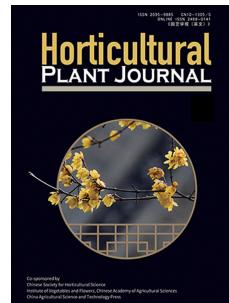


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Methyl salicylate-induced *CitERF92* promotes the accumulation of PMFs in citrus

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ABSTRACT

Polymethoxylated flavones (PMFs) are a class of flavonoid compounds predominantly present in citrus and are renowned for their extensive biological activity (antiobesity, antioxidant and cardiovascular protection). Recent advancements have elucidated the biosynthetic pathway of PMFs, although the transcriptional regulatory mechanisms involved are not yet fully understood. In this study, we used PMF-rich Bingtangcheng (*Citrus sinensis* Osbeck) fruit (BTC) and treated it with exogenous methyl salicylate (MeSA). Our study demonstrates that MeSA treatment dynamically regulates the biosynthesis of PMF monomers in citrus peels during low-temperature storage (10 °C), with 0.2 mmol · L⁻¹ MeSA combined with 14-day storage effectively enhancing PMF accumulation. The transcriptomic analyses demonstrated that MeSA treatment activated the expression of essential genes associated with the PMF biosynthetic pathway, including *FNSII*, *F3'H*, and *COMT*. Additionally, MeSA also activated the biosynthesis and signaling pathways of other phytohormones. A transcription factor, *CitERF92*, from the ERF family B3 group, was significantly induced by MeSA and strongly correlated with the expression of *CitFNSII-1*. Dual-luciferase assays and electrophoretic mobility shift assays demonstrated that *CitERF92* could bind to and significantly activate the promoter activity of *CitFNSII-1*. Subsequent transient overexpression experiments confirmed that *CitERF92* markedly increased the content of PMF monomers in the BTC peel. These results reveal the *CitERF92-CitFNSII-1* module as a novel regulator of PMF biosynthesis, enhancing the understanding of the regulatory network that controls PMF production in citrus.

Keywords: Citrus; Methyl salicylate; Polymethoxylated flavones; ERF; Transcriptional regulation

1. Introduction

Citrus is globally acknowledged as one of the most significant fruits, appreciated by consumers for its diverse varieties, enjoyable flavors, good taste and high nutritional value, including vitamins, phenols, and flavonoids (Singh et al., 2020). Among the nutrients in citrus, flavonoids are central due to their broad biological activities and importance to plant ecophysiology. Notably, polymethoxylated flavones (PMFs), a subclass distinguished by methoxy modifications, demonstrate multi-target therapeutic effects through antioxidant, cardiovascular protective, anti-obesity, and antitumor mechanisms (Choi et al., 2011; Barreca et al., 2017; Duan et al., 2017; Wang et al., 2021a). Recent years have seen an increased research focus on the plant-derived flavonoids.

In citrus, flavonoids are synthesized through the phenylpropanoid pathway (Zhao et al., 2020). The metabolic pathway of flavonoids has two parts: the biosynthesis of naringenin, which forms the flavonoid skeleton (upstream pathway), and modifications (e.g. glycosylation and methylation) for the formation of various types of flavonoids (downstream pathway) (Sharma et al., 2019; Zheng et al., 2023; Shang et al., 2024). Citrus is a rich source of O-methylated flavonoids, structural modifications that reduce polarity and enhance lipophilicity, thereby contributing to their superior bioactivity. PMFs are predominantly enriched in citrus peels, with the most common monomers being sinensetin, nobiletin, tangeretin, 5-demethylnobiletin, isosinensetin, heptamethoxyflavone, and 5,6,7,4'-tetramethoxyflavone (Peng et al., 2021). In addition to their important pharmacological functions, PMFs

have been found to exhibit antifungal activity against fungi such as *Colletotrichum gloeosporioides* (Almada-Ruiz et al., 2003) and *Penicillium. digitatum* (Ortuño et al., 2006). Therefore, as a reflection of the nutritional value of citrus fruits, PMFs warrant further investigation into their regulation and biosynthesis mechanisms.

Phytohormones and their derivatives serve as both endogenous signals for fruit growth and development, and as regulators of various aspects of fruit quality during postharvest exogenous treatments (Baswal et al., 2020; Habibi et al., 2020; Niu et al., 2024). Moreover, phytohormone treatments enhance fruit quality by mediating crosstalk interactions among various phytohormones (Wu et al., 2020; Li et al., 2022). Salicylic acid (SA) and its derivative are reported frequently as endogenous plant growth regulators. They have been shown to induce a wide variety of metabolites and physiological responses, influencing plant growth and development (Asghari and Aghdam, 2010). SA and MeSA are even more widely applied in the postharvest preservation of citrus. For example, exogenous SA treatment could improve the antioxidant activity of navel orange during storage (Huang et al., 2008), reduce the rot rate of *Citrus unshiu* (Zhu et al., 2016), increase the levels of health-promoting compounds (Baswal et al., 2021), and prolong the cold storage life of citrus fruits (Baswal et al., 2020). MeSA was proven effective for maintaining quality and maintaining higher anthocyanin in blood oranges (Habibi et al., 2020).

Additionally, treatments with SA and MeSA have been shown to activate phenylpropanoid pathways. Exogenous application of SA enhances the expression of *phenylalanine ammonia lyase* (PAL) and *chalcone synthase* (CHS), leading to increased flavonoid accumulation in watermelon (Liu et al., 2023a). Similarly, MeSA has been found to stimulate the accumulation of total flavonoids by increasing the activity of PAL, CHS, cinnamate 4-hydroxylase (C4H), 4-coumarate: coenzyme A ligase (4CL), and chalcone isomerase (CHI) (Li et al., 2019; Yang et al., 2023). However, in citrus, the biosynthesis of PMFs is controlled by a series of crucial enzymes, especially the downstream synthetase and modification enzymes such as flavone synthase II (FNSII), flavonoid hydroxylase (FH) and caffeoyl coenzyme A / caffeic acid O-methyltransferase (COMT/CCoAOMT) (Liao et al., 2023; Liu et al., 2023b; Zheng et al., 2023; Peng et al., 2024; Wen et al., 2024). Expression of these downstream enzymes determines the metabolite flow from the phenylpropane pathway to the production of flavanones and PMFs, but there are few reports on their regulatory mechanisms.

With the elucidation of flavonoid biosynthesis in citrus, we are attempting to establish a bridge between MeSA and PMFs. This study analyzed the effects of MeSA on PMF content in citrus by investigating treatment concentrations and storage durations. It was found that the $0.2 \text{ mmol} \cdot \text{L}^{-1}$ MeSA treatment significantly increased the contents of PMFs after 14 d of storage. The differentially expressed genes (DEGs) enrichment analysis revealed that MeSA treatment activated phenylpropanoid biosynthesis, flavonoid biosynthesis, as well as hormone biosynthesis and signaling pathways. Additionally, a positive regulator of PMF biosynthesis, *CitERF92*, was identified. Both *in vitro* and *in vivo* experiments demonstrated that MeSA promotes the accumulation of PMFs in citrus peel by activating the *CitERF92-CitFNSII-1* module. This study elucidates the mechanisms underlying the regulation of post-harvest fruit quality by MeSA and its impact on PMFs. It encompasses both treatment methodologies and molecular aspects, thereby enhancing the theoretical framework of PMF biosynthesis. Furthermore, this research offers valuable insights for improving post-harvest quality and facilitating the genetic breeding of citrus.

2. Materials and Methods

2.1. Plant materials and fruit treatment

'Bingtangcheng' fruits (*Citrus sinensis* Osbeck) were harvested from the experimental base of the Quzhou Academy of Agriculture and Forestry Science in Zhejiang Province, China, at 240 days after flowering. Without mechanical wounding and disease, healthy fruits were selected for MeSA treatment.

The MeSA was purchased from Sangon Biotech (Shanghai, China), and diluted to different concentration

gradients. Injections were made on the opposite sides of each fruit, with one side contained MeSA solutions and the other one was water as a control. The injection area had a diameter of approximately 4 cm, with 5 mL of solution injected into each area. After injection, the injection part of the fruit peels was sampled after 2 d, 10 d, and 14 d of storage at 10 °C (Fig. S1). A total of nine samples were prepared in this experiment, which were divided equally into three biological replicates. Upon sampling, the peels of the fruit from the injection area were separated, sliced into small pieces, and mixed. Pretreatment experiments revealed that MeSA treatments at concentrations of 0.05 mmol · L⁻¹ and 0.2 mmol · L⁻¹ effectively altered the content of PMFs in fruit peels.

2.2. Chemical sources

Isosinensetin, sinensetin, nobiletin, and tangeretin were obtained from Yuanye (Shanghai, China). 5,6,7,4'-tetramethoxyflavone and heptamethoxyflavone were obtained from SinoStandards (Chengdu, China). The chromatographic grade acetonitrile for HPLC was purchased from Tedia (St. Fairfield, OH, USA), formic acid at chromatographic grade was obtained from Aladdin (Shanghai, China), and the methanol for HPLC was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. PMF extraction and detection

Citrus flavonoids were extracted by the same method as in a previous study (Zhao et al., 2021) with minor modifications. 0.1 g of citrus peels was extracted three times with 0.5 mL of 80% ethanol by sonication and then combined with the supernate (~1.5 mL). The compounds were separated at room temperature by acetonitrile (solvent A) and water (solvent B, containing 0.1% formic acid) with the following gradients at a flow rate of 1 mL · min⁻¹, 0–5 min, 20% of A; 5–10 min, 20%–27% of A; 10–15 min, 27% of A; 15–25 min, 27%–40% of A; 25–35 min, 40%–60% of A; 35–40 min, 60%–80% of A; 40–42 min, 80%–100% of A; 42–45 min, 100%–20% of A; 45–50 min, 20% of A. An HPLC-DAD system (Waters Crop., Milford, MA, USA) coupled with a Sunfire C18 ODS column (4.6 mm × 250 mm, 5 µm; Waters) was used for flavonoids detection. The PMFs were monitored at 330 nm.

2.4. RT-qPCR analysis.

Total RNA extracted from BTC fruit peels at 2 d, 10 d, and 14 d (including both control and MeSA treatment) was used for RT-qPCR. EASYspin Plus Plant RNA Kit (Beijing, China) was used for total RNA extraction, and HiScript III 1st Strand cDNA Synthesis Kit with gDNA wiper (Vazyme, Nanjing, China) was used to remove the genomic DNA and to synthesize the cDNA. RT-qPCR was performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and CFX96™ Real-time PCR System (Bio-Rad, Hercules, CA, USA). The citrus β-actin gene, validated for stable expression under experimental conditions (Pillitteri et al., 2004), served as a housekeeping gene for normalization, and fold change was calculated using the 2^{-ΔCt} method. The gene-specific oligonucleotide primers were listed in Table S1, with the primers tested and confirmed by product resequencing.

2.5. Total RNA extraction and RNA sequencing

BTC fruits treated with 0.2 mmol · L⁻¹ MeSA and stored after 14 d were selected for transcriptome analysis because of the positive promotional effect on PMF content. The total RNA was extracted using the RNAPrep Pure Plant Kit (Tiangen, Beijing, China) and quantified for concentration, with three biological replicates per experimental group. For RNA-seq library construction, 1 µg of total RNA was used for reverse transcription per sample, with three biological replicates set up. The libraries were sequenced on the Illumina NovaSeq 6000 sequencing platform by Biomaker Technologies Co., Ltd. (Beijing, China). Adapter sequences and low-quality sequence reads were removed from the raw data. These clean reads were aligned to the *Citrus sinensis* v1.0 genome database (<http://citrus.hzau.edu.cn/download.php>) using Hisat2 tools.

2.6. Analysis of transcriptome sequencing data

The basic information about clean reads and clean data was presented in Table S2, indicating the high quality

of RNA-seq and the reliability of the data. The fragments per kilobase of transcript per million fragments mapped value was used to quantify the gene expression levels. Genes with an adjusted P-value < 0.05 and Fold Changes ≥ 1.5 found by DESeq2 were assigned as differentially expressed. The trends in the overall change of enrichment pathways were analyzed by Gene Set Enrichment Analysis (GSEA). RNA-seq data of BTC fruits at different developmental stages are available under accession number PRJNA924350 (Zheng et al., 2023). The cis-acting elements of DEGs promoter were predicted by PlantCARE (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and visualized by TBtools (Chen et al., 2020) after selection and redundancy removal.

2.7. Dual-luciferase assays

The coding sequence (CDS) of *CitERF92* was cloned into the pGreen II 0029 62-SK vector, while the promoter of *CitFNSII-1* was cloned into the pGreen II 0800-LUC vector using the primers listed in Table S1. The recombinant vectors were transformed into *Agrobacterium tumefaciens* (GV3101::pSoup) and the cultures were resuspended in infiltration buffer (10 mmol · L⁻¹ MES, 10 mmol · L⁻¹ MgCl₂, 200 μmol · L⁻¹ acetosyringone) to OD₆₀₀ = 0.8. The *CitERF92*-SK and *CitFNSII-1pro*-LUC were mixed in a ratio of 10:1 (v/v) for transient expression in *Nicotiana benthamiana* leaves. The luminescence of firefly luciferase (LUC) and *Renilla* luciferase (REN) was measured using the GloMax96 instrument (Promega, Madison, WI) after the reaction of the Dual-Luciferase Report Assay System (Promega, Madison, WI).

2.8. Recombinant protein purification and EMSA assay

The cDNA library from BTC peel was used as a template to amplify the *CitERF92* CDS after removing the terminator. This sequence was then constructed into the pGEX-4T-1 vector with a GST tag using homologous recombination. The primers are provided in Table S1. The transcription factor *CitERF92* protein were purified using the Beyotime GST-tagged protein purification kit (Shanghai, China), following the manufacturer's instructions.

A probe labeled with 3'-Biotin was synthesized by Hangzhou GeneBio Biotechnology Company (Hangzhou, China), while primers with the same sequence but without 3'-Biotin labeling were used as competition probes (Table S1). The EMSA was conducted using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The luminescence reaction was carried out using the kit, and images were captured using a ChemiDoc™ XRS+ (Bio-Rad, USA) instrument.

2.9. Transient overexpression of *CitERF92*

The *CitERF92* CDS was inserted into the pBI121 vector after removing the terminator, and the primers for cloning are shown in Table S1. The transient overexpression assay was performed according to Liao et al., (2023). *Agrobacterium tumefaciens* strain EHA105 carrying the recombinant *CitERF92*-pBI121 plasmid was cultured in LB medium for 48 h and resuspended in infiltration buffer (10 mmol · L⁻¹ MES, 10 mmol · L⁻¹ MgCl₂, 150 μmol · L⁻¹ acetosyringone, pH 5.6) to an OD₆₀₀ of 0.8. BTC fruits with uniform maturity (240 days after flowering) were selected for injection. Injections were administered to the opposite sides of the equatorial plane of each fruit. One side was injected with *CitERF92*-pBI recombinant plasmid, while the other side was injected with pBI vector as a control. The diameter of the injection site was 4 cm, and the injection volume for each side was approximately 5 mL. Each fruit was set as a biological replicate, with a total of 10 replicates. The injected fruits were placed in a climatic chamber at 25 °C with a photoperiod of 16 h light/8 h dark. 5 days later, the injected pericarp tissue was removed, cut into small pieces, and frozen in liquid nitrogen before being stored at -80 °C for future use.

2.10. Statistical analysis

All data were obtained from at least three replications, and error bars represent the standard error of the mean (SEM). ChemBiodraw Ultra version 18.0 was used to generate chemical structural formulas. The correlation analysis between the content of monomer PMFs and gene expression was performed and visualized by Chiplot

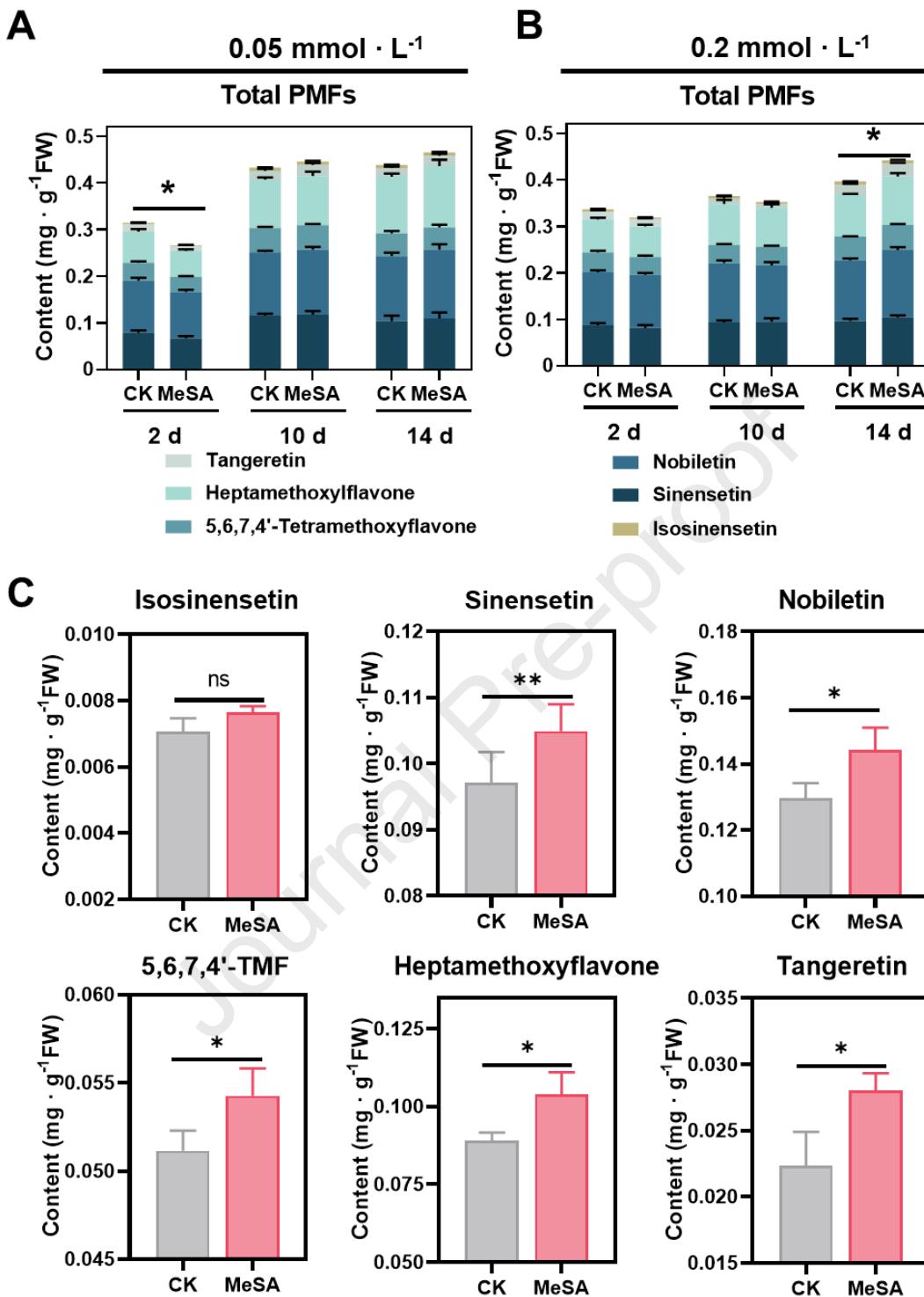
(<https://www.chiplot.online/>), with a confidence level of 95.0% (* $P < 0.05$), 99.0% (** $P < 0.01$) or 99.9% (***) $P < 0.001$). The heat map was performed by Chiplot as well. GraphPad Prism 8.0 (GraphPad Software; San Diego, CA, USA) was used to plot other figures. The significant differences between treatments were determined using paired two-tailed Student's *t*-tests. The asterisks indicate significant differences: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3. Results

3.1. Effects of MeSA treatment on PMF content of BTC fruit peels

MeSA has been reported to play a crucial role in the postharvest preservation of citrus fruits (Baswal et al., 2020). To investigate the effects of exogenous MeSA treatment on the content of PMFs, we applied two concentrations of MeSA (0.05 mmol · L⁻¹ and 0.2 mmol · L⁻¹) to the peels of mature 'Bingtangcheng' (BTC) fruit, along with storage durations of 2 days, 10 days, and 14 days. The PMF compounds in the BTC fruit peels, both with and without MeSA treatment, were detected and quantified using HPLC. As shown in the Fig. S2, A, six main PMF compounds were detected and identified. By comparing the results with authentic standards and reported studies (Zheng et al., 2023), these compounds were confirmed to be isosinensetin (P1), sinensetin (P2), nobiletin (P3), 5,6,7,4'-tetramethoxyflavone (P4), heptamethoxyflavone (P5), and tangeretin (P6) (Table S3). The chemical structures of these PMFs are illustrated in Fig. S2, B. Among the six PMFs, nobiletin was the most abundant, with concentrations ranging from 0.099 mg · g⁻¹ FW to 0.148 mg · g⁻¹ FW (Table S4).

The results presented in Fig. 1, A and B indicate that total PMF content increased over time during storage. After 14 days, the total PMF content of MeSA-treated group reached its maximum level of 0.458 mg · g⁻¹ FW. Remarkably, compared to the controls, the total PMF content was significantly lower in the 0.05 mmol · L⁻¹ group at 2 d (Fig. 1, A). Upon storage for 14 d, an increase in total PMF content was observed in the 0.2 mmol · L⁻¹ treatment group (Fig. 1, B), whereas the up-regulation was not significant in the 0.05 mmol · L⁻¹ treatment group (Fig. 1, A). Further analysis showed that, after 14 days of storage, five PMF monomers in the 0.2 mmol · L⁻¹ MeSA treatment group were significantly upregulated, except for isosinensetin, which had a very low content (Fig. 1, C). These results demonstrate that MeSA treatment for 14 days has a significant effect on PMF content of BTC fruit peels. The PMF content is correlated with the concentration of MeSA treatment and the duration of storage following treatment. Notably, the combination of 0.2 mmol · L⁻¹ MeSA treatment and storage for 14 days exhibits a pronounced promoting effect on the PMF content in the peels of BTC fruit.

**Fig. 1 Content of PMFs after MeSA treatment in the peels of BTC fruit**

(A) Content ($\text{mg} \cdot \text{g}^{-1}\text{FW}$) of total PMFs during storage with $0.05 \text{ mmol} \cdot \text{L}^{-1}$ MeSA treated. (B) Content ($\text{mg} \cdot \text{g}^{-1}\text{FW}$) of total PMFs during storage with $0.2 \text{ mmol} \cdot \text{L}^{-1}$ MeSA treated. (C) Content ($\text{mg} \cdot \text{g}^{-1}\text{FW}$) of PMF monomers at $0.2 \text{ mmol} \cdot \text{L}^{-1}$ 14 d treatment group. 5,6,7,4'-TMF, 5,6,7,4'-tetramethoxyflavone. The values of content are listed in Table S4. Values are means \pm SEM ($n = 4$). * $P < 0.05$ (Student's paired t-test).

3.2. Differential gene expression and functional pathway analysis in response to MeSA

It has been confirmed that PMFs increased in BTC peels treated with $0.2 \text{ mmol} \cdot \text{L}^{-1}$ MeSA at the 14-day time

point (Fig.1). For further investigating the potential mechanism in response to exogenous MeSA treatment for the PMF biosynthesis pathway, RNA-seq analysis was carried out. As shown in Fig. 2, A, 273 differentially expressed genes (DEGs) were detected between the MeSA treatment and sterile water treatment groups, including 202 up-regulated DEGs and 71 down-regulated DEGs. As shown in Fig. 2, B, the 273 DEGs were categorized into 27 GO terms across three main categories: 15 biological processes, 3 cellular components, and 9 molecular functions. Notably, most terms showed a higher abundance of upregulated DEGs than downregulated DEGs. DEGs related to biological processes were primarily involved in cellular processes, metabolic processes and response to stimulus. DEGs associated with cellular components were primarily linked to cellular anatomical entity, while the molecular function-related DEGs were mainly involved in binding, catalytic activity and transporter activity. The result of KEGG enrichment analysis is shown in Fig. 2, C, DEGs related to the phenylpropanoid biosynthesis, flavonoid biosynthesis and isoflavonoid biosynthesis pathways were significantly enriched (marked with red pentacle), which are considered to participate in flavonoid metabolism. Notably, these DEGs were significantly enriched in several phytohormone biosynthesis pathways, including brassinosteroid biosynthesis, alpha-linolenic acid metabolism, zeatin biosynthesis and cysteine and methionine metabolism pathways (marked with blue pentacle), which are involved in the biosynthesis of brassinolide (BR), jasmonic acid (JA), cytokinin (CTK) and ethylene (ET), respectively. DEGs related to plant-pathogen interaction pathways were also significantly enriched, consistent with previous studies showing that the exogenous application of MeSA enhances fruit resistance (Yang et al., 2023).

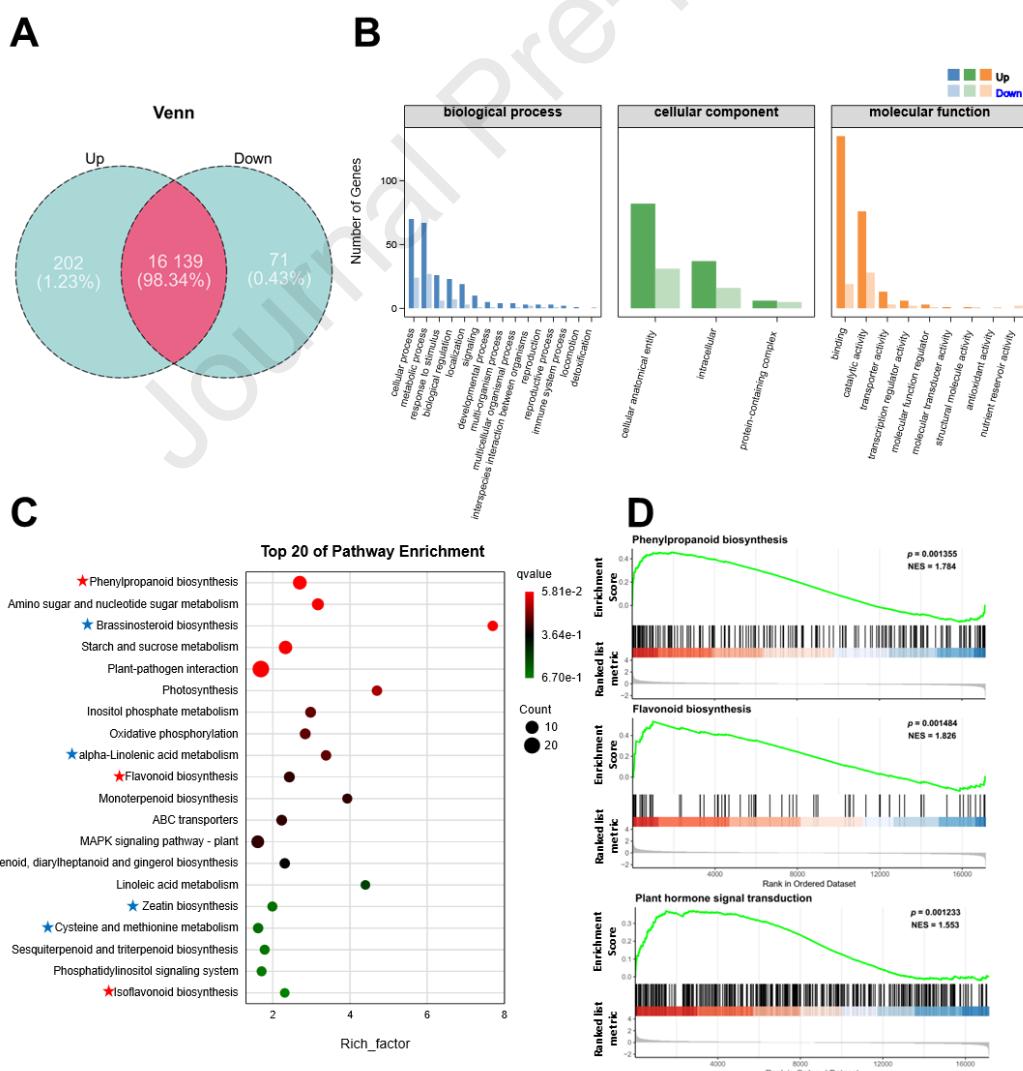


Fig. 2 The transcriptome analysis of MeSA treatment at 0.2 mmol · L⁻¹ 14 d treatment group

(A) The number of differentially expressed genes (DEGs). (B) GO classification of DEGs. (C) The top 20 enriched KEGG pathways of DEGs, with the flavonoid metabolism and phytohormones biosynthesis pathway marked by red and blue pentacle, respectively. (D) The GSEA analysis of DEGs involved in the phenylpropanoid biosynthesis, flavonoid biosynthesis, and plant hormone signal transduction

We further performed GSEA to observe the overall expression of these pathways. As shown in Fig. 2, D, after MeSA treatment, the pathways of phenylpropanoid biosynthesis, flavonoid biosynthesis, and phytohormone biosynthesis and signal transduction were significantly enriched ($\text{NES} > 1$, $P < 0.05$), with enrichment scores (ES) greater than 0, indicating that these three pathways were upregulated in response to MeSA treatment. Based on the above analysis, we focused on the pathways involved in phenylpropanoid biosynthesis, flavonoid biosynthesis, and phytohormone biosynthesis and signaling. We speculate that these pathways are crucial in mediating the MeSA-induced regulation of PMF biosynthesis.

3.3. Analysis of DEGs related to PMF biosynthesis pathways

The phenylpropanoid biosynthesis pathway was significantly enriched in the Top 20 KEGG pathways and is also involved in the biosynthesis of precursors in the PMF metabolic pathway. We identified and analyzed the DEGs enriched in the phenylpropanoid biosynthesis and flavonoid biosynthesis pathways. The results are shown in the Table S5, and Fig. 3, A shows the PMF biosynthesis pathway and related DEGs. PAL, a key upstream enzyme in the phenylpropanoid pathway, had its encoding gene (*Cs9g09020*) significantly up-regulated, while the gene encoding CHS (*Cs3g19350*) was down-regulated (Fig. 3, A; Table S5). FNSII has been identified as the key enzyme involved in the biosynthesis of PMFs in citrus (Zheng et al., 2023), and the related gene *Cs5g18660* (which is characterized as *CitFNSII-1*) was significantly up-regulated. Besides, one gene encoding F3'H (*orange1.1g03164*) showed a significant up-regulation trend, and two genes (*Cs8g14890* and *orange1.1g04984*) were down-regulated. COMT is a crucial enzyme in PMF biosynthesis; five members (*Cs5g13580*, *Cs5g16860*, *Cs5g18010*, *Cs5g18040*, and *Cs5g18050*) were significantly up-regulated, and one member (*orange1.1t04506*) exhibited significant down-regulation. RT-qPCR analysis of the *PAL*, *FNSII-1*, *F3'H*, and *COMT* genes demonstrated that their expression patterns were generally consistent with the trends observed in the RNA-seq data (Fig. S3). Moreover, the genes encoding cinnamyl-alcohol dehydrogenase (*Cs3g10950* and *Cs9g09020*) and shikimate O-hydroxycinnamoyltransferase (*Cs8g13150*) also showed an up-regulated trend (Table S5), indicating that MeSA might activate other phenylpropane branch pathways in addition to flavonoid biosynthesis.

Promoter analysis was employed to explore the regulatory mechanisms of these key DEGs, and Fig. 3, B shows the presence of cis-acting elements in the promoter regions. They contained a variety of hormone response elements, including methyl jasmonate (MeJA)-responsive element, abscisic acid (ABA)-responsive element, salicylic acid (SA)-responsive element, gibberellin (GA)-responsive element, and auxin (IAA)-responsive element (Fig. 3, B). Compared to other DEGs, the promoter region of *Cs5g18660* (*CitFNSII-1*) contains a relatively small number of cis-acting elements (14); however, it has a higher proportion of hormone-responsive elements (7/14), including two salicylic acid response elements (TCA-element). These cis-acting elements could play a role in responding to MeSA and regulate the expression of *Cs5g18660* (*CitFNSII-1*) (Fig. 3, B). Additionally, they are responsive to light, drought, stress, and low temperature. The genes encoding F3'H (*orange1.1t04984.1*, *orange1.1t03164.1*) have flavonoid biosynthesis regulation cis-acting elements (TTTTTACGGTTA). Thus, we presume that the application of exogenous MeSA treatment might activate genes associated with the phenylpropanoid and flavonoid biosynthesis pathways via hormone response elements, resulting in higher PMF content.

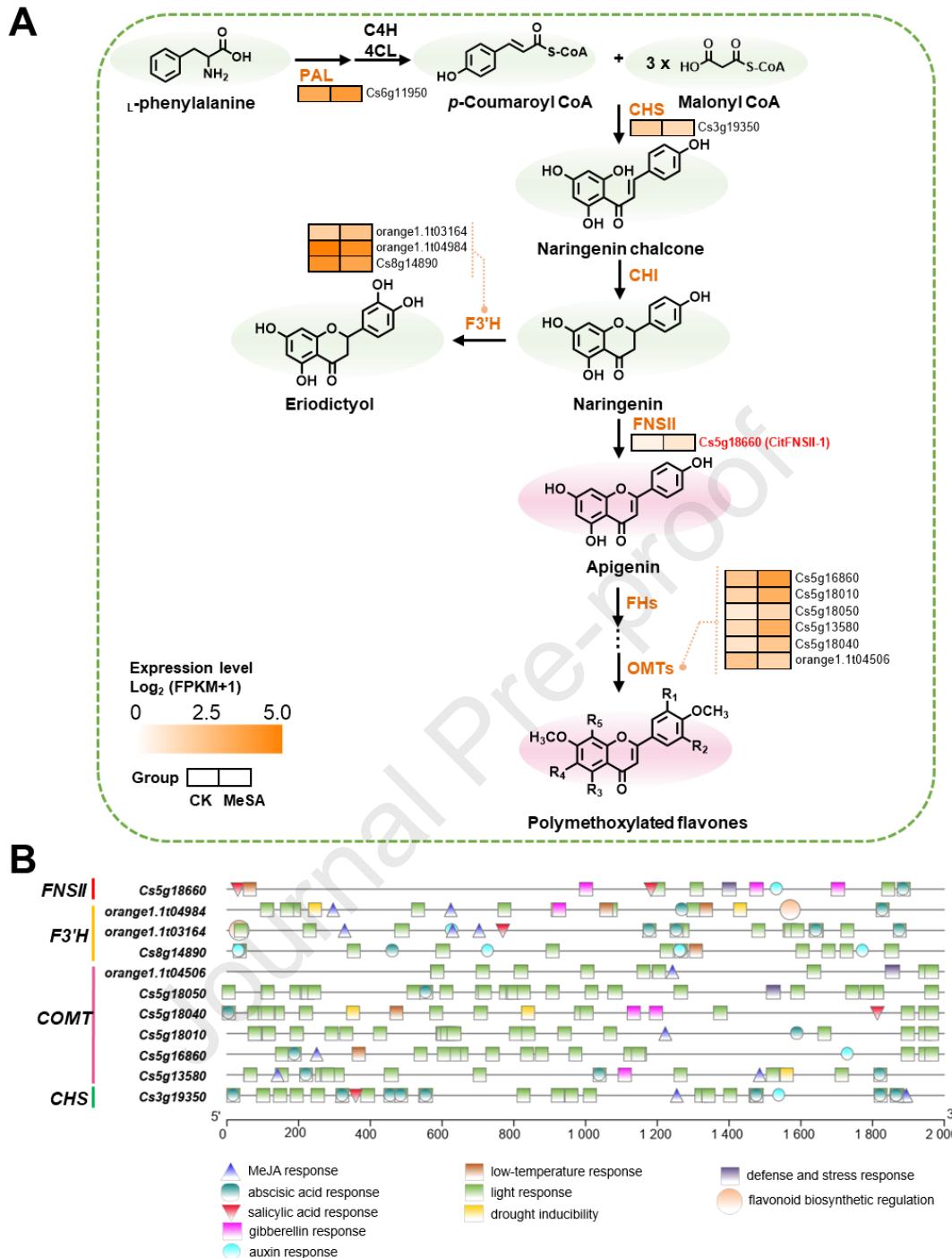


Fig. 3 Analysis of DEGs related to PMF biosynthesis pathway

(A) The DEGs were identified in the phenylpropanoid and flavonoid metabolism pathways. The color gradient from white to orange indicates the relative expression levels from low to high. Compounds with a green background represent metabolites from the phenylpropanoid and flvanones pathways, while compounds with a pink background represent metabolites from the PMF biosynthesis pathway. (B) Analysis of cis-acting elements in DEGs promoter with the visualization was performed by TBtools. PAL, phenylalanine ammonia-lyase; FNSII, flavone synthase II; F3'H, flavonoid 3'-monooxygenase-like; COMT, caffeic acid 3-O-methyltransferase; CHS, chalcone synthase.

3.4. Analysis of DEGs related to phytohormone biosynthesis and signal transduction

It has been reported that there are antagonistic or cooperative interactions between phytohormones, which may regulate the production of plant secondary metabolites (Ullah et al., 2022). Therefore, we screened the DEGs

involved in phytohormone biosynthesis and signal transduction, and the results are shown in Table S6. These DEGs are derived from different phytohormone biosynthesis and signal transduction pathways, including jasmonic acid (JA, 4 DEGs), brassinosteroid (BR, 6 DEGs), ethylene (ET, 2 DEGs), cytokinin (CTK, 2 DEGs), SA (5 DEGs), and IAA (1 DEG). All these DEGs were significantly up-regulated after the MeSA treatment and the expression heatmap of DEGs is shown in Fig. 4, A. However, the degrees of up-regulation of these DEGs induced by MeSA differed, with SA-related genes being up-regulated more significantly (Table S6). The SA-related DEGs were annotated to encode SA 3-hydroxylase (S3H) (*Cs9g14480*, *Cs9g14500*, *Cs9g14520*) and SA-dependent methyltransferase (SAMT) (*Cs1g24440*), which could hydroxylate and methylate salicylic acid, respectively (Table S6). The BR-related DEGs were the most prevalent, yet their expression levels were low (Table S7), which is due to the low levels of BRs present in plants (Bajguz and Tretyn, 2003). RT-qPCR was employed to validate six key genes, including *LOX* (JA-related, *orange1.1t04376*), *MYC2* (JA-related, *Cs4g14510*), *ACO* (ET-related, *Cs2g20590*), *S3H* (SA-related, *Cs9g14480*), *SAMT* (SA-related, *Cs1g24440*), and *UGT76B1* (CTK-related, *Cs7g32220*). The expression profiles of these genes were found to be consistent with the RNA-seq data (Fig. S3).

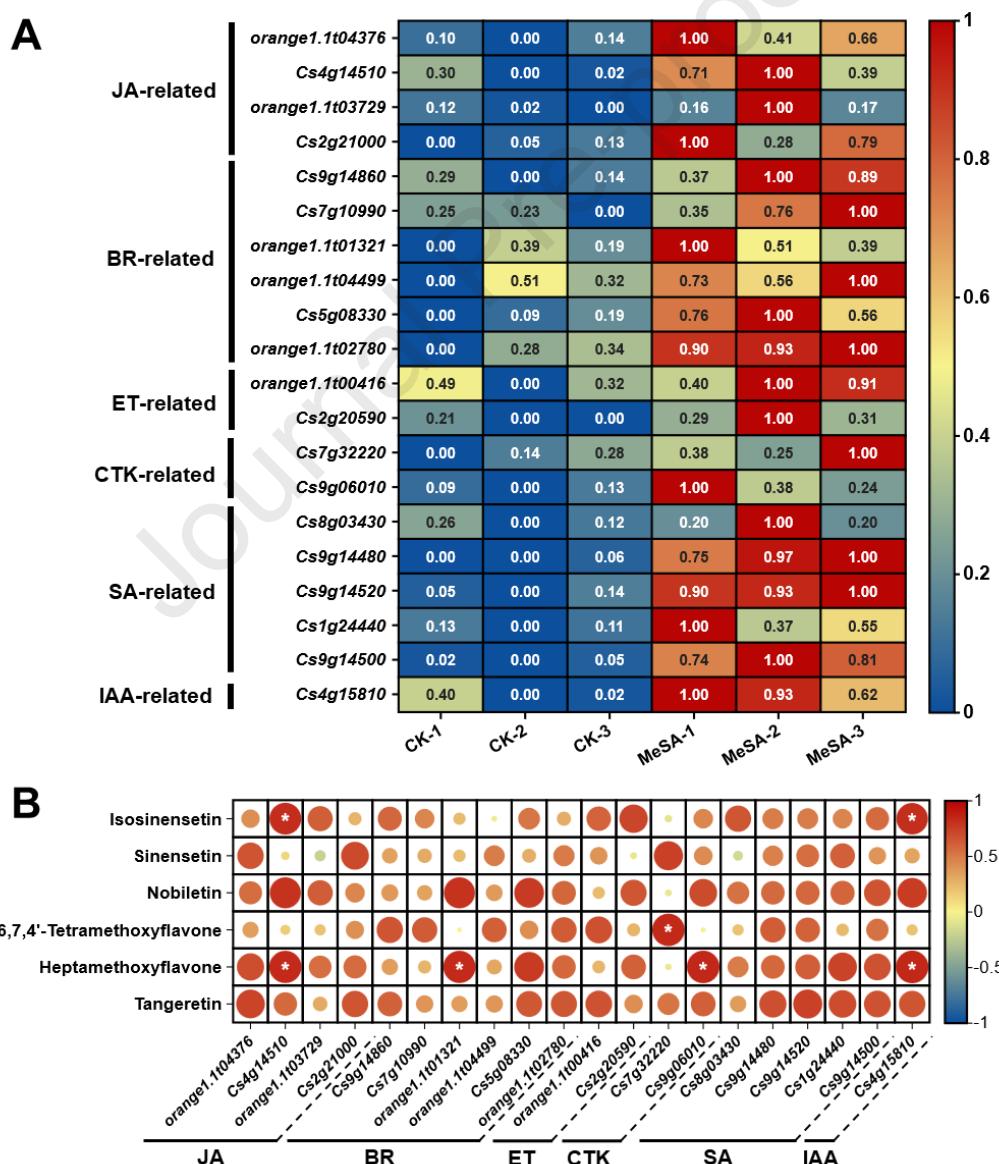


Fig. 4 Analysis of DEGs related to phytohormone biosynthesis and signal transduction pathways

- (A) Expression levels of DEGs involved in the phytohormone metabolisms. FPKM values listed in Table S7 are used for heatmap display after normalized by row.
(B) Correlation analysis between the expression levels of these DEGs and the contents ($\text{mg} \cdot \text{g}^{-1}$) of monomer flavonoid. The analysis was performed by Pearson's correlation coefficient. The asterisks indicate significant differences: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Furthermore, we analyzed the correlation between these DEG expressions and the content of PMF monomers. After MeSA treatment, the expression profiles of specific phytohormone pathway-associated genes exhibited a significant positive correlation with PMF accumulation, with the most pronounced correlation observed in heptamethoxyflavone (Fig. 4, B). These results suggested that the MeSA treatment induces the expression of endogenous hormone-related genes in citrus peels, and might have positive synergistic action on the biosynthesis of PMFs.

3.5. Analysis of candidate transcription factors mediating MeSA-induced PMF biosynthesis in citrus peel

Plant hormones affect the expression of other functional genes by regulating the activity of transcription factors (TFs). Among all DEGs, nine differentially expressed TFs were identified, including *Cs7g10990* (annotated as BRI1) and *Cs4g14510* (annotated as MYC2), which were significantly enriched in phytohormone biosynthesis and signal transduction pathways (Fig. 4, A). We analyzed the expression levels of other differentially expressed TFs to identify key TFs involved in flavonoid biosynthesis. Fig. 5, A showed that eight differentially expressed TFs from the AP2/ERF, WRKY, bHLH, TALE and CPP families were identified among the DEGs, which were predicted to bind the *CitFNSII-1* promoter region. After MeSA treatment, the expression levels of *CitERF24* and *CitTXC2* were down-regulated, while the other six TFs (*CitERF92*, *CitWRKY33*, *CitWRKY70*, *CitWRKY52*, *CitBEL1-like* and *CitMYC2*) were significantly up-regulated.

To explore the connection between these TFs and the PMF biosynthesis pathway, we chose *CitFNSII-1* (*Cs5g18660*), which has been characterized and induced by MeSA treatment, for correlation analysis. Fig. 5, B illustrates that the expression pattern of *CitERF92* is significantly correlated with *CitFNSII-1* (* $P < 0.05$). Therefore, we selected *CitERF92* as a candidate TF for further analysis. The multiple sequence alignment of *CitERF92* with homologous genes from *Arabidopsis* and other plant species revealed that *CitERF92* contains a conserved APETALA2 (AP2) domain, thus classifying it within the ERF subfamily of the AP2/ERF family (Fig. S4). Previous research has confirmed that *CitERF32/33* (Zhao et al., 2021) and *CitERF003* (Wan et al., 2023) are involved in the regulation of flavonoid biosynthesis in citrus. Phylogenetic analysis compared to *Arabidopsis* ERFs indicates that *CitERF92* is distinct from *CitERF32/33* and *CitERF003*, showing higher homology with *AtERF1A* and *AtERF2* from the B-3 subgroup of the ERF subfamily (Fig. 5, C). During fruit development, *CitERF92* expression in BTC peels initially goes up and then declines, with the peak expression occurring 80 days after flowering (DAF). 80 DAF onward, its transcript abundance remained significantly higher in the flavedo compared to the albedo (Fig. 5, D). *CitFNSII-1* displayed a delayed expression peak relative to *CitERF92*, with pronounced tissue-specific divergence between flavedo and albedo (Fig. 5, E). Total PMF accumulation patterns (Fig. 5, F; Table S8) mirrored this spatial regulation, consistent with the coordinated expression of *CitFNSII-1* and *CitERF92*. Notably, the primary PMF accumulation phase (30-80 DAF) demonstrated temporal overlap with the peak expression period of *CitERF92* (60-100 DAF), suggesting potential regulatory coordination during the early-mid development.

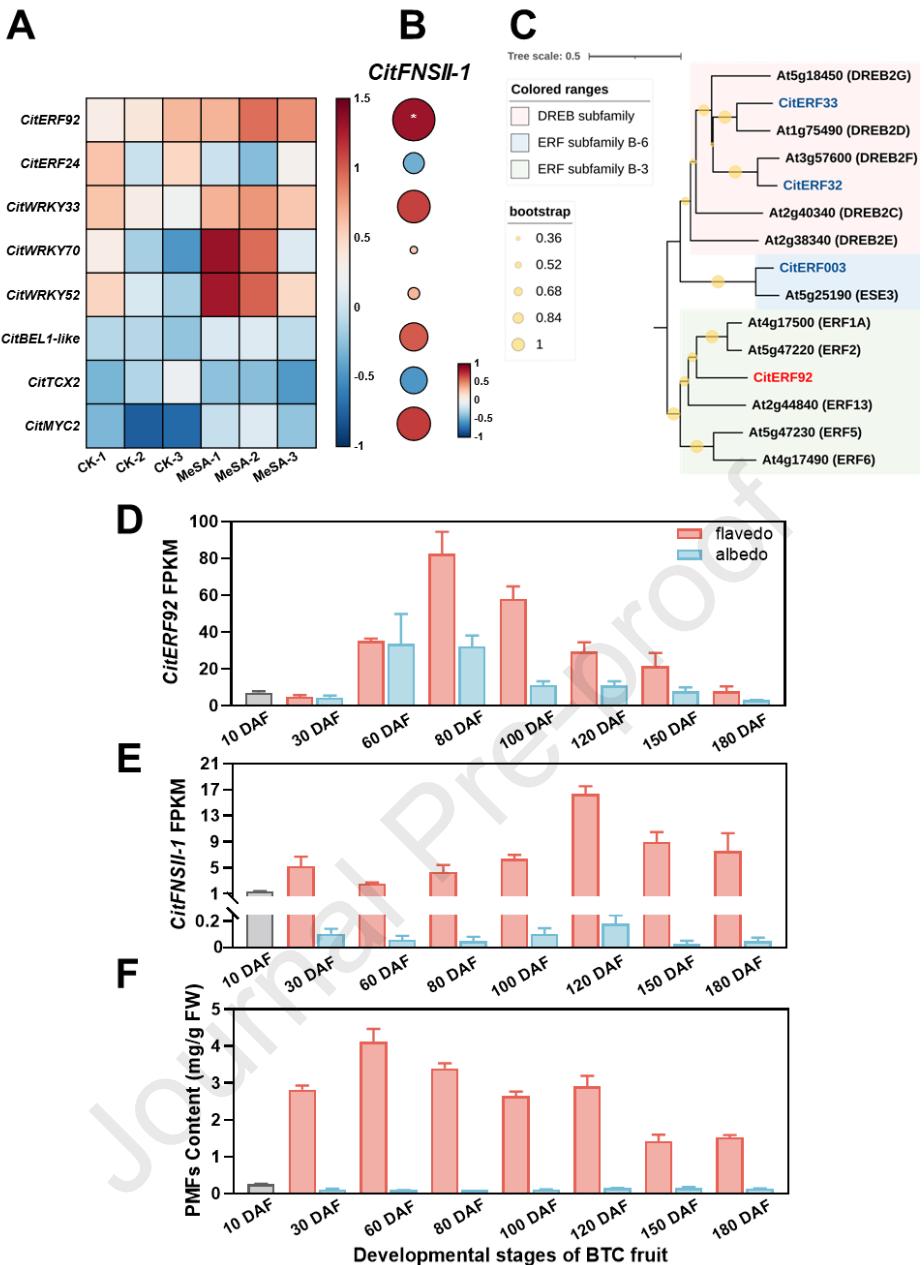


Fig. 5 Identification of PMF biosynthesis-related transcription factors in response to MeSA treatment

(A) Expression heatmap of differentially expressed transcription factors. FPKM values are used for the heatmap after the log10 conversion. (B) Correlation analysis between the expression levels of candidate transcription factors and *CitFNSII-1*. *CitFNSII-1* corresponds to the *Cs5g18660* gene of DEGs and has been reported to participate in flavone biosynthesis in citrus (Zheng et al., 2023). The analysis was performed by Pearson's correlation coefficient, *P < 0.05. (C) Phylogenetic analysis of CitERF92 and other ERF members from citrus and *Arabidopsis*. The evolutionary tree was constructed using the Neighbor-Joining method with 1,000 replicate bootstrap support, with bootstrap values indicated by orange dots whose sizes represent the corresponding values. CitERF32/33 were reported by Zhao et al. (2021); CitERF003 was reported by Wan et al. (2023). (D) Relative expression levels of *CitERF92* in the flavedo and albedo at developmental stages. (E) Relative expression levels of *CitFNSII-1* in the flavedo and albedo at developmental stages. (F) Total PMF content in the flavedo and albedo at developmental stages.

3.6. *CitERF92* binds to and activates the *CitFNSII-1* promoter

CitERF92 was cloned and inserted into a reporter vector for dual-luciferase assay. The result was shown in Fig. 6, A, which indicated that *CitERF92* functioned as a transcriptional activator and activated the promoter activity of *CitFNSII-1* with 1.96-fold promotion. The *CitERF92* protein was further purified for electrophoretic mobility shift

assay (EMSA) (Fig. S5). Results presented in Fig. 6, B demonstrated that the recombinant CitERF92 protein binds to the biotin-labeled *CitFNSII-1* probe, resulting in a shifted band. Furthermore, increasing the concentration of the specific competitor leads to a gradual weakening of this band, indicating the specificity of the CitERF92 protein binding to the *CitFNSII-1* promoter. These results indicate that exogenous MeSA treatment stimulates *CitFNSII-1* gene expression by upregulating *CitERF92*, leading to increased PMF levels in citrus.

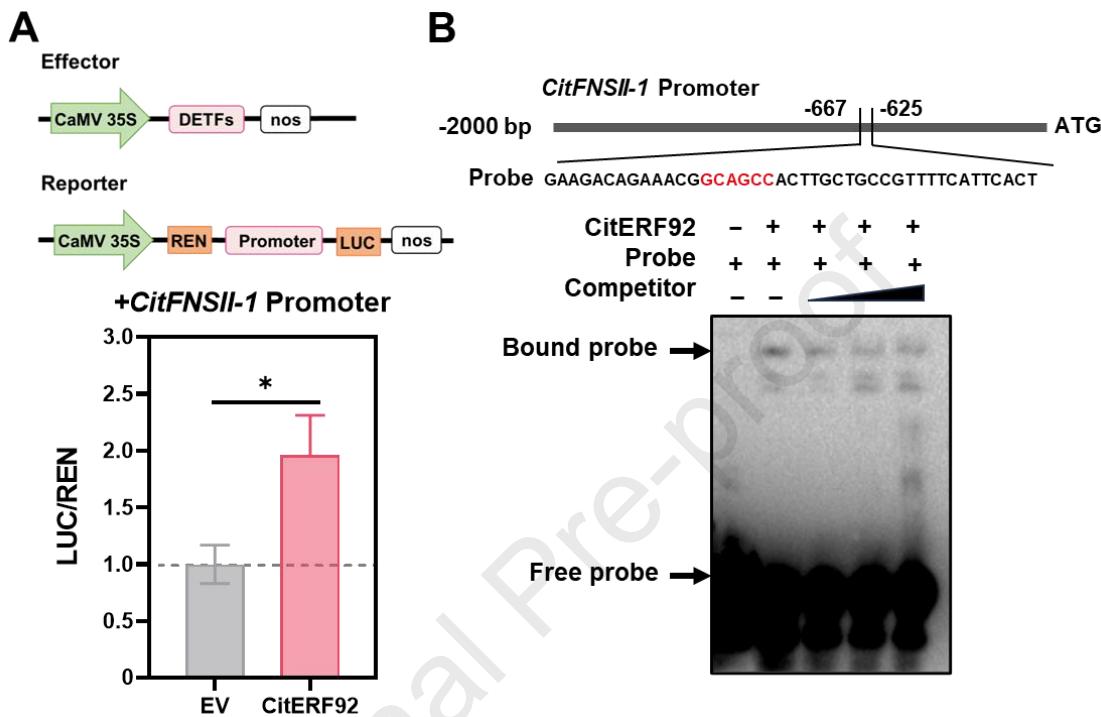


Fig. 6 CitERF92 activates *CitFNSII-1* and binds to its promoter

(A) Regulatory effects of *CitERF92* on the promoter of *CitFNSII-1*. The ratio of LUC/REN of the empty vector (EV) plus promoter was set as 1. Values are means ± SEM ($n = 3$). * $P < 0.05$ (Student's paired t-test). (B) EMSA of 3' biotin-labelled dsDNA probes with the *CitERF92*-binding protein.

3.7. Effect of overexpressing *CitERF92* in BTC fruit peel

To verify the promoting effect of *CitERF92* on PMF biosynthesis, we conducted a transient overexpression experiment by injecting suspensions containing *CitERF92*-pBI121 and control (pBI121) into the peel of BTC fruit. As shown in Fig. 7, A, *CitERF92* expression was significantly elevated in the overexpression group versus controls, confirming transient system efficacy. Moreover, *CitFNSII-1* exhibited a 2.9-fold upregulation, while PMF monomer accumulation was quantified in Fig. 7, B. However, the concentration of isosinensetin was too low for accurate quantification, so only the changes in the contents of the other five PMF monomers were analyzed. Compared to the control, the contents of sinensetin, nobiletin, 5,6,7,4'-tetramethoxyflavone (5,6,7,4'-TMF), heptamethoxyflavone, and tangeretin were all found to be increased to varying degrees, with increases of 24.0%, 28.0%, 24.7%, 30.1%, and 27.2%, respectively. The transient overexpression experiment provided direct evidence that *CitERF92* acts as a positive regulatory factor in the biosynthesis of PMFs by activating the transcription of *CitFNSII-1* in citrus.

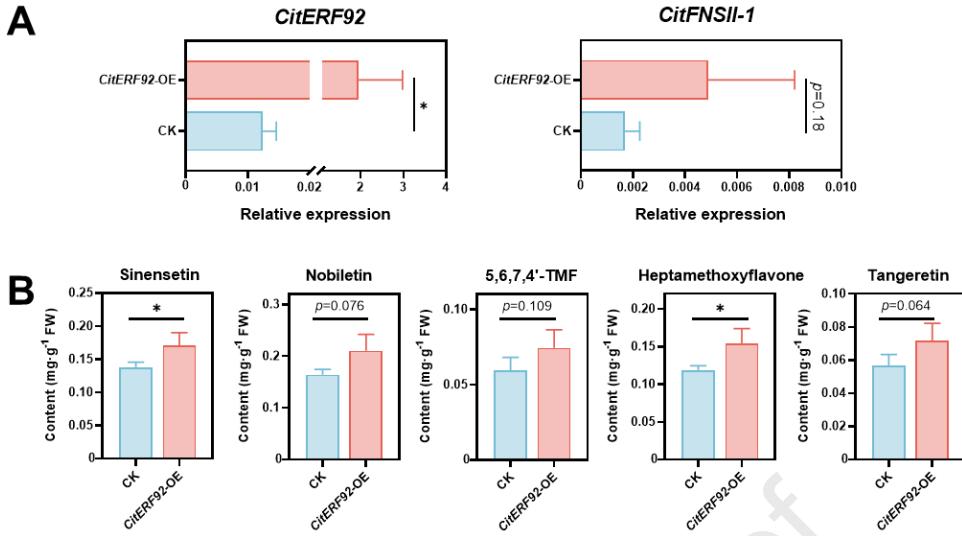


Fig. 7 Transient overexpression of *CitERF92* increased the content of PMF monomer in the peels of BTC

(A) The expression pattern of *CitERF92* and *CitFNSII-1* after *CitERF92-pBI121* was infiltrated. (B) Changes in the content of PMF monomers after *CitERF92* overexpressed. Values are means \pm SEM ($n = 10$). * $P < 0.05$ (Student's paired *t*-test).

4. Discussion

4.1. Exogenous MeSA activates the PMF biosynthesis pathway

SA and its derivatives have been shown to have the potential to improve the post-harvest quality of fruits and vegetables. MeSA is a class of volatile compounds that can be synthesized from SA by SAMT and, as a natural phytohormone, is generally considered safe (GRAS) for fruits (Fan et al., 2021). Previous studies have frequently used MeSA as a preservative to enhance fruit quality and extend storage time, and the effect of MeSA treatment concentrations varies (Fan et al., 2021). In citrus, PMFs are a specific type of flavonoid (Chen et al., 2015), and are well-known for their beneficial biological activities (Choi et al., 2011; Duan et al., 2017; Wang et al., 2021a). Furthermore, PMFs exhibit significantly enhanced ROS-scavenging capacity attributed to their structural methoxy groups (Wang et al., 2018).

Previous studies indicate that amino acid profiles at 10°C remain similar to day 0 after 14 days of storage, suggesting minimal quality alteration within this period (Matsumoto and Ikoma, 2012). To study the effect of MeSA on the regulation of PMF biosynthesis in citrus more comprehensively, two concentrations (0.05 mmol · L⁻¹, and 0.2 mmol · L⁻¹) of MeSA solutions and three points of storage duration (2 d, 10 d, and 14 d) were applied in BTC fruits. Compared to the control, the total PMFs initially showed a decreasing trend at 2 d, followed by an increase at 14 d during storage (Fig. 1, A), indicating that longer storage durations positively influence the accumulation of PMFs, but cannot be ruled out that progressive water loss in citrus peels increases dry matter concentration. These findings suggest that the regulation of PMF biosynthesis by MeSA might be a dynamic process, as reported for tea plants that were sprayed with foliar applications of MeSA (Li et al., 2019). Additionally, an appropriate concentration is important for postharvest treatments of fruits and vegetables (Habibi et al., 2020). This study demonstrated that the total PMF content in the 0.05 mmol · L⁻¹ MeSA treatment group exhibited a more pronounced decrease at 2 d, whereas the 0.2 mmol · L⁻¹ MeSA treatment group showed a significantly higher upregulation at 14 d (Fig. 1, A and B). Furthermore, under the 0.2 mmol · L⁻¹ MeSA treatment with 14 d, the PMF monomers (including nobletin, tangeretin, sinensetin, 5,6,7,4'-tetramethoxyflavone, and heptamethoxyflavone) were significantly upregulated (Fig. 1, C), indicating that this treatment is highly effective in enhancing the nutritional quality of citrus fruits and providing a critical breakthrough for value-added utilization of

citrus by-products. As functional components specifically accumulated in peels, elevated PMF levels substantially improve the development potential of citrus-derived secondary products.

The biosynthesis of flavonoids is derived from the phenylpropane pathway (Zhao et al., 2020). PAL is the first rate-limiting enzyme in this pathway, and several studies have reported that exogenous MeSA treatment considerably enhances PAL activity (Habibi et al., 2020; Li et al., 2017). Our transcriptome data showed that PAL (*Cs6g11950*) was up-regulated, suggesting that the flavonoid pathway was activated by MeSA in the first step. Similarly, the activity of C4H, 4CL CHS, and CHI has been reported to be activated by MeSA in tea leaves and winter jujube (Li et al., 2019; Yang et al., 2023). However, we found that a DEG encoding CHS (*Cs3g19350*) was down-regulated after treatment (Fig. 3, A). We detected the relative expression of the upstream genes *CitCHS1*, *CitCHS2* and *CitCHI* in various MeSA treatment groups using RT-qPCR. The findings indicated a lack of activation in gene expression, which was inversely related to flavonoid monomer levels (Fig. S6). The transcription level of *CHS* is reported to be inhibited by high concentrations of cinnamic acid (Loake et al., 1991), so the accumulation of cinnamic acid caused by the up-regulation of PAL might be one of the reasons for the inhibition expression of CHS. Consequently, we assumed that other significant genes might be involved in how MeSA regulates citrus flavonoid biosynthesis.

The biosynthesis of PMFs represents a branch of the flavonoid biosynthesis pathway (Zhao et al., 2020). Within the PMF biosynthesis branch, naringin is converted to flavone (apigenin) by FNSII and subsequently transformed into various PMFs by flavone hydroxylase (FH) and O-methyltransferase (OMT) (Fig. 3, A). In our study, *FNSII-1* (*Cs5g18660*), *F3'H* (*orange1.1t03164*) and *COMT* (*Cs5g16860*, *Cs5g18010*, *Cs5g18050*, *Cs5g13580*, and *Cs5g18040*) were up-regulated after MeSA treatment (Fig. 3, A), thereby promoting the biosynthesis of various monomeric PMFs (Fig. 1, C). Notably, *Cs5g18660* (*CitFNSII-1*) was identified as a pivotal enzyme in citrus for catalyzing the production of PMF precursors (Zheng et al., 2023). Although the upregulation fold of *CitFNSII-1* is not the highest among DEGs, its well-defined function merits further investigation. In brief, our findings indicate that exogenous MeSA chiefly enhances PMF production in citrus by increasing the expression of downstream genes, including FNS, F3'H, and COMT. This is the first report of such an effect in citrus and holds significant implications for further exploration of the transcriptional regulatory mechanisms underlying PMF biosynthesis.

4.2. Crosstalk between MeSA and other phytohormones and their role in maintaining quality during PMF accumulation

Phytohormones, as essential signaling molecules, coordinate intrinsic developmental processes and integrate environmental signals through hormone crosstalk, which includes synergistic, antagonistic, and additive interactions (Aerts et al., 2021). Analysis of the promoter regions of DEGs involved in the PMFs biosynthesis pathway revealed a widespread distribution of hormone response elements. This suggests that MeSA treatment may integrate signals from other hormones to co-regulate these DEGs. In our study, MeSA treatment increased the *SAMT* (*Cs1g24440*) expression, and the expression of three *S3H* (*Cs9g14480*, *Cs9g14500*, *Cs9g14520*) also increased (Table S6). It is known that SA and MeSA undergo SABP/SAMT-mediated interconversion (Ding and Ding, 2020), whereas S3H maintains SA homeostasis through metabolic conversion to 2,3-DHBA, preventing hyperaccumulation (Zhang et al., 2013). Therefore, we hypothesize that the application of exogenous MeSA suppresses the accumulation of endogenous SA in citrus peels through S3H- and SAMT-mediated metabolic regulation (Fig. S7). As an endogenous resistance signal, SA activates the expression of pathogenesis-related genes 1 (*PR1*) to strengthen plant resistance (Kong et al., 2020). In this study, MeSA treatment upregulated the expression of *PR1* (*Cs8g03430*) gene and enhanced fruit resistance against postharvest diseases including *Penicillium italicum* and *Penicillium digitatum* infection (Ortuño et al., 2006).

JA biosynthetic genes (*LOX*, *HPL*, *OPR*) and the *MYC2* transcription factor demonstrated coordinated upregulation (Fig. 4, A), showing positive correlation with PMF accumulation (heptamethoxyflavone, Fig. 4, B).

This observation aligns with SA-JA synergy in flavonoid induction (Ullah et al., 2022). Notably, MeSA-induced *CitWRKY70* overexpression (Fig. 5, A) may mediate SA/JA crosstalk by positively regulating SA-responsive genes (e.g., *PR1*) while suppressing JA signaling (Fig. S7) (Li et al., 2004). Therefore, it is hypothesized that MeSA treatment enhances PMF accumulation in citrus through a JA-dependent pathway, ultimately improving postharvest storage quality.

Brassinosteroids (BRs) are novel steroidal growth regulators and present at low concentrations across the plant kingdom (Bajguz and Tretyn, 2003). The involvements of BRs in flavonoid metabolism have been documented in *Arabidopsis thaliana*, *Vitis vinifera*, and *Malus domestica* (Liang et al., 2020; Wang et al., 2021b; Xi et al., 2013). BR signaling components—three cytochrome P450 genes (*orange1.1t01321/4999/2780*), *BRI1* (*Cs9g14860/7g10990*), and *BAK1* (*Cs5g08330*)—were activated (Fig. 4, A), supporting BRs' role in flavonoid metabolism. Brassinosteroid-INSENSITIVE 2 (BIN2), the downstream target of *BRI1* and *BAK1*, functions as a negative regulator of SA-responsive genes, thereby establishing an antagonistic crosstalk between BR and SA signaling pathways (Kim et al., 2022).

Furthermore, we found an up-regulation in the expression of *UGT76B1* (*Cs7g32220*) and *IPT5* (*Cs9g06010*) (related to cytokinin biosynthesis); as well as *YUC2* (*Cs4g15810*) (related to IAA biosynthesis) (Fig. 4, A). Crucially, these phytohormone-related genes positively correlated with flavonoid accumulation (Fig. 4, B), indicating exogenous MeSA promotes PMF biosynthesis through integrated hormonal regulation.

4.3. MeSA induces the *CitERF92-CitFNSII-1* module to promote the accumulation of PMFs in citrus

Ethylene induces the biosynthesis of flavonoids in fruits, such as pears, apples, and strawberries (An et al., 2018; Given et al., 1988; Yao et al., 2017). Ethylene response factors (ERFs), which belong to the AP2/ERF family, are crucial in various biological processes and for how plants respond to environmental stresses (Nakano et al., 2006). Furthermore, ERF has been reported to function as a central hub mediating interactions among diverse hormone signaling response factors (Hu et al., 2022). Previous studies have shown that certain ERFs regulate the flavonoid biosynthesis. For example, Ni et al. (2019) demonstrated that overexpression of *Pp4ERF24* or *Pp12ERF96* in pear enhances anthocyanin accumulation; while An et al. (2018) found that *MdERF3* is associated with anthocyanin biosynthesis in apples. Moreover, the regulation of flavonoids by ERFs is influenced by phytohormones, primarily JAs and ethylene. For instance, *FtERF-EAR3* from buckwheat is induced by JA and suppresses the expression of *FtF3H*, thus inhibiting flavonoid biosynthesis (Ding et al., 2022); similarly, *ZbERF3* is induced by ethylene and promotes anthocyanin biosynthesis in pepper fruits (Zhang et al., 2024). Although numerous studies have reported that SA affects flavonoid biosynthesis, the relationship between salicylic acid and ERFs expression levels remains unclear. Our study found that exogenous MeSA treatment not only increased the PMF content in citrus but also induced the expression of two ERF members (*CitERF92* and *CitERF24*), suggesting that ERF transcription factors mediate the regulation of citrus PMF biosynthesis by MeSA.

In citrus, Zhao et al. (2021) identified two ERF subfamily members, *CitERF32/33*, as being involved in the regulation of citrus flavonoid biosynthesis. These transcription factors promoted the transcription of the upstream gene *CHI1* and increased the total flavonoid content in 'Oukan'. Wan et al. (2023) found that *CitERF003* in 'Newhall' mutant functions as a positive regulator of flavonoids. Additionally, it is predicted that ERFs regulate key genes involved in the biosynthesis of flavonoid during the development of *Citrus aurantium* fruit (Chen et al., 2022). In this study, we identified a MeSA-induced *CitERF92* among differentially expressed transcription factors. The expression of *CitERF92* demonstrated a significant positive correlation with the expression levels of *CitFNSII-1* and was highly expressed in flavedo tissue (PMF-rich), indicating that it is a candidate transcription factor that plays a role in regulating PMF biosynthesis (Fig. 5). We analyzed the evolutionary relationship between *CitERF92* and *CitERF32/33*, and *CitERF003* in citrus, and their homologous genes in *Arabidopsis*. Fig. 5, C shows that *CitERF92* belongs to the ERF subfamily B-3 subgroup. Members of the ERF B3 subgroup are believed to regulate

plant disease resistance by coordinating or antagonizing the integration of SA, JA, and ethylene signaling pathways (Pré et al., 2008; Zang et al., 2021). In our study, we found that MeSA treatment significantly up-regulated the expression of genes associated with ethylene biosynthesis, such as *ACS* (*orange1.1t00416*) and *ACO* (*Cs2g20590*). This suggests that MeSA could enhance the biosynthesis of endogenous ethylene. Furthermore, *CitERF92* may regulate gene expression within the PMF biosynthesis pathway by integrating signals from both MeSA and ethylene.

CitFNSII-1/2 catalyze the formation of flavone compounds and are key enzymes that influence the flux of the flavonoid biosynthesis pathway toward PMFs (Zheng et al., 2023). Previous studies showed that $1 \text{ mmol} \cdot \text{L}^{-1}$ MeSA treatment on BTC peel followed by 6 d of storage led to a decrease in PMF content, and was primarily driven by *CitFNSII-2* (Zheng et al., 2023). Similarly, we observed a reduction in PMF accumulation at 2 d after MeSA treatment, but a significant increase at 14 d. Transcriptomic analysis revealed that MeSA treatment induced the expression of *CitFNSII-1* at 14 days (Fig. 3, A). The regulation of PMF content by MeSA is a dynamic process, which may be attributed to the differential responses of *CitFNSII-2* (at 6 d) and *CitFNSII-1* (at 14 d) to MeSA signaling. The promoter region of *CitFNSII-1* contains numerous hormone-responsive elements, suggesting that hormone signals regulate its expression (Fig. 4, B). As a co-expressed transcription factor of *CitFNSII-1*, *CitERF92* has been shown to bind and activate the promoter region of *CitFNSII-1* *in vitro* (Fig. 6). Furthermore, transient overexpression experiments in BTC peel confirmed that *CitERF92* promotes PMF biosynthesis by activating the transcription of *CitFNSII-1* (Fig. 7).

In this work, we found that exogenous MeSA has a dynamic regulatory effect on the biosynthesis of PMFs in citrus peel during storage. Exogenous MeSA significantly increased the level of PMF monomers in citrus, which was dependent on the treatment method ($0.2 \text{ mmol} \cdot \text{L}^{-1}$ at 14 d of storage). Transcriptome analysis revealed that MeSA promoted the accumulation of PMFs mainly by increasing the expression of downstream key genes (*FNS*, *F3'H*, and *COMT*). Moreover, genes associated with biosynthesis and signal transduction of phytohormones (JA, BR, ET, CTK, SA, IAA) were activated. This suggests that these phytohormones might have a synergistic interaction with MeSA in the flavonoid biosynthesis of citrus. It was also discovered that *CitERF92*, an ethylene response factor, acts as a positive regulator in the MeSA-mediated biosynthesis of PMFs by promoting the expression of *CitFNSII-1*. These results offer a theoretical and molecular basis for the mechanism of MeSA-mediated PMF accumulation and provide a new perspective for citrus post-harvest quality improvement (Fig. 8).

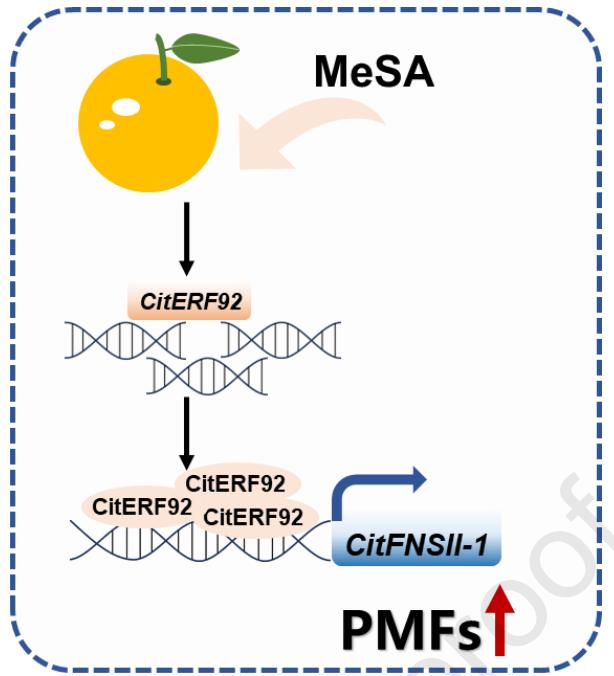


Fig. 8 Schematic diagram of the MeSA-induced *CitERF92*-*CitFNSII-1* module regulating PMF biosynthesis in citrus

CitERF92 is induced by exogenous MeSA and activates the expression of *CitFNSII-1* by binding to its promoter. As a key enzyme in the PMF biosynthesis pathway, *CitFNSII-1* directly enhances the PMF content in the peel of BTC fruit.

Acknowledgments

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Data availability

The RNA-Seq data reported in this article can be obtained in the Sequence Read Archive (SRA) under the project number PRJNA1215551 and PRJNA924350 (Zheng et al., 2023).

Declaration of interests

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.

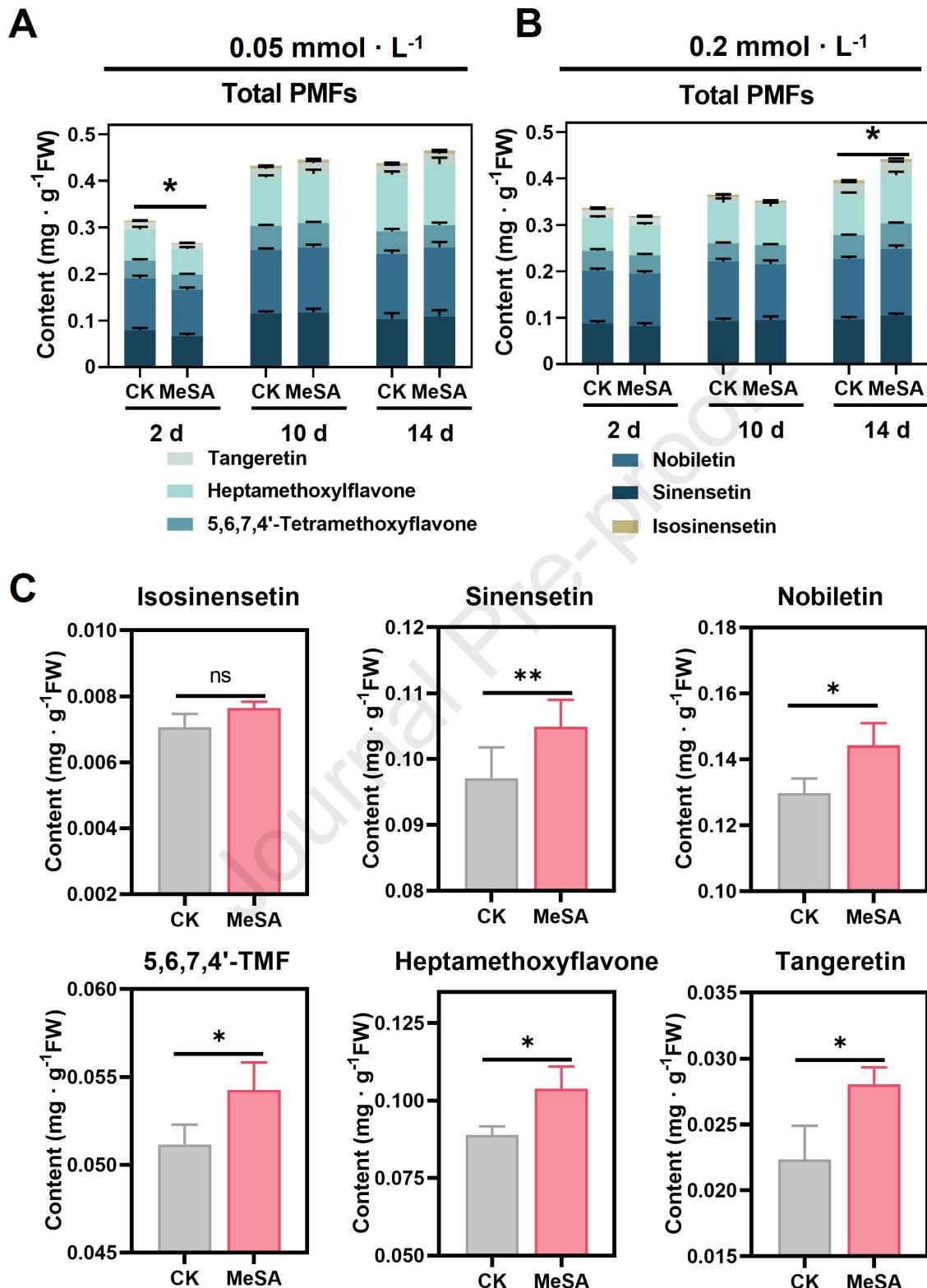
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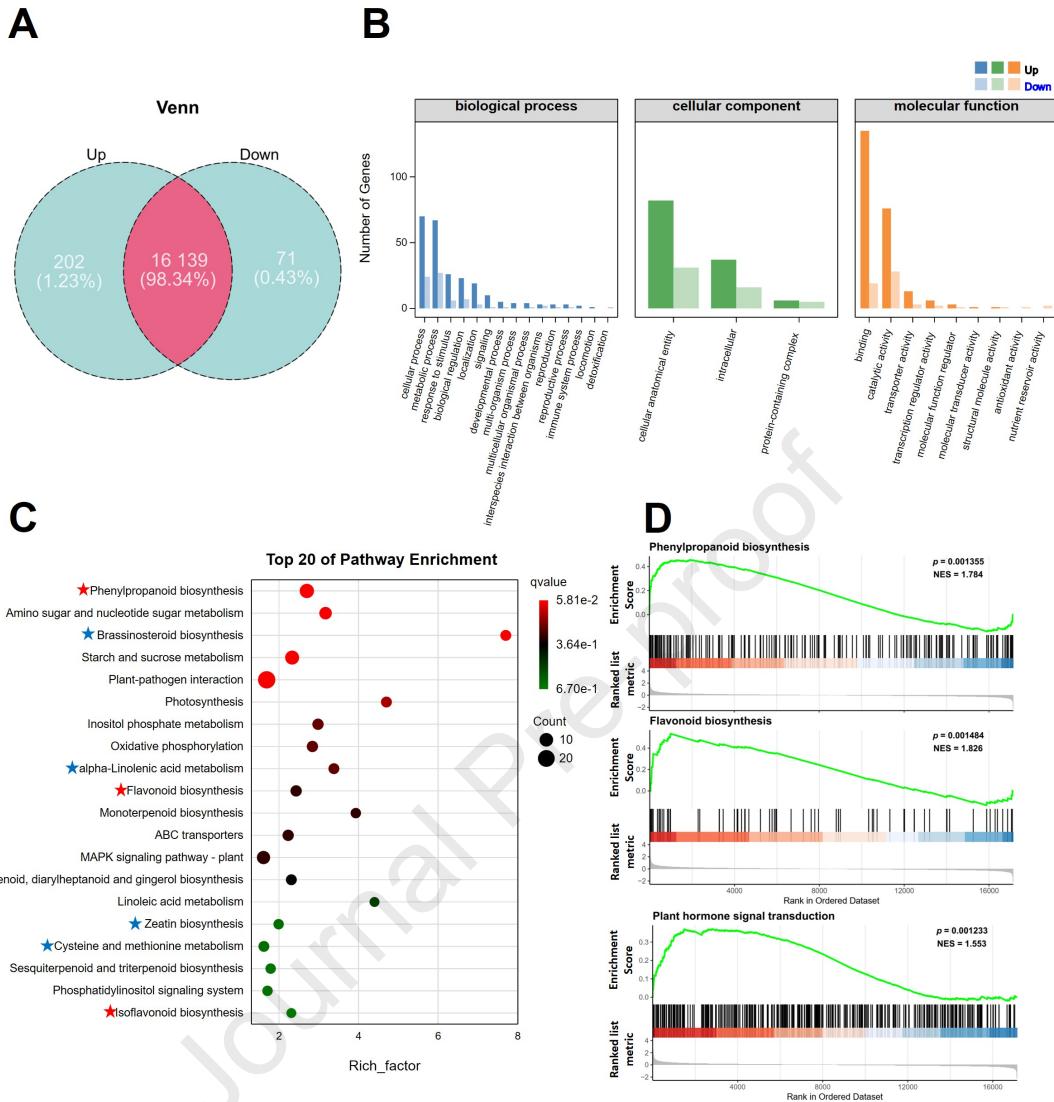
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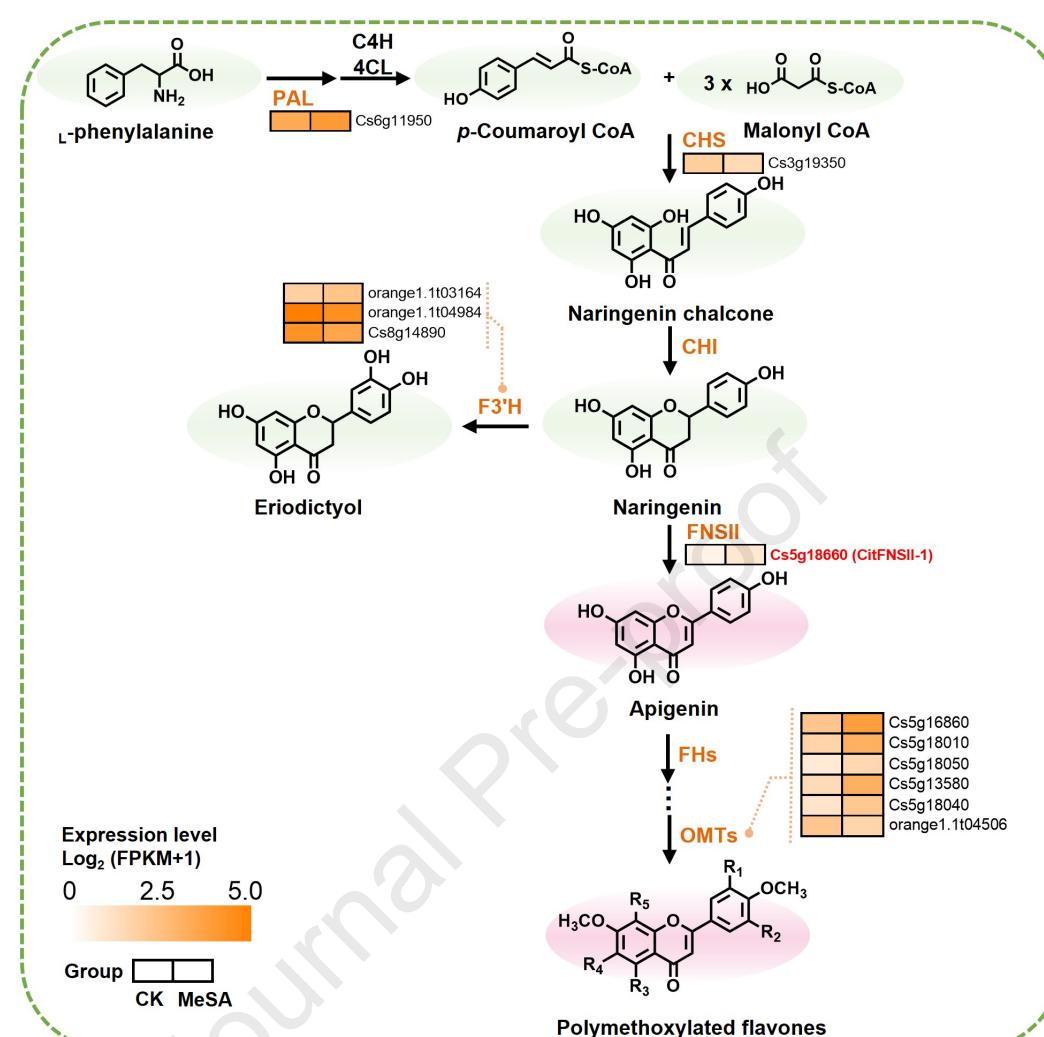
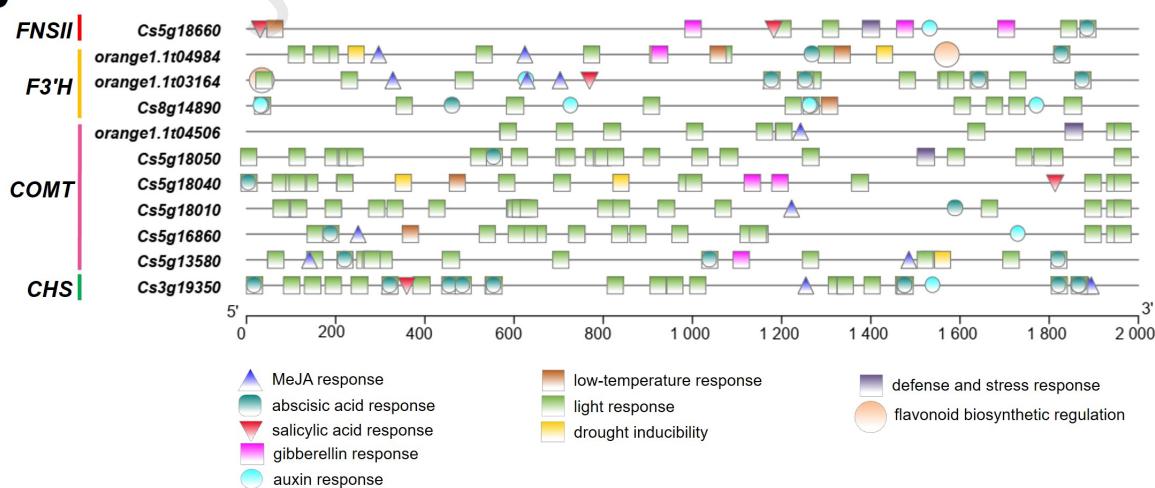
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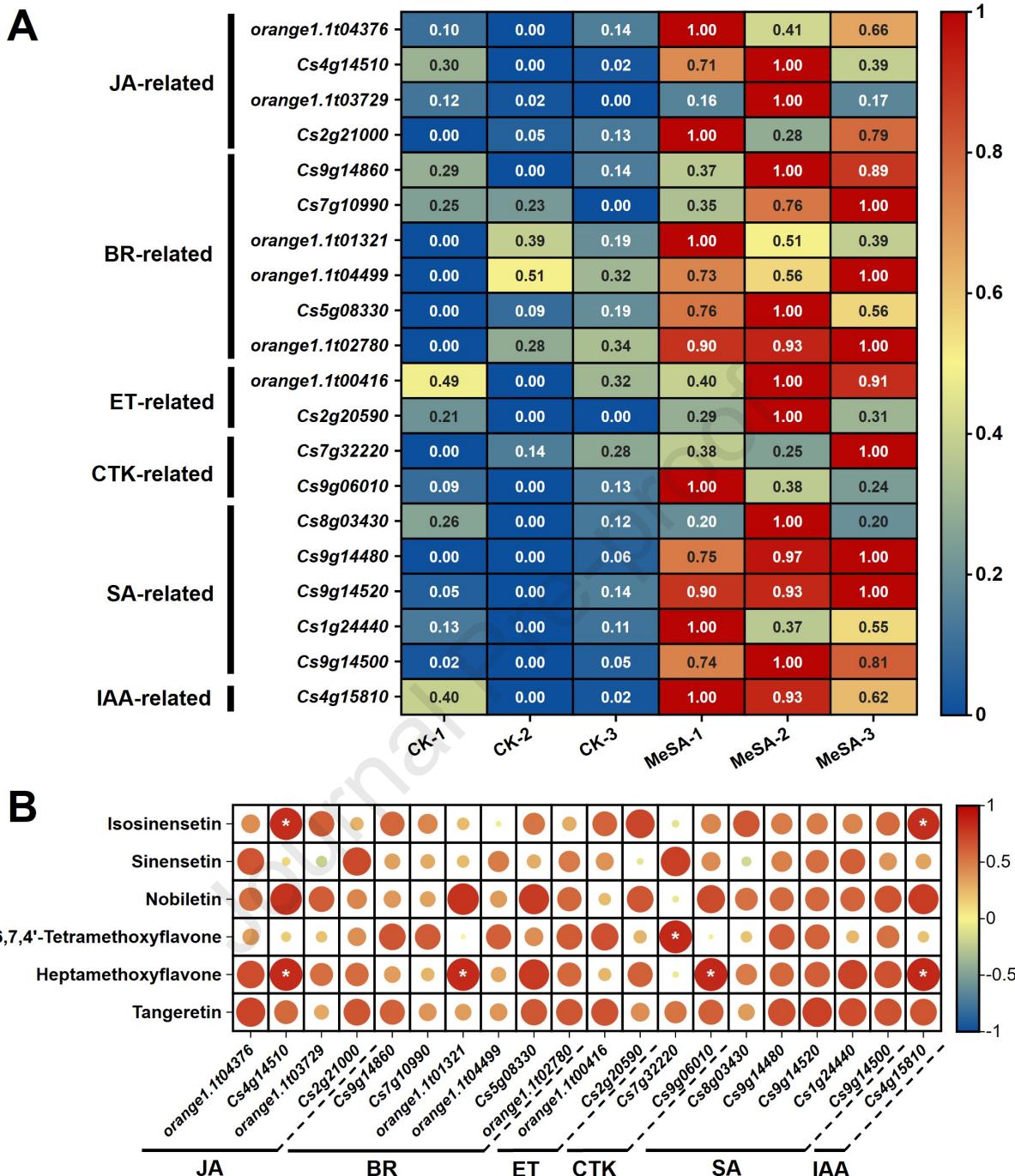
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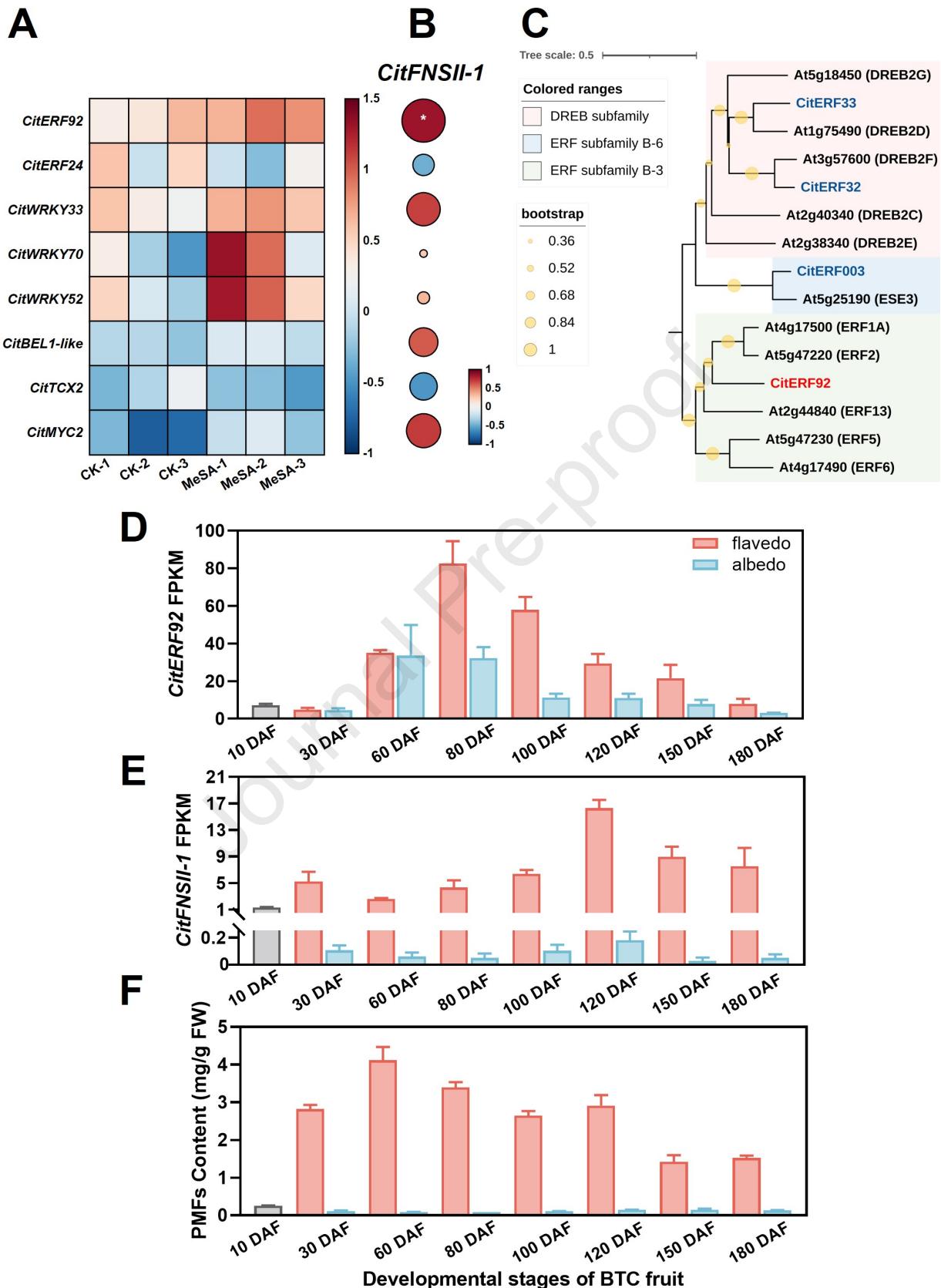
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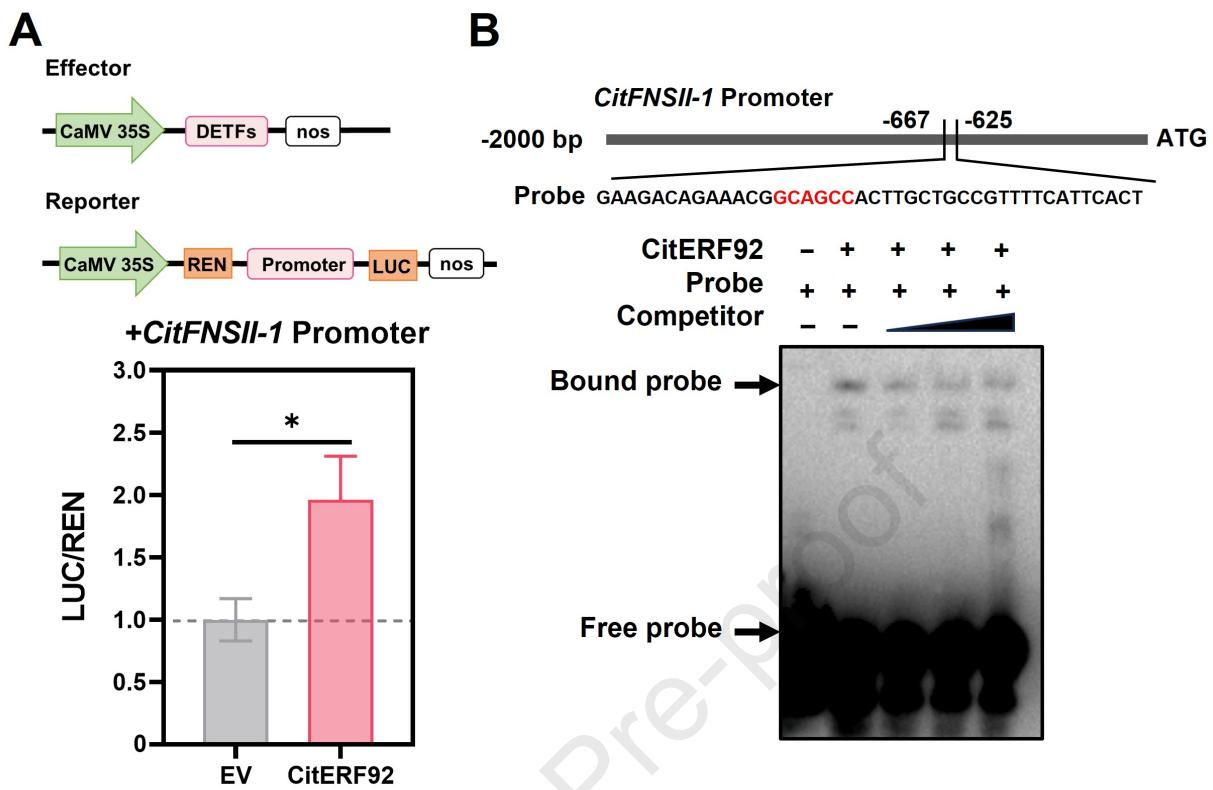


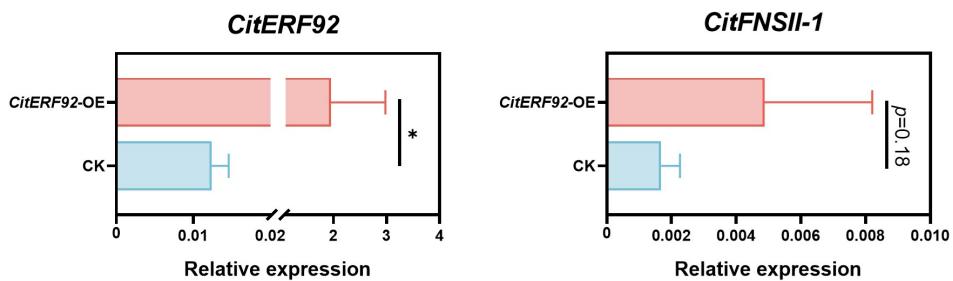
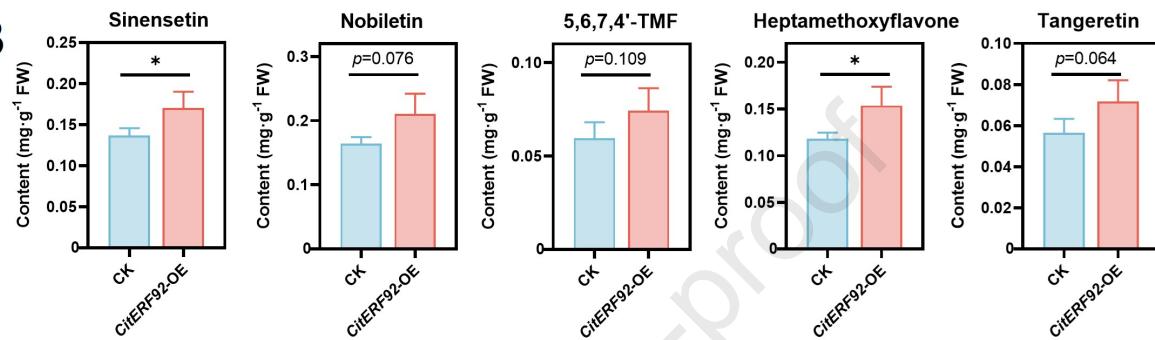
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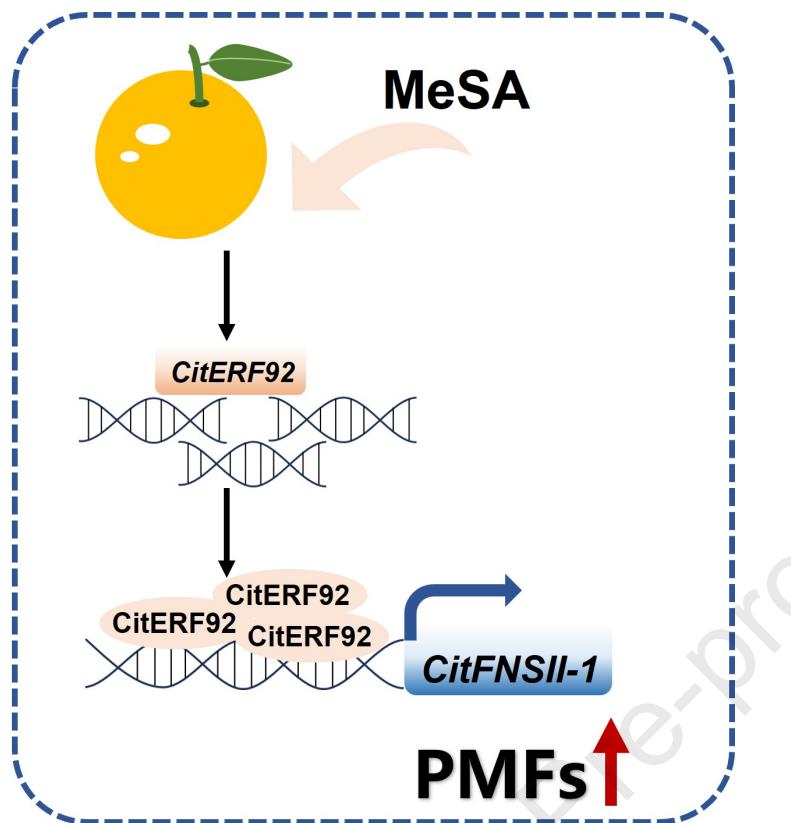




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