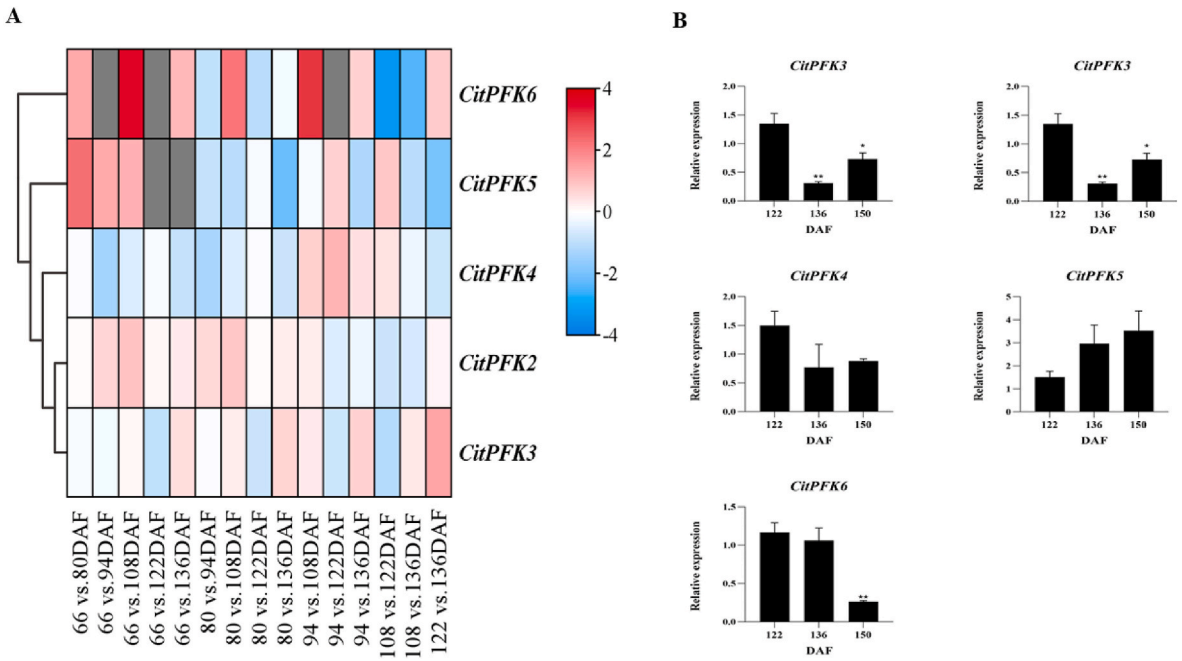

Fig. 4. Expression profiles of the *CitPFK* genes in different fruit development and ripening stages. **A.** Expression profiles of *CitPFK* genes in six fruit development stages: 66, 80, 94, 108, 122, and 136DAF. The log_2FC values obtained from the pairwise comparison of samples were used to visualize the heatmap. The transcript abundances were represented by color scales ranging from blue (low) to red (high). **B.** Expression profiles of CitPFK genes in three ripening stages of citrus fruit. Error bars show the mean from n = 3 biological replicates ±SE. Asterisks indicate significant differences (*P < 0.05, **P < 0.01) according to one-way ANOVA tests with post-hoc Tukey test.

3.6. CitPFK gene expression profiles in response to exogenous GA and SA postharvest treatments

The expression of CitPFK genes in response to the application of exogenous phytohormones was investigated using two plant hormones: salicylic acid (SA) and gibberellic acid (GA). The transcript levels from five PFK genes were measured using the RT-qPCR method. The results revealed that the PFK genes responded to the external application of these phytohormones (Fig. 5). Specifically, after 8 days of SA treatment, almost all the genes had much higher transcript levels ($P < 0.05$). *CitPFK3* showed a general increase in transcript levels, whereas the other four genes declined after 8 days of SA application.

In GA-treated fruits, the transcript abundance of *CitPFK2* and *CitPFK5* genes differed noticeably from the other genes, with their levels

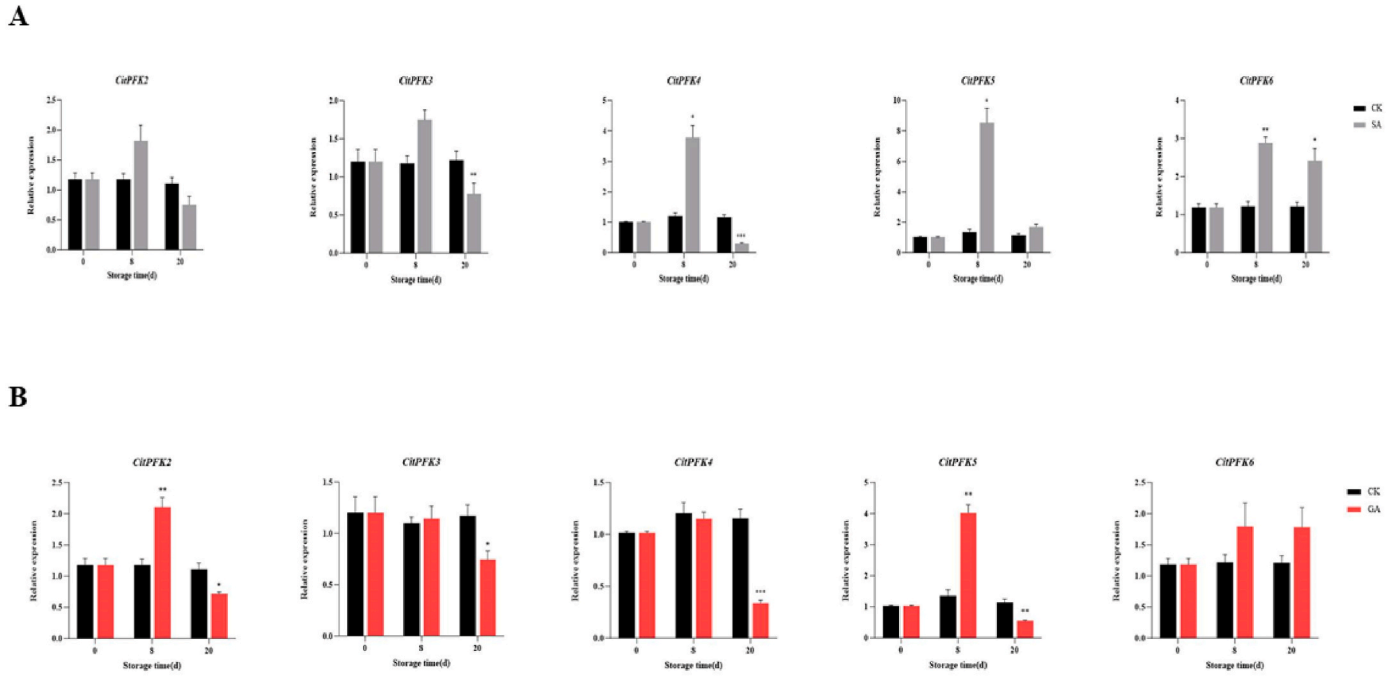

Fig. 5. Expression profiles of the CitPFK genes in response to SA and GA postharvest hormonal treatments. A. CitPFK gene expression in responses to salicylic acid (SA), B. CitPFK gene expression in responses to gibberellic acid (GA). Error bars show the mean from n = 3 biological replicates ±SE. Asterisks indicate significant differences (*P < 0.05; **P < 0.01; ***P < 0.001) according to Student's t-test.

significantly elevated at eight days of treatment. However, at 20 days after treatment, the expression of all genes, except for *CitPFK6*, displayed a significant decrease ($P < 0.05$). Our research indicates that exogenous SA and GA influenced the expression of CitPFK genes during postharvest storage.

3.7. Functional characterization of CitPFK3 in citrus using VIGS

To study the role of *CitPFK3* in citrus fruit, we obtained a 224 bp sequence from the 5' untranslated region of the *CitPFK3* gene and performed virus-induced gene silencing (VIGS). The fruits were injected with the transient VIGS construct and an empty vector during maturation (129 days after flowering, DAF) (Fig. 6A). Eight days post-injection, the transcript level of *CitPFK3* in the TRV2-PFK3-injected fruits was downregulated by 92% compared to the TRV2-TRV1 (control) inoculated fruits (Fig. 6B and C). To ensure that the reduction of target enzyme activity was altered by silencing, the activities of the PFK enzyme were additionally tested in both controls and transformed fruits. Our results indicated a significant reduction in the PFK enzyme activity by 70.9 % in transgenic fruits (Fig. 6E). These results demonstrate that gene silencing of *CitPFK3* was effectively achieved using the predicted silencing fragment and associated siRNA.

To further validate the impact of *CitPFK3* silencing, we analyzed the fructose, glucose, and sucrose levels in the citrus fruits injected with the empty TRV2 vector and TRV2: PFK3 eight days post-injection. The results revealed that suppression of *CitPFK3* significantly increased fructose and glucose levels by 24.9% and 33.4%, respectively (Fig. 6D). In contrast, there was no significant change in sucrose content between the VIGS-silenced fruits and the control. Our results provide clear evidence for the pivotal role of *CitPFK3* in the hexose sugar buildup during citrus fruit development.

3.8. Suppression of the CitPFK3 gene upregulated expression and activity of the PFP gene

To explore whether the increased hexose sugars in the *CitPFK3* mutant are directly associated with alterations in gene expression in the sugar-metabolism pathway, we analyzed a subset of sugar metabolism genes for their expression levels both upstream and downstream of the glycolysis/gluconeogenesis pathway. The genes we examined included two phosphoenolpyruvate carboxykinases (ATP) (CICLE_v10000510mg and CICLE_v10025088mg), diphosphate-dependent phosphofructokinase (CICLE_v10000595mg), glucose-6-phosphate isomerase (CICLE_v10000722mg), pyruvate dehydrogenase E2 component (CICLE_v10001084mg), hexokinase (CICLE_v10008086mg), pyruvate decarboxylase (CICLE_v10011317mg), fructose-bisphosphate aldolase, class I (CICLE_v10012049mg), phosphoglucomutase (CICLE_v10014721mg), and two pyruvate kinases (CICLE_v10014963mg and CICLE_v10014977mg).

Quantitative RT-PCR results showed no significant change in gene expression levels, except for diphosphate-dependent phosphofructokinase (PFP) in VIGS-silenced fruits compared to the control fruits (Fig. 7). The transcript levels of glucose-6-phosphate isomerase (PGI), pyruvate kinase (PK1), and phosphoenolpyruvate carboxykinase (PEPCK1) were slightly increased but not significantly.

On the other hand, phosphoglucomutase (PGM), pyruvate dehydrogenase E2 component (PDEC), pyruvate decarboxylase (PDC), pyruvate kinase (PK2), and phosphoenolpyruvate carboxykinase (PEPCK2) exhibited a slight decrease in the transgenic fruits. The expression patterns of hexokinase (HK) and fructose-bisphosphate aldolase (FBA) were similar in both the control and transformed fruits (Fig. 7).

We measured the PFP enzyme activity in both mutant and control fruits to provide additional confirmation for our findings. We found that the transgenic fruits exhibited significantly higher PFP enzyme activity than the control fruits (Fig. 6F). These results suggest that the decrease in *CitPFK3* gene expression led to an activation of the PFP gene, compensating for the reduced *CitPFK3* activity (Fig. 6E) and ultimately contributing to the observed increase in hexoses.

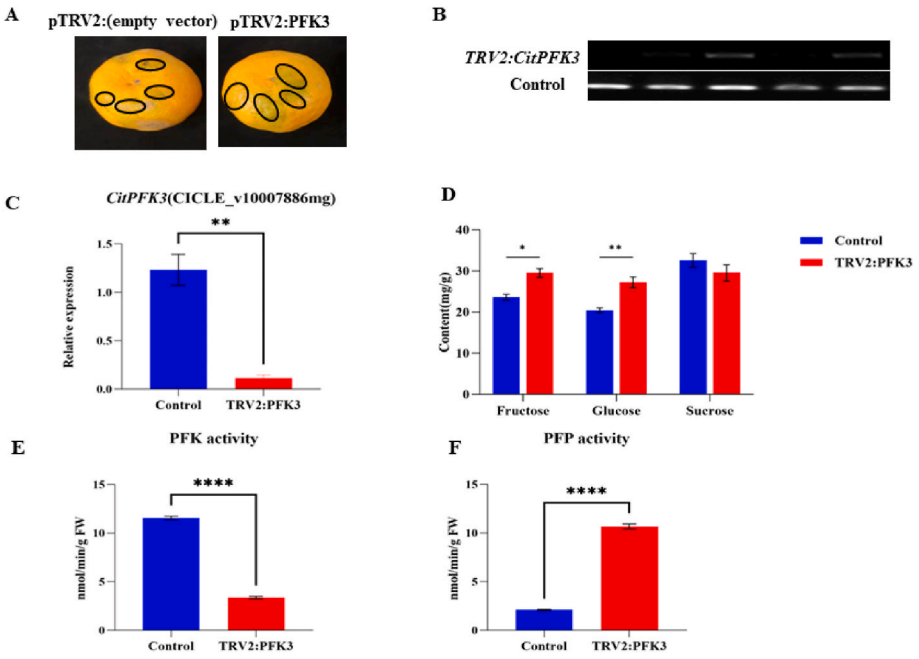

Fig. 6. VIGS transformation in citrus fruits. A. Transient assays in Dafen4 cultivar using control (empty vector of TRV2) or pTRV2:*CitPFK3*. The black circles indicate the injection sites. **B.** *CitPFK3* expression levels in mutant fruits and control by real-time PCR. **C.** The relative expression levels of *CitPFK3* in the citrus pulp of transformed fruits using RT-qPCR. **D.** The content of soluble sugars. **E.** PFK enzyme activities. **F.** PFP enzyme activities. Error bars show the mean from n = 3 biological replicates ±SE. Asterisks represent significant differences (*$P < 0.05$, **$P < 0.01$ ****$P < 0.0001$) according to the two-tailed Student's t-test.

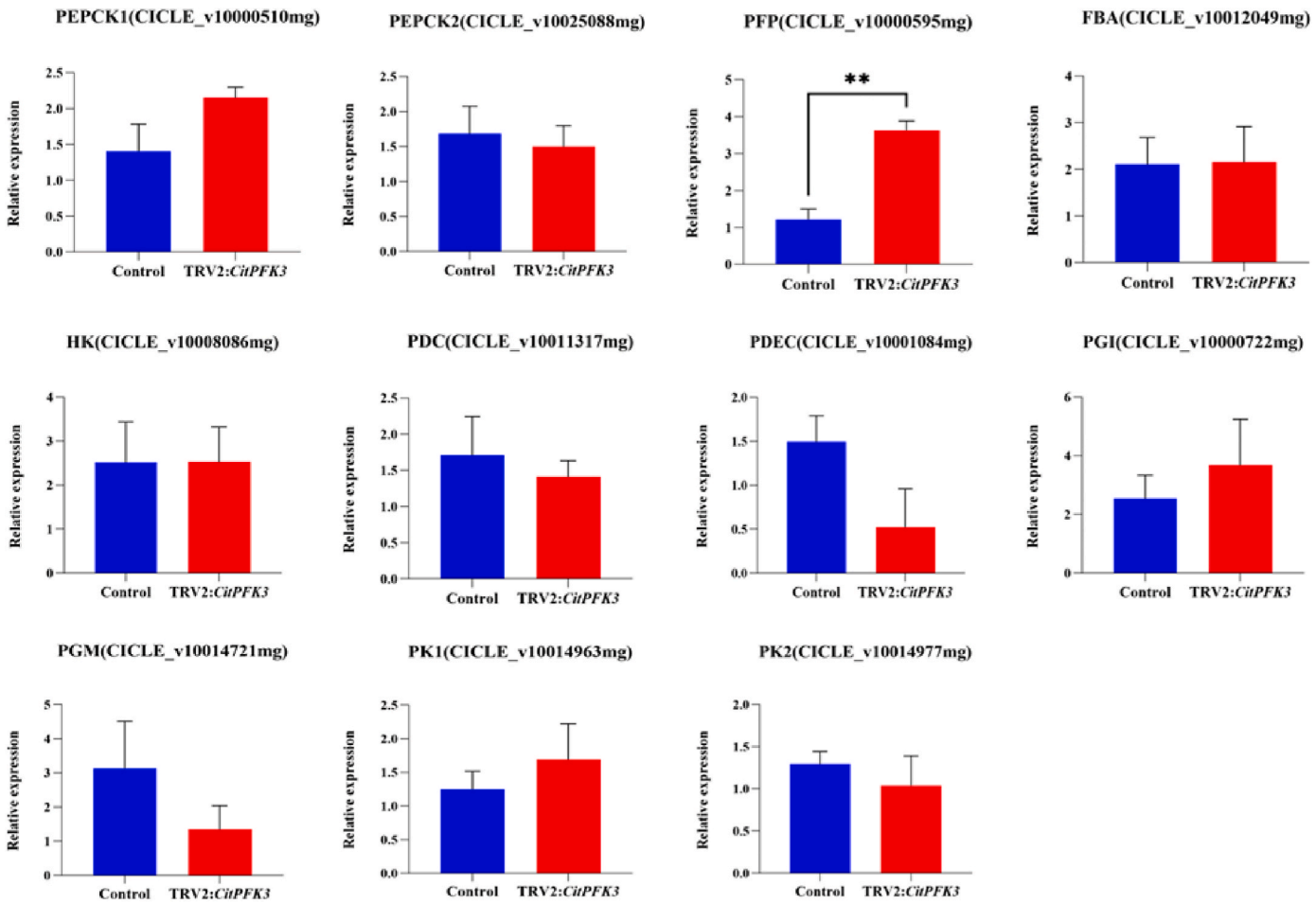

Fig. 7. The effect of virus-induced gene silencing of *CitPFK3* on the relative expression levels of sugar-responsive genes in the glycolysis/gluconeogenesis pathway. Error bars show the mean from n = 3 biological replicates ±SE. Asterisks represent significant differences (**P < 0.01) according to Student's t-test.

4. Discussion

4.1. Identification and characterization of the phosphofructokinase gene family

The Phosphofructokinase (PFK) gene family has been extensively studied in various plants, including Chinese white pear (Lü et al., 2019), Arabidopsis (Mustroph et al., 2007), cassava (Wang et al., 2021), rice (Mustroph et al., 2013), sugarcane (Zhu L. et al., 2013), Cotton (Mehari et al., 2022) and Northern red oak (Kim et al., 2023). However, its characterization in citrus remains unexplored. Therefore, we thoroughly examined this gene family in citrus, encompassing phylogenetic analysis, chromosomal mapping, gene structure, conserved protein domains, cis-regulatory elements, and transcript profiles.

In the *Citrus clementina* genome, we identified 8 PFK genes, comprising five ATP-PFK and three PFP genes. Our phylogenetic analysis classified these genes into distinct subfamilies: PFK-A, PFK-B, PFK-C under PFK and PFP-α, PFP-β under PFP (Fig. 1), consistent with classifications observed in other plants such as cotton and Arabidopsis (Mehari et al., 2022; Mustroph et al., 2007). Subcellular localization analysis showed that four CitPFK genes localize to chloroplasts, three to the cytoplasm, and one to mitochondria, consistent with cassava findings (Wang et al., 2021). *CitPFK3* shares homology with *AtPFK1* (At4g29220) and belongs to the PFK-A subgroup. Conserved phosphofructokinase domains, as demonstrated by motif analysis of CitPFKs, were found to be necessary for catalyzing the crucial glycolysis step of phosphorylating fructose-6-phosphate to fructose-1,6-bisphosphate (Mustroph et al., 2007).

Further examination of cis-regulatory elements in the promoters of the CitPFK gene revealed enrichment in elements associated with stress, hormone response, and light response (Supplementary Table S3). This diversity underscores the functions of CitPFK genes in plant growth and their responsiveness to stress. Notably, the presence of phytohormonal elements, including ABRE, GARE-motif, and ERE, suggests that PFK genes are involved in the ripening of citrus fruits, which is consistent with the regulatory roles played by these plant hormones in fruit physiology (Feng et al., 2021; Fuentes et al., 2019; Kumar et al., 2013).

4.2. The expression profiles of CitPFKs during fruit development in citrus

Citrus fruits undergo three stages of growth and ripening: cell division, expansion, and ripening (Bain, 1958). The ATP-dependent 6-phosphofructokinase expression illustrated a dynamic pattern during citrus fruit development. Our study exhibited significant variability in gene expression patterns, demonstrating their dynamic roles during citrus fruit development. *CitPFK6* demonstrated significant upregulation in early stages (66 vs. 80 DAF) and downregulation in later stages, indicating its involvement in early developmental processes. *CitPFK5* showed slight upregulation across all stages, indicating consistent activity throughout citrus fruit development. *CitPFK4* predominantly exhibited downregulation, implying a diminishing role as development progresses. CitPFK2's mixed expression pattern suggests a context-dependent function, while *CitPFK3*'s consistent upregulation implies increasing importance during maturation stages (Katz et al., 2011; Lin et al., 2015). During the initial stages of fruit development, there is active cell division and growth; therefore, there is a high demand

for energy and glycolytic intermediates. Later, during the maturation phases, the need goes down, indicating a shift during ripening toward sugar accumulation and flavor compound synthesis (Cercos et al., 2006; Feng et al., 2021). These findings give insight into the complex regulatory networks governing gene expression during citrus fruit development.

The study of ATP-dependent 6-phosphofructokinase genes across three stages of fruit ripening (122, 136, and 150 DAF), as determined by RT-qPCR, revealed distinct expression patterns among the five genes. *CitPFK2* and *CitPFK3* exhibited a notable decrease in expression at 136 DAF, followed by an increase at 150 DAF. This fluctuation suggests a potential regulatory mechanism influencing their transcription during later stages of fruit development, possibly related to metabolic shifts or signalling pathways activated during ripening. In contrast, *CitPFK4* maintained consistent expression levels throughout the three stages, indicating a stable regulatory profile across the developmental timeline studied. This stability could imply a steady requirement for its enzymatic activity in metabolic processes critical for fruit ripening.

CitPFK5 demonstrated a gradual increase in expression over the ripening stages, signifying its role in metabolic pathways that intensify as fruit maturation progresses. This gradual upregulation may correspond to the increasing demand for ATP and metabolic intermediates necessary for the final stages of fruit maturation. Conversely, *CitPFK6* showed a decreasing trend in expression, with transcript levels slightly reduced at 136 DAF and a sharp drop at 150 DAF. This decline suggests a potential downregulation of metabolic pathways associated with this gene during late ripening stages, possibly indicating a shift in energy and carbon utilization as the fruit matures (Katz et al., 2011). These findings uncover the differential regulation of the CitPFK gene family throughout citrus fruit ripening. Each gene likely fulfills distinct roles in metabolic pathways and cellular processes crucial for the development and maturation of citrus fruits. Further research could delve deeper into the specific metabolic functions and regulatory mechanisms governing these PFK genes to understand better their contributions to fruit quality and yield in citrus production.

4.3. Exogenous SA and GA treatments modify the expression of CitPFKs during postharvest storage of citrus fruits

Phytohormones play a critical role in the development of fruit and its storage after harvesting. They regulate important metabolites, such as sugar, which influence fruit quality. Moreover, numerous studies have been conducted, and phytohormones have been demonstrated to improve postharvest quality and prolong shelf life (Al-Qurashi and Awad, 2019; Shahzad et al., 2024). Studies in grape berries demonstrate hormone-induced upregulation of sugar metabolism genes (Murcia et al., 2018). Gibberellic acid delays ripening, decreasing levels as the fruit matures (Porat et al., 2001; Zhou et al., 2022). Our research revealed that exogenous GA application significantly influences ATP-dependent 6-phosphofructokinase regulation during postharvest. Notably, *CitPFK2* and *CitPFK5* transcript levels rise after GA treatment, likely due to GA-responsive elements in their promoters (Porat et al., 2001; Zhou et al., 2022)

It has been demonstrated that salicylic acid (SA) increases sugar accumulation in postharvest fruits, including citrus fruits. (Baswal et al., 2020; Haider et al., 2020; Shahzad et al., 2024; Zhu et al., 2016). We noticed significant increases in the expression levels of almost all CitPFK genes following SA treatment, indicating that SA boosts glycolytic activity to meet the heightened energy demands during stress responses. Sugars play a key role in cell metabolism by providing sufficient energy for the cell to fight against pathogens. SA is an essential signaling molecule in systemic acquired resistance (SAR), a defense mechanism that offers persistent defense against various infections. SAR requires substantial energy to manufacture pathogenesis-related proteins and secondary metabolites (Zhu et al., 2016). Salicylic acid ensures enough ATP and metabolic intermediates to enable these defensive activities by activating the activity of glycolytic enzymes like PFK. The activation of these genes also indicates effective responses through SA-responsive cis-acting elements induced by exogenous SA application (Niu et al., 2023).

ATP-dependent 6-phosphofructokinase, an enzyme responsible for phosphorylating fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (FBP), plays a crucial role in this process (Perby et al., 2022). Our investigation into CitPFK gene expression, particularly their responses to GA and SA, provides promising insights into manipulating genes involved in sugar metabolism. Our research enhances our understanding of how these plant hormones modulate CitPFK genes, which are critical in crucial metabolic processes for fruit development and quality maintenance. Our results demonstrate that gene promoters containing cis-acting elements responsive to these hormones react to their exogenous application. Collectively, leveraging these plant hormones in postharvest applications holds the potential to enhance fruit quality and prolong shelf life.

4.4. Fructose and glucose increase in transformed fruits, but sucrose remained unchanged mainly by CitPFK3 knockout

Fruit sweetness is a crucial aspect of fruit quality, influenced by the type and amount of soluble sugars and organic acids present. The ratio of major sugars also plays a significant role. During ripening, citrus fruits typically experience a rise in sugar levels and reduced organic acids (Dong et al., 2019). Citrus fruits accumulate mainly sucrose and monosaccharides such as glucose and fructose (Bean, 1960). Sugar accumulation peaks in the later phases of fruit development and continues during ripening (Chen et al., 2023; Fang et al., 2023; Komatsu et al., 2002; Lin et al., 2015).

Our research indicates that *CitPFK3* mutants had higher fructose and glucose accumulation (Fig. 6D), likely due to reduced glycolytic flux from missing plastid PFK3 catalysis. While free hexoses are not direct PFK substrates, their elevated levels suggest feedback regulation suppressing hexose phosphorylation before PFK action (Van Der Merwe et al., 2010). Similar PFK inhibition in potatoes and Arabidopsis led to hexose phosphate accumulation and sucrose synthesis (Claassen et al., 1993; Perby et al., 2022).

Fructose, prominent in acidless citrus varieties, substitutes citric acid and can be derived directly from glucose via gluconeogenesis (Albertini et al., 2006). Both fructose and glucose are derived from starch and sucrose metabolism and are catabolized through glycolysis, where phosphofructokinase catalyzes fructose-6-phosphate to fructose-1, 6-bisphosphate (Smyth and Black, 1984). PFKs are critical in determining glycolysis direction and are associated with fructose metabolism during ripening (Jiang et al., 2023). In various fruits, including citrus, pears, and peaches, PFK upregulation correlates with maturation (Desnoues et al., 2014; Jiang et al., 2023; Katz et al., 2011).

Interestingly, *CitPFK3* silencing didn't significantly alter sucrose content, possibly due to compensatory activation of PFP, a reversible glycolytic enzyme (Mustroph et al., 2007). Despite this, glycolysis persisted in *CitPFK3*-silenced fruits, evidenced by stable sugar metabolism gene expression (Fig. 7). Transgenic tobacco studies highlight PPi-PFK's glycolytic role in phosphate shortage (Fernie et al., 2002). Glycolysis, pivotal in sugar metabolism, activates during citrus ripening and is regulated by enzymes like hexokinase, phosphofructokinase, and pyruvate kinase, which adjusts sugar and acid balance. In potatoes and Arabidopsis, PFK suppression reduces enzyme activity without impacting sucrose levels (Cercos et al., 2006; Lin et al., 2015). Similarly, our study found that *CitPFK3* suppression decreased PFK activity in mutant fruits. Compensatory PFP activation supports glycolytic continuity, warranting further investigation into PFP and PFK roles during citrus ripening.

This work lays a foundation for future research on the phosphofructokinase gene family in citrus. Further research, such as overexpression, RNA interference (RNAi) or CRISPR/Cas9-mediated

knockout experiments, could help to fully uncover the functions of *CitPFK3* and other PFK isoforms in citrus.

5. Conclusion

Our study unveils five ATP-PFK gene members from the *Citrus clementina* genome, highlighting their differential expression patterns and structural features. *CitPFK3* is critical in hexose sugar accumulation during citrus fruit ripening, underscoring its pivotal role in fruit quality regulation. The expression of CitPFK genes throughout fruit development provides a valuable experimental dataset and a dependable reference for future functional analysis of these genes in citrus. By delving into hormonal influences on these genes, we deepen our understanding of how plant hormones orchestrate metabolic processes crucial for fruit development and postharvest maintenance. This insight sheds light on regulatory mechanisms in non-climacteric fruits like citrus and underscores potential strategies for enhancing postharvest performance and storage longevity.

Author contributions

Mingbao Luan: Conceptualization, funding acquisition, resources, supervision, validation, writing-review, and editing. Yong Deng: Conceptualization, project administration, supervision, validation, and writing-review and editing. Sophia Nyamusi Ochiki: Conceptualization, investigation, data curation, validation, formal analysis, visualization, and writing- original draft, review, and editing. Tianxin Chen: Conceptualization, formal analysis, and writing-review and editing. Zhixin Meng: Methodology, formal analysis, and writing-review and editing. Jiahao Zhou and Zexin Gao: Methodology and writing-review and editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for *[Journal name]* and was not involved in the editorial review or the decision to publish this article.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.109235.

Data availability

Data will be made available on request.

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