

Functional Characterization of a Highly Efficient UDP-Glucosyltransferase CitUGT72AZ4 Involved in the Biosynthesis of Flavonoid Glycosides in Citrus

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ABSTRACT: Citrus is an important dietary source of flavonoid glycosides, and UDP-glycosyltransferases (UGTs) are the key enzymes responsible for their glycosylation. In this study, a genome-wide analysis of the *CitUGT* gene family was conducted to identify *CitUGTs* that contribute to flavonoid 4'-O-glucosides biosynthesis. Our analysis identified 136 *CitUGTs* in the *Citrus clementina* genome, classifying them into 18 phylogenetic groups (A–R) and 25 families. This classification was strongly supported by consistent gene structures and motif patterns. Moreover, we identified a *CitUGT* gene (*Ciclev10025462m*, designated *CitUGT72AZ4*) that encodes flavonoid 4'-O-glucosyltransferase for the first time in citrus. This enzyme preferentially glycosylated the 4'-OH group of multiple flavonoids, exhibiting higher catalytic efficiency toward quercetin and three flavones *in vitro*. Virus-induced gene silencing of *CitUGT72AZ4* significantly decreased the accumulation of flavonoid 4'-O-glucosides. These results indicated that *CitUGT72AZ4* participated in the biosynthesis of flavonoid 4'-O-glucoside in citrus. Overall, our findings provide valuable insights into the *CitUGT* gene family and contribute to its functional characterization.

KEYWORDS: flavonoid glycoside, UDP glycosyltransferase, glycosylation, citrus, gene expression

1. INTRODUCTION

Flavonoids are a diverse group of secondary metabolites found extensively throughout the plant kingdom. Numerous studies have shown that flavonoids are essential not only for promoting plant growth and defending against a variety of biotic and abiotic stresses^{1,2} but also for providing nutritious compounds for humans.³ These compounds can be categorized into six distinct subgroups, including flavanones, flavones, flavonols, isoflavones, flavanols, and anthocyanidins.⁴ In plants, flavonoids are commonly present in the form of *O*- or *C*-glycosides, and glycosylation is a pivotal modification for the biosynthesis of flavonoid glycosides, enhancing their stability, solubility, and bioactivity, as well as contributing to their structural and functional diversity.^{5,6}

Glycosylation of flavonoids is usually catalyzed by UDP glycosyltransferase (UGT), which transfers a glucose moiety from UDP sugars to various small-molecule acceptors, such as flavonoids, terpenes, and alkaloids.⁷ These enzymes are largely characterized by a highly conserved sequence of 44 amino acids at their C-terminus, called the plant secondary product glycosyltransferase box (PSPG box), which is believed to serve as the sugar donor binding site.⁸ Conversely, the sequences at the N-terminus varied greatly, which are considered to be involved in the recognition of different acceptors. Rapid advances in genomics and bioinformatics have facilitated the identification of numerous UGTs in various species.⁹ Phylogenetic analysis has classified 107 UGT genes in *Arabidopsis thaliana* into 14 groups (A–N),¹⁰ and four additional groups (O, P, Q, R) were later found in *Prunus*

persica,¹¹ *Vitis vinifera*,¹² *Zea mays*,¹³ and *Camellia sinensis*.¹⁴ Previous studies have revealed that several UGTs display strict position specificity when they catalyze the glycosylation of flavonoids. For instance, NmF4'GT (*Nemophila menziesii*) specifically glycosylated the 4'-OH position of apigenin, and the product was further glycosylated at their 7-OH positions by NmF4'G7GT.¹⁵ However, some UGTs could glycosylate flavonoids at multiple hydroxyl positions. For example, FaGT6 (*Fragaria × ananassa*) could glycosylate quercetin at their 3-OH, 7-OH, 3'-OH, and 4'-OH positions.¹⁶

Citrus (*Citrus reticulata* Blanco) is one of the most extensively cultivated fruits, containing abundant bioactive compounds, such as flavonoids, dietary fiber, and carotenoids. Flavonoid glycosides are essential bioactive compounds in citrus, which have attracted great attention owing to their beneficial effects on human health, such as antioxidant,¹⁷ anti-inflammatory,¹⁸ and antifungal activities.¹⁹ For example, quercetin 4'-O-glucoside, isolated from *Citrus aurantifolia* leaves, showed a superior free radical scavenging activity compared to vitexin 7-O-arabinoside and isovitexin.²⁰ Narirutin 4'-O-glucoside, identified in tangelo (*C. × tangelo*) juice, exhibited antimicrobial activity by effectively inhibiting the

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growth of *Staphylococcus aureus*.²¹ Moreover, citrus is well-known for its high abundance of flavonoid glycosides, with some compounds (e.g., naringin and neohesperidin) contributing to the bitterness of citrus fruits and impacting its flavor characteristics.²² So far, few *CitUGTs* related to flavonoid glycoside biosynthesis have been discovered in citrus. For instance, *CitUGT89AK1* has been characterized as flavonoid 7-O-glycosyltransferase that catalyzed the glycosylation of flavonoid aglycones at their 7-OH positions to form the corresponding flavonoid 7-O-glucosides (e.g., naringenin 7-O-glucoside and hesperetin 7-O-glucoside).²³ These products were further glycosylated to form bitter neohesperidosides (e.g., neohesperidin and naringin) and non-bitter rutinosides (e.g., hesperidin and narirutin) by 1,2-rhamnosyltransferase (1,2RhaT) and 1,6-rhamnosyltransferase (1,6RhaT), respectively.^{24,25} Recently, *CgUGT4*, encoding flavonoid 7-O-glycosyltransferase, together with *Cg1,2RhaT* and acyltransferases (*CgATs*), has been shown to form a metabolic gene cluster responsible for melittidin biosynthesis in pummelo.²⁶ However, the specific *CitUGTs* responsible for flavonoid 4'-O-glucoside biosynthesis has not yet been identified in citrus.

The availability of genomic, transcriptomic, and metabolomic data has made it possible to identify *CitUGTs* involved in the flavonoid glycoside biosynthesis in citrus. Here, we conducted an exhaustive genome-wide investigation of the *CitUGT* gene family, covering their phylogenetic relationships, gene structures, conserved motifs, chromosomal locations, gene duplications, and synteny analysis. Through phylogenetic and transcriptome analysis, we identified a *CitUGT* gene (*Ciclev1002S462m*, designated *CitUGT72AZ4*) that encodes flavonoid 4'-O-glucosyltransferase for the first time in citrus and confirmed its function in flavonoid 4'-O-glucosides biosynthesis by *in vitro* and *in vivo* assays. Our study provides valuable information on the *CitUGT* gene family, facilitating their functional characterization.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Standards including naringenin, hesperetin, eriodictyol, isosakuranetin, apigenin, luteolin, chrysoeriol, acacetin, diosmetin, quercetin, kaempferol, and isorhamnetin were purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, China). Naringenin 7-O-glucoside, naringenin 4'-O-glucoside, hesperetin 7-O-glucoside, eriodictyol 7-O-glucoside, isosakuranetin 7-O-glucoside, apigenin 7-O-glucoside, apigenin 4'-O-glucoside, luteolin 7-O-glucoside, luteolin 4'-O-glucoside, luteolin 3'-O-glucoside, chrysoeriol 7-O-glucoside, acacetin 7-O-glucoside, diosmetin 7-O-glucoside, quercetin 7-O-glucoside, quercetin 4'-O-glucoside, quercetin 3'-O-glucoside, kaempferol 7-O-glucoside, isorhamnetin 7-O-glucoside, and UDP-glucose were obtained from CFW Laboratories Inc. (Walnut, CA). Chromatographic acetonitrile and methanol were acquired from Tedia (Fairfield, OH).

2.2. Identification and Phylogenetic Analysis of *CitUGTs*. To identify putative *CitUGT* members, a simple hidden Markov model (HMM) search was conducted on the *C. clementina* genome using the UGT domain (PF00201) from InterPro (<https://www.ebi.ac.uk/interpro/entry/pfam/#table>). Meanwhile, a local BLASTP search was executed with the known UGT protein sequences from *A. thaliana* as queries. After merging the results from the HMM and BLASTP searches and removing redundant protein sequences, the conserved domains of each putative *CitUGT* protein were further validated by the NCBI Batch CD-Search (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) and SMART (<http://smart.embl-heidelberg.de/>). The physical and chemical properties of *CitUGT* proteins were analyzed by the Protein Parameter Calc program in TBtools,²⁷ and their subcellular localization was predicted using the BUSCA Web server tool (<https://busca.biocomp.unibo.it/>). Amino

acid sequences from *CitUGTs* and various plant UGTs were aligned by the ClustalW program in MEGA 11.0.²⁸ The phylogenetic tree was generated via the neighbor-joining approach with 1000 bootstrap replicates and visualized by the iTOL online program (<https://itol.embl.de/>).

2.3. Analysis of the Gene Structure and Conserved Motif.

The exon–intron structures of *CitUGTs* were visualized by using the GFF3 file of the *C. clementina* genome in TBtools. Furthermore, conserved motifs in the putative *CitUGT* proteins were identified through the online MEME v.5.5.5 tool (<http://meme-suite.org/tools/meme>), which allowed for a maximum of 14 motifs. Structural domains of the *CitUGT* proteins were analyzed with NCBI Batch CD-Search, identifying their types and locations. Additionally, TBtools was employed to create a comprehensive image that integrated the phylogenetic tree, gene structure, structural domains, and conserved motifs.²⁷

2.4. Chromosomal Location, Gene Duplication, and Synteny Analysis. The genomic positions of *CitUGTs* were derived from the genome annotation file of *C. clementina*, and their physical map on the chromosomes was created by using TBtools. MCScanX software was utilized to examine the intraspecies synteny relationships of *CitUGTs* within *C. clementina*,²⁹ and the OneStepMCScanX-SuperFast function in TBtools was used to analyze the interspecies synteny analyses of *UGTs* between *C. clementina* and *A. thaliana*, *Oryza sativa*, *V. vinifera*, *M. domestica*.³⁰ Duplication events within the *CitUGT* gene family were investigated and visualized using the Advanced Circos and DualSyntePlot program in TBtools.²⁷ Additionally, a Simple K_a/K_s Calculator was utilized to determine the K_a/K_s ratios of UGT duplicate pairs.²⁷ The divergence times for these pairs were estimated using the formula $T = K_s/2\lambda \times 10^{-6}$ million years ago (MYA), where λ was set to 1.5×10^{-6} .³¹

2.5. Expression Pattern Analysis of *CitUGTs*. The transcriptome data from our previous report³² were employed to analyze the *CitUGT* expression patterns across nine distinct tissues of “Ponkan” (*C. reticulata* Blanco.), including root (RO), main stem (MS), flavedo (FL), mature leaf (ML), albedo (AL), tangerine pith (TP), segment membrane (SM), juice sacs (JS), and seed (SE). The FPKM values were utilized to assess the transcript abundance of *CitUGTs*, and expression heatmaps were plotted by TBtools.²⁷

2.6. Recombinant Protein Expression and Purification. The full-length coding sequences of *CitUGT72AZ4* and *CitUGT84J10* were inserted into the pET32a (+) vector. Following sequencing confirmation, the recombinant plasmids, along with the empty vector, were transferred to *Escherichia coli* BL21 (DE3) cells. Recombinant protein expressions were carried out with slight modifications to previously reported methods.³³ Once the cell culture reached an optical density (OD_{600}) of 0.6–0.8, isopropyl β -D-thiogalactoside (IPTG) was added at a final concentration of 1 mM to induce protein expression. The cultures were maintained at 16 °C with shaking at 150 rpm for 20–24 h. Cells were collected by centrifugation at 3900g for 10 min. The fusion proteins were purified as described by Xie et al.,³⁴ verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and measured by the BCA assay kit (FUDE Biotech, Hangzhou, China).

2.7. Enzymatic Activity Assay. Enzymatic assays were executed in a 100 μ L reaction system, which included 100 mM Tris-HCl buffer (pH 7.5, 2 mM DTT), 400 μ M flavonoid substrates, 1.5 mM UDP-glucose, and 20 μ L of purified protein at 30 °C. After 16 h of incubation, enzymatic reactions were halted by adding 100 μ L of ice-cold methanol. The supernatants were centrifuged at 13,000 rpm for 40 min before analysis with an Agilent 1290 ultra-HPLC (Agilent Technologies, Santa Clara, CA). Products were separated on an Inertsil C18 ODS column (4.6 mm × 250 mm, 5 μ m) at a flow rate of 1 mL/min. Detection was carried out using a mobile phase of 0.1% (v/v) phosphoric acid in water (A) and acetonitrile (B) with the following gradient elution program: 0 min, 20% B; 5 min, 20% B; 10 min, 27% B; 15 min, 27% B; 25 min, 40% B; 35 min, 60% B; 40 min, 80% B; 42 min, 100% B; 45 min, 100% B; 50 min, 20% B; 55 min, 20% B. Enzymatic products were detected at 280 nm with a 10 μ L injection volume, and their structures were confirmed via liquid

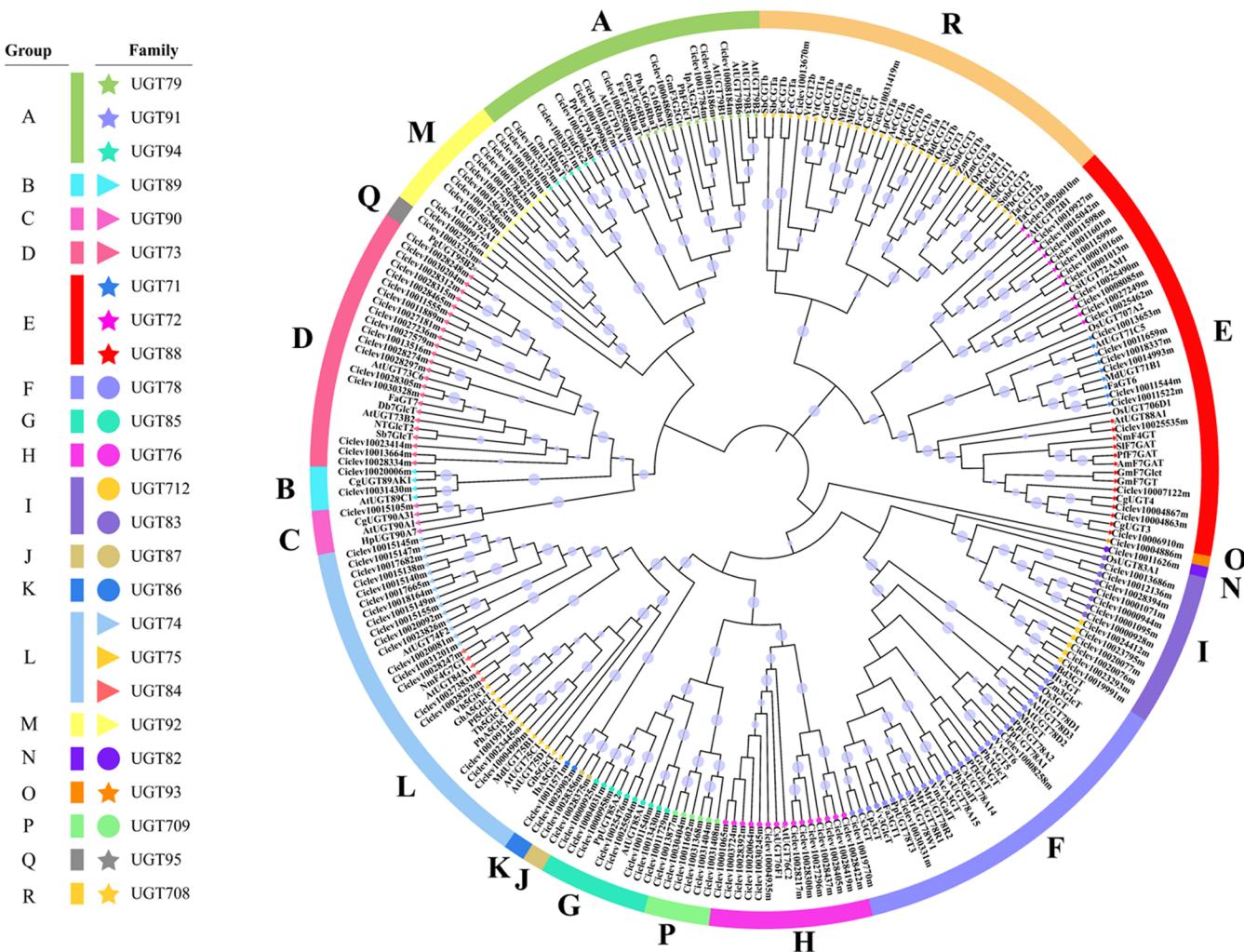


Figure 1. Phylogenetic analysis of UGT family genes from *C. clementina*. A neighbor-joining phylogenetic tree was built by aligning the protein sequences of 136 CitUGTs with several functional UGTs from various plant species. The abbreviations for species names are as follows: At, *A. thaliana*; Nt, *Nicotiana tabacum*; Os, *O. sativa*; Gm, *Glycine max*; Fa, *Fragaria × ananassa*; Am, *Antirrhinum majus*; Sb, *Scutellaria baicalensis*; Sl, *Scutellaria laeteviolacea*; Pf, *Perilla frutescens*; Db, *Dorotheanthus bellidiformis*; Ph, *Petunia hybrid*; Zm, *Z. mays*; Hv, *Hordeum vulgare*; Vm, *Vigna mungo*; Md, *Malus × domestica*; Pp, *P. persica*; Mr, *Morella rubra*; Vv, *V. vinifera*; Ac, *Actinidia chinensis*; Csi, *C. sinensis*; Mt, *Medicago truncatula*; Bd, *Brachypodium distachyon*; Gh, *Glandularia × hybrid*; Ih, *Iris × hollandica*; Th, *Torenia hybrid*; Si, *Setaria italica*; Phe, *Phyllostachys heterocycle*; Gu, *Glycyrrhiza uralensis*; Am, *Arisaema erubescens*; Lp, *Landoltia punctata*; Ps, *Pistia stratiotes*; Vt, *Viola tricolor*; Cm, *C. maxima*; Cs, *C. sinensis*; Cp, *C. paradisi*; Fc, *Fortunella crassifolia*; Cr, *C. reticulata*; Nm, *N. menziesii*; Pg, *Punica granatum*. A light blue circle at the center of each branch marks bootstrap values over 50%. These CitUGTs were categorized into 18 groups (A–R) and 25 families, each represented by different color strips and symbols. Accession numbers for UGTs from various plants are detailed in Table S8.

chromatography/mass spectrometry (LC-MS/MS) as previously reported.³⁵

To elucidate the optimum reaction conditions of CitUGT72AZ4, UDP-glucose served as the sugar donor, and naringenin served as the sugar acceptor. The optimal reaction temperature was tested between 10 and 60 °C at pH 7.0, while the optimum pH was evaluated between pH 4.0 and 10 in four different buffers at 30 °C, including sodium citrate buffer (100 mM, pH 4.0–6.0), phosphate buffer (100 mM, pH 6.0–7.5), Tris-HCl buffer (100 mM, pH 7.0–9.0), and Na₂CO₃-NaHCO₃ buffer (100 mM, pH 8.5–10.0). The kinetic parameters of acceptors and sugar donors for CitUGT72AZ4 were measured using UDP-Glo Glycosyltransferase Assay Kit from Promega (Madison, WI), following the protocols of Ren et al. and Sheikh et al.^{33,36} To determine the kinetic parameters of acceptors, assays were conducted in a 50 μL reaction mixture containing 1 μg of purified protein, 650 μM UDP-glucose, 0.1 M Tris-HCl buffer (pH 7.0), and various concentrations of the flavonoid substrate (0–500 μM). For the kinetic parameters of sugar donors, 200 μM naringenin was used as the acceptor, and 0–1 mM UDP-glucose was used as the

sugar donor. Reactions were performed at 37 °C for 30 min and then terminated with an equal volume of UDP Detection Reagent. Kinetic parameters were estimated using nonlinear regression to fit the Michaelis–Menten equation.

2.8. Virus-Induced Gene Silencing in Citrus. To verify the function of *CitUGT72AZ4* *in vivo*, virus-induced gene silencing (VIGS) tests were conducted on the seedlings of “Jincheng” (a citrus cultivar known for its high content of flavonoid glycosides) following a previous protocol.³⁷ A 353 bp fragment of CDS from *CitUGT72AZ4* was constructed into the TRV2 vector using primers shown in Table S9. *Agrobacterium tumefaciens* EHA105 harboring TRV1 and TRV2 constructs were cultured in Luria Broth (LB) until OD₆₀₀ of 1.2. After centrifugation, the supernatant was removed and the cells were resuspended in a fresh medium (pH 5.6, 10 mM MES, 10 mM MgCl₂, 150 μM acetosyringone). The cell suspensions of TRV1 and TRV2 were combined in a 1:1 volume ratio and subsequently injected into the germinated seedlings through vacuum infiltration (−100 kPa, 1 min). The seedlings were cocultured with *A. tumefaciens* in the dark for 3 days and then grown in soil for 40 days.

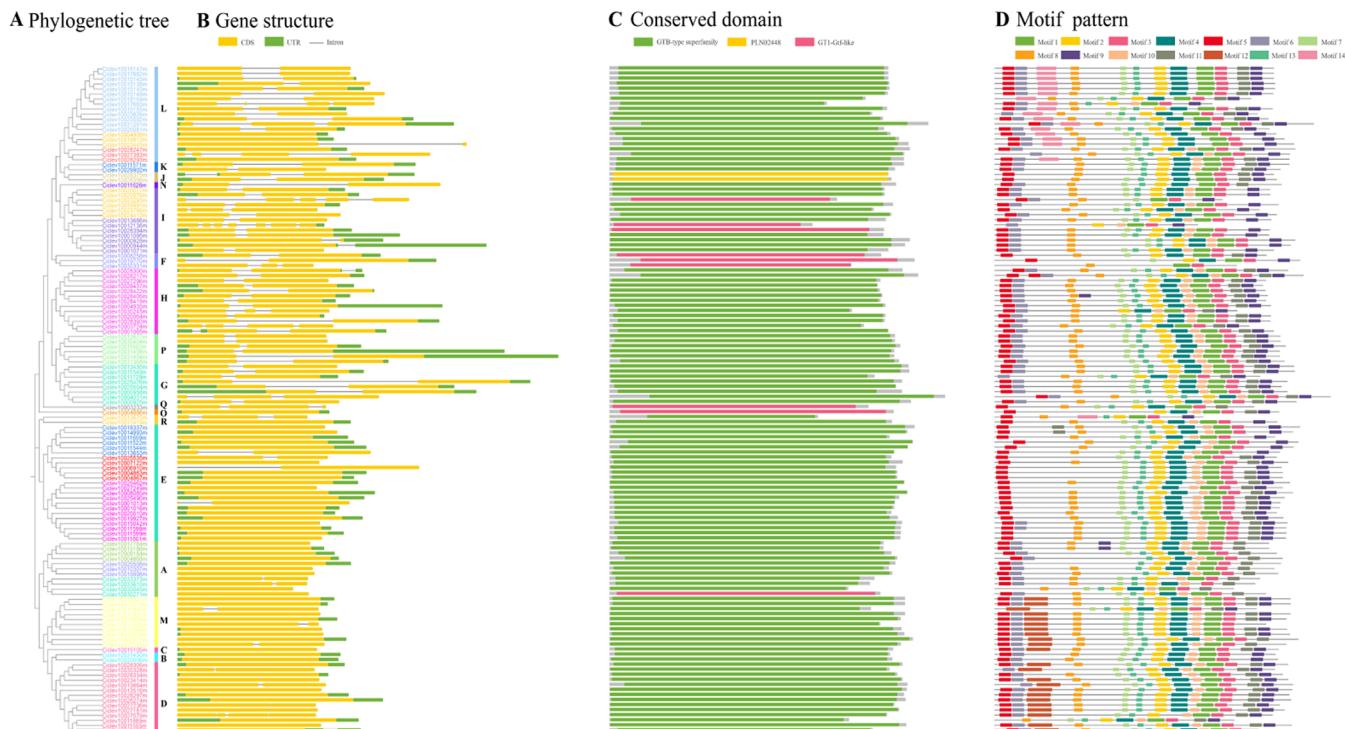


Figure 2. Diagram of phylogenetic relationships (A), gene structure (B), conserved domains (C), and motif patterns (D) of CitUGTs. The 18 phylogenetic groups are indicated by the same color used in Figure 1. Coding sequences are shown in yellow boxes, untranslated regions are shown in green boxes, and introns are shown by black lines without boxes. Fourteen distinct motifs are depicted in different colored boxes, and their weblogs are shown in Figure S2.

Seedlings injected with TRV1 and an empty TRV2 served as control (CK). LC-MS/MS was used to analyze flavonoid glycosides from the aerial parts of each plant.

2.9. RNA Extraction and Quantitative Real-Time PCR Analysis. The Easy Plant RNA Extraction Kit (Easy-Do Biotech Co., Ltd.) was utilized to isolate total RNA from VIGS samples, followed by the elimination of genomic DNA and reverse transcription into cDNA. Quantitative real-time PCR (qRT-PCR) was conducted following established protocols.³⁸ The citrus β -actin (Accession No. Ciclev10025866 mg) acted as the housekeeping gene for monitoring mRNA abundance and the $2^{-\Delta Ct}$ method was applied to quantify expression levels.

2.10. Metabolite Analysis by HPLC and LC-MS. Metabolomics data from our prior study³² were utilized to examine the accumulation patterns of flavonoid glycoside across nine distinct tissues of “Ponkan”. For the VIGS experiment, a sample power (0.2 g) was sonicated for 60 min in 1 mL of 70% methanol. The supernatant was then gathered by centrifuging at 13,000 rpm for 30 min and then used for LC-MS analysis. The quantification of targeted flavonoid glycosides was carried out using an Agilent 6470 triple quadrupole mass spectrometer (Agilent Technologies), operating in negative ionization mode as described previously.³⁴ Acetonitrile (A) and water with 0.1% formic acid (B) served as the mobile phase with a flow rate of 0.2 mL/min. The gradient used for elution was as follows: 0 min, 5% A; 3 min, 20% A; 12 min, 60% A; 15 min, 95% A; 17 min, 95% B. MassHunter Workstation software was utilized for data analysis.

2.11. Statistical Analysis. Graphics were plotted by GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA). Structural formulas were depicted with ChemDraw 14.0 (PerkinElmer, Waltham, MA). Data were represented as mean \pm SE from a minimum of three biological replicates. Statistical significance was evaluated using one-way ANOVA followed by Tukey’s test, with bars labeled by different letters indicating significant differences ($P < 0.05$).

3. RESULTS

3.1. Identification and Phylogenetic Analysis of *CitUGT* Gene Family in *Citrus*. Identification of *CitUGT* gene family members was achieved through both local BLASTP and HMM search against the *C. clementina* genome (v1.0). After redundant and incomplete sequences were eliminated, 141 *CitUGT* protein sequences with the conserved PSPG box were obtained. These proteins were encoded by 136 *CitUGT* genes due to alternative splicing events (Table S1). Subsequently, all of the identified *CitUGTs* were assigned systematic names by the UGT Nomenclature Committee. Analysis of the physical and chemical properties revealed that *CitUGTs* exhibited a broad range of characteristics. Specifically, the coding sequences of *CitUGTs* spanned from 1020 to 1686 bp, resulting in proteins with lengths from 339 to 561 amino acids. These proteins had molecular weights extended from 38.17 to 63.21 kDa and theoretical isoelectric points extended from 4.91 to 8.54. Analysis of subcellular localization suggested that the majority of *CitUGTs* were predicted to be located in the plasma membrane (88.97%), followed by the chloroplast (4.41%), organelle membrane (3.67%), and endomembrane system (2.94%).

Our phylogenetic analysis categorized 136 *CitUGTs* into 18 distinct groups. This classification included 14 conservative groups (A–N) previously recognized in *A. thaliana*, as well as four novel groups (O, P, Q, and R) observed in other plant species (Figure 1 and Table S1). *CitUGT* members were primarily concentrated in groups A–L, though their numbers varied considerably across these groups (Figure S1). Remarkably, group E had the most *CitUGTs* (28 members), followed by group L (19 members), group D (18 members), group H (13 members), group I (13 members), and group A

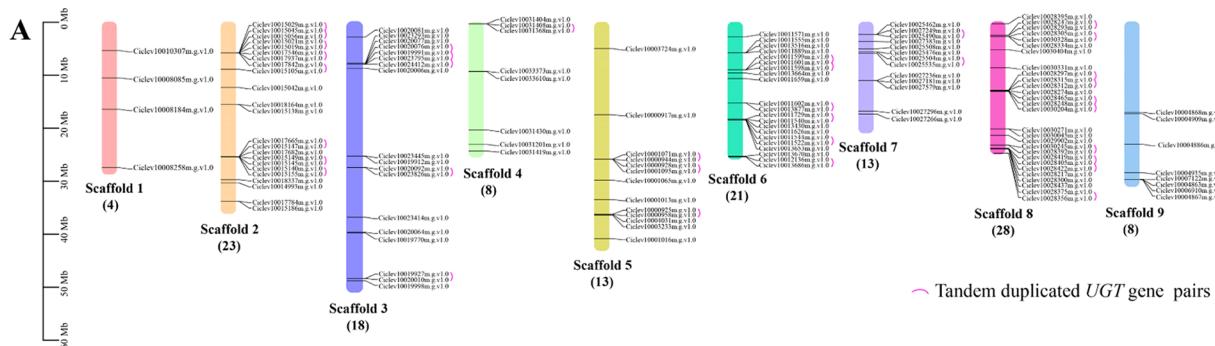
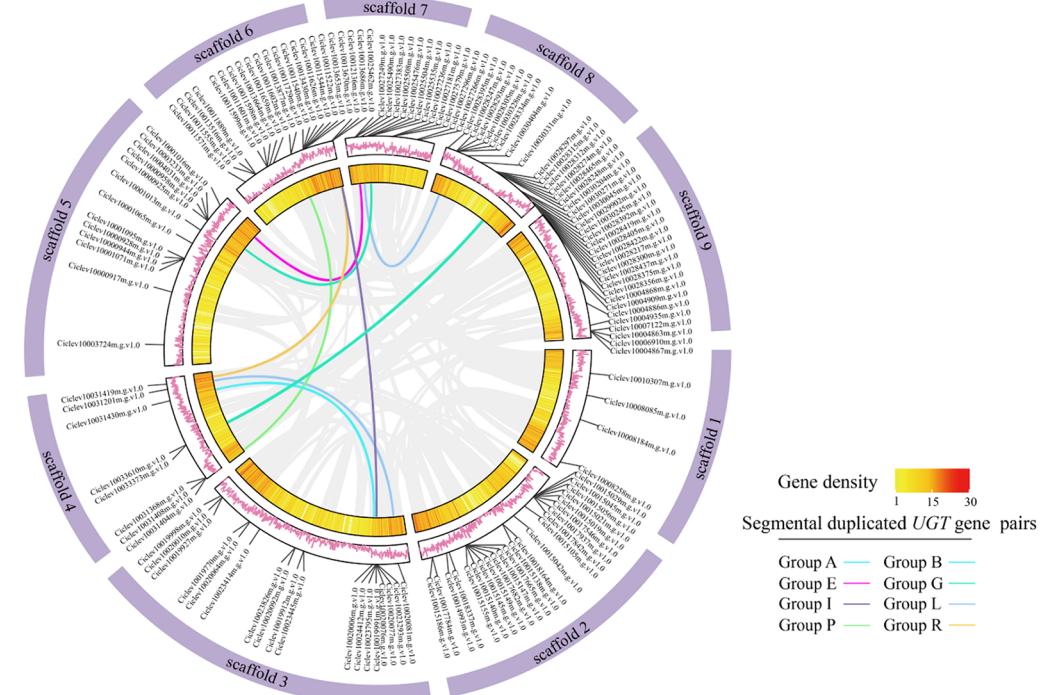
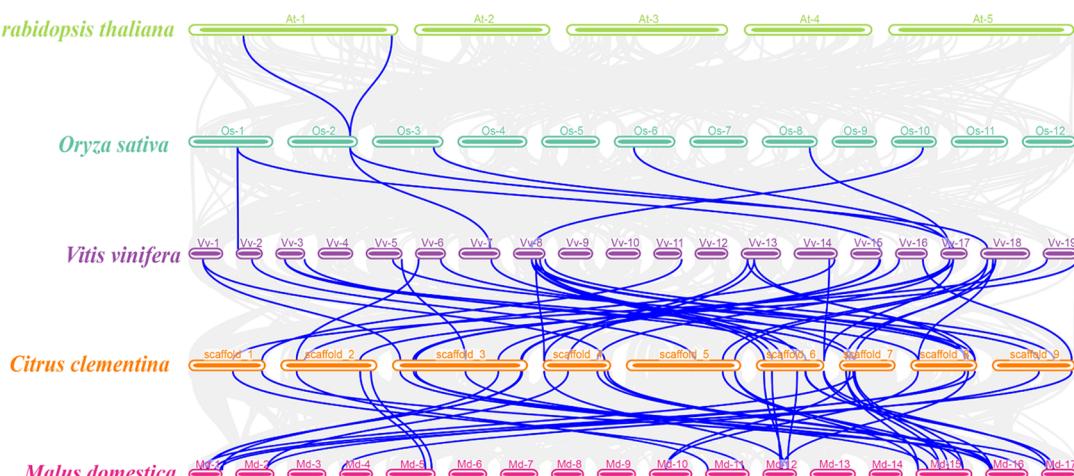
**B****C***Arabidopsis thaliana*

Figure 3. Chromosomal locations and synteny analysis of *CitUGTs*. (A) Chromosomal distribution and tandem duplication of *CitUGTs*. The scaffold names and numbers of *CitUGTs* are shown at the bottom of the nine scaffolds. The scale bar on the left indicates the scaffold lengths, and the pink lines indicate tandem duplicated gene pairs. (B) Collinearity analysis of the *CitUGT* gene family within *C. clementina*. The collinear blocks of *C. clementina* are indicated by light gray lines in the circle, and the segmentally duplicated gene pairs are highlighted by the same color used in Figure 1. The external track rendered in purple denotes various scaffolds, and the internal track depicted as a yellow-orange heatmap indicates the gene density. (C) Syntenic blocks of UGTs between *C. clementina* and the other four species, while blue lines indicate orthologous UGT gene pairs.

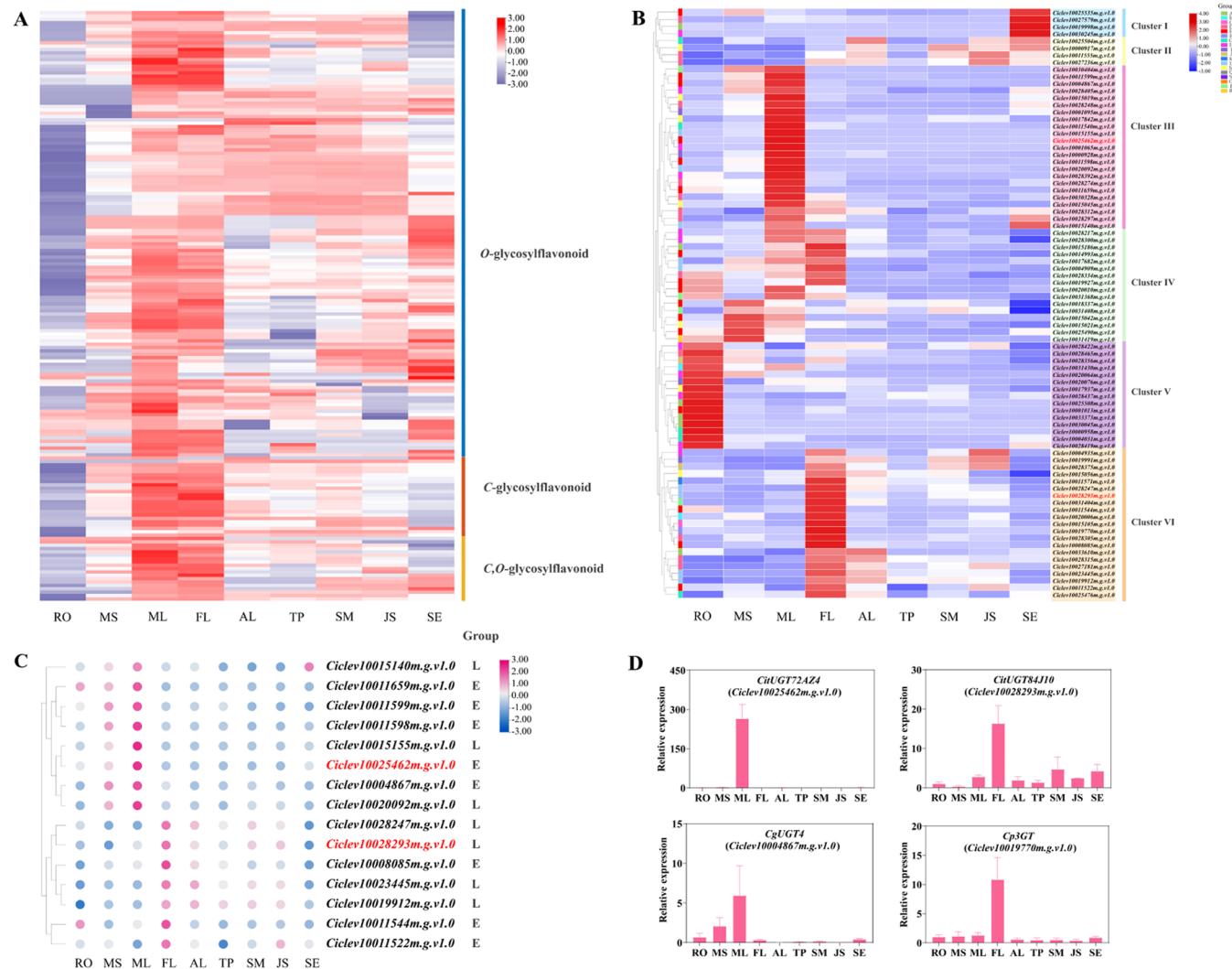


Figure 4. Flavonoid glucosides accumulation and *CitUGT* expression in various tissues of "Ponkan". (A) Accumulation patterns of flavonoid glucosides in multiple tissues. A heatmap illustrates the flavonoid glucosides accumulated in the tissues of "Ponkan", including root (RO), main stem (MS), mature leaf (ML), flavedo (FL), albedo (AL), tangerine pith (TP), segment membrane (SM), juice sacs (JS), and seed (SE). Relative contents were log₂-transformed and normalized for hierarchical clustering. High abundance was shown in red, while low abundance was shown in blue. The blue line indicates O-glycosylflavonoids, the red line indicates C-glycosylflavonoids, and the orange line indicates C, O-glycosylflavonoids. (B) Expression patterns of 83 *CitUGTs* in diverse tissues of "Ponkan". TBtools was used for hierarchical clustering analysis of the expression patterns of 83 *CitUGTs*, with a color scale representing expression levels. (C) Expression patterns of 15 *CitUGTs* from group E and L in various tissues of "Ponkan". High expression levels are shown in pink, while low levels are shown in blue. The red font indicates the two candidate genes (*Ciclev10025462m.g.v1.0* and *Ciclev10028293m.g.v1.0*). (D) qRT-PCR analysis of the expression patterns of four *CitUGTs* in various tissues. *Ciclev10025462m.g.v1.0* and *Ciclev10028293m.g.v1.0* were designated as *CitUGT72AZ4* and *CitUGT84J10*, respectively. *Cp3GT* and *CgUGT4* have been identified as encoding flavonoid 3-O-glycosyltransferase and 7-O-glycosyltransferase, respectively.

(11 members). Furthermore, these 18 phylogenetic groups could be further divided into 25 families based on the identity of amino acid sequences (>40%), and each group contained one or multiple families (Figure 1 and Table S2). Groups A, E, I, and L were multifamily groups comprising three families (e.g., UGT79, UGT91, and UGT94), three families (e.g., UGT71, UGT72, and UGT88), two families (e.g., UGT83 and UGT712), and three families (e.g., UGT74, UGT75, and UGT84), respectively. However, the remaining 14 groups (e.g., B, C, D, F, G, H, J, K, M, N, O, P, Q, and R) are composed of a single family (e.g., UGT89, UGT90, UGT73, UGT78, UGT85, UGT76, UGT87, UGT86, UGT92, UGT82, UGT93, UGT709, UGT95 and UGT708). Notably, *CitUGT89AK1* and *CgUGT4*, identified as flavonoid 7-O-glycosyltransferases, were clustered into group B (UGT89 family) and E (UGT88

family), respectively.^{23,26} *Cp3GT*, a flavonoid 3-O-glycosyltransferase,³⁹ was clustered into group F (UGT78 family), while *FcCGT*, a flavonoid C-glycosyltransferase⁴⁰ was clustered into group R (UGT708 family).

3.2. Analysis of the Conserved Motif and Gene Structure of *CitUGT* Gene Family. To elucidate the structural and functional features of the *CitUGT* gene family, we carried out a detailed analysis focusing on conserved motifs and gene structures. Utilizing the MEME online tools, we identified 14 conserved motifs, designated as motifs 1–14 (Figure S2). Our analysis revealed that *CitUGT* members within the same family shared similar motif patterns. The positions of these motifs remained consistent, typically following the order of motif 5–6–14–8–7–13–2–4–1–3–11–9 in most *CitUGT* members. Notably, motif 1 was

identified as the PSPG box, which is essential for glycosyl donor binding. Motif 12 was unique to members of groups D and M, whereas motif 14 was specific to groups L, K, and R. These specific motifs likely contribute to functional diversity among *CitUGT* family members in citrus.

Gene structure analysis is crucial for understanding the evolutionary relationship of the gene family. Our intron–exon structure analysis showed that *CitUGTs* contained 1–7 exons with varying numbers of introns (Figure 2 and Table S2). Among the 136 *CitUGTs*, 57 *CitUGTs* lacked introns, 63 *CitUGTs* possessed a single intron, and the remaining 16 included 2–6 introns. The highest count of intron-free genes was observed in group E (19 members), followed by groups D (13 members) and M (10 members). Phylogenetically close *CitUGT* members displayed similar exon–intron structures, corresponding with conserved motif patterns. These observations indicated that the phylogenetic relationships of the *CitUGT* gene family are closely associated with their gene structures.

3.3. Chromosomal Location, Gene Duplication, and Synteny Analysis of *CitUGT* Gene Family. Based on the genome annotation of *C. clementina*, we mapped the chromosomal locations of the *CitUGT* gene family to summarize their distribution. The 136 *CitUGTs* were unevenly dispersed across nine scaffolds (1–9) (Figure 3A). The highest number of *CitUGTs* were distributed on scaffold 8 with 28 genes, followed by scaffold 2 with 23 genes and scaffold 6 with 21 genes. The remaining were spread across other scaffolds, each containing 4–18 genes.

Our analysis revealed that 46 *CitUGT* gene pairs originated tandem duplication events, and some of these pairs were located closely together, forming paralogous clusters on various scaffolds (Figure 3A and Table S3). Notably, scaffolds 8 and 6 each contained more than three clusters, indicating that these regions might be potential hotspots for duplication. Additionally, 9 *CitUGT* gene pairs were identified as originating from segmental duplication events (Figure 3B and Table S3). These results implied that tandem duplication might be the main driving force behind the extensive expansion of the *CitUGT* gene family. To assess evolutionary pressures on the *CitUGT* gene family, we examined the nonsynonymous (K_a) and synonymous (K_s) substitution rates for these gene pairs. The results indicated that the K_a/K_s values of all 46 tandem and seven of the nine segmental duplicated gene pairs were below 1, which implies that most *CitUGTs* may have undergone extensive purifying selection to eliminate harmful mutations (Table S4). The divergence time for the tandem duplications was estimated to date from 69.51 and 1.06 MYA, whereas segmental duplication was estimated to date from 219.07 and 60.41 MYA.

To gain insight into the evolutionary relationships of the *CitUGT* gene family, we created syntenic maps for *C. clementina* along with four other plant species: *A. thaliana*, *O. sativa*, *V. vinifera*, and *M. domestica*. We identified 39, 36, 20, and 9 orthologous *UGT* gene pairs between *C. clementina* and *V. vinifera*, *M. domestica*, *A. thaliana*, *O. sativa*, respectively (Figure 3C). This result indicated that *C. clementina* had a closer evolutionary relationship with *V. vinifera* and *M. domestica*.

3.4. Tissue-Specific Expression Patterns of *CitUGT* Gene Family. The expression patterns of *CitUGTs* were investigated using transcriptome data from 9 distinct “Ponkan” tissues. After filtering the missing and low values, 83 *CitUGTs*

(61.03%) were shown to have an expression (FPKM value >10) in at least one tissue (Table S5). These *CitUGTs* were further categorized into six distinct clusters according to their expression patterns in various tissues (Figure 4B). It was found that *CitUGTs* in clusters I, III, V, and VI showed tissue-specific expression patterns, whereas those in clusters II and IV did not present particular expression patterns. Notably, a total of 23, 21, 15, and 5 *CitUGTs* in clusters III, VI, V, and I showed high expression levels in leaves, flavedo, roots, and seed, respectively. Furthermore, 44 *CitUGTs* highly expressed in either leaf or flavedo were concentrated in group E (8 genes), D (8 genes), L (7 genes), and M (4 genes) (Table S5). These findings implied that *CitUGT* members from groups E–M might participate in the glycosylation of flavonoids, especially those found in the leaf and flavedo tissues of citrus plants. Additionally, to confirm the accuracy of transcriptome data of “Ponkan”, we employed qRT-PCR to assess two *CitUGTs* known for their roles in flavonoid glycosides (Figure 4D). The results demonstrated that *Cp3GT* (98.93% amino acid sequence identity to *Ciclev10019770m.g.v1.0*) was highly expressed in the leaf, while *CgUGT4* (98.13% amino acid sequence identity to *Ciclev10004867m.g.v1.0*) was highly expressed in the flavedo. The qRT-PCR results aligned with the FPKM values derived from RNA-seq, thereby validating the reliability of the transcriptomic data.

3.5. Identification of *CitUGTs* Involved in Flavonoid Glycoside Accumulation. To probe the accumulation patterns of flavonoid glycosides in various tissues of “Ponkan”, a high-throughput quantification was employed. A sum of 177 flavonoid glycosides were found in multiple tissues of “Ponkan”, including 134 O-glycosylflavonoids, 24 C-glycosylflavonoids, and 19 C, O-glycosylflavonoids (Figure 4A and Table S6). It is evident that O-glycosylflavonoids accounted for the largest proportion (75.14%) of flavonoid glycosides in all tested tissues, and most of them were highly accumulated in leaf and flavedo while not in the root, such as naringenin 4'-O-glucoside, luteolin 7-O-rutinoside, and quercentin 7-O-rutinoside-4'-O-glucoside. Similar to O-glycosylflavonoids, the majority of C-glycosylflavonoids and C, O-glycosylflavonoids exhibited similar accumulation patterns in these tissues.

Group E and L are two major groups of the *UGT* gene family both in citrus and model plant species.^{11,12} Several *UGTs* in groups E and L have been proven to be involved in the glycosylation of flavonoids.^{26,41} In this study, 15 *CitUGTs* from groups E and L were highly expressed in either leaf or flavedo, consistent with the abundant flavonoid glycosides in these tissues (Figure 4C). Among them, *Ciclev10025462m.g.v1.0* (designated as *CitUGT72AZ4*) exhibited the highest expression levels in the leaf, while *Ciclev10028293m.g.v1.0* (designated as *CitUGT84J10*) displayed the highest expression levels in the flavedo, suggesting that they were likely to participate in the biosynthesis of flavonoid glycosides (Table S6). Furthermore, *CitUGT72AZ4* and *CitUGT84J10* have been annotated to encode UDP glycosyltransferase, and they were clustered into Group E (*UGT72* family) and Group L (*UGT84* family), respectively (Table S1). Therefore, these two *CitUGTs* were selected as candidate genes potentially engaged in the biosynthesis of flavonoid glycosides.

3.6. Functional Characterization of Recombinant *CitUGTs*. *CitUGT72AZ4* and *CitUGT84J10* were isolated from the cDNA library of “Ponkan” and subsequently inserted into the pET32a (+) expression vector. The open reading

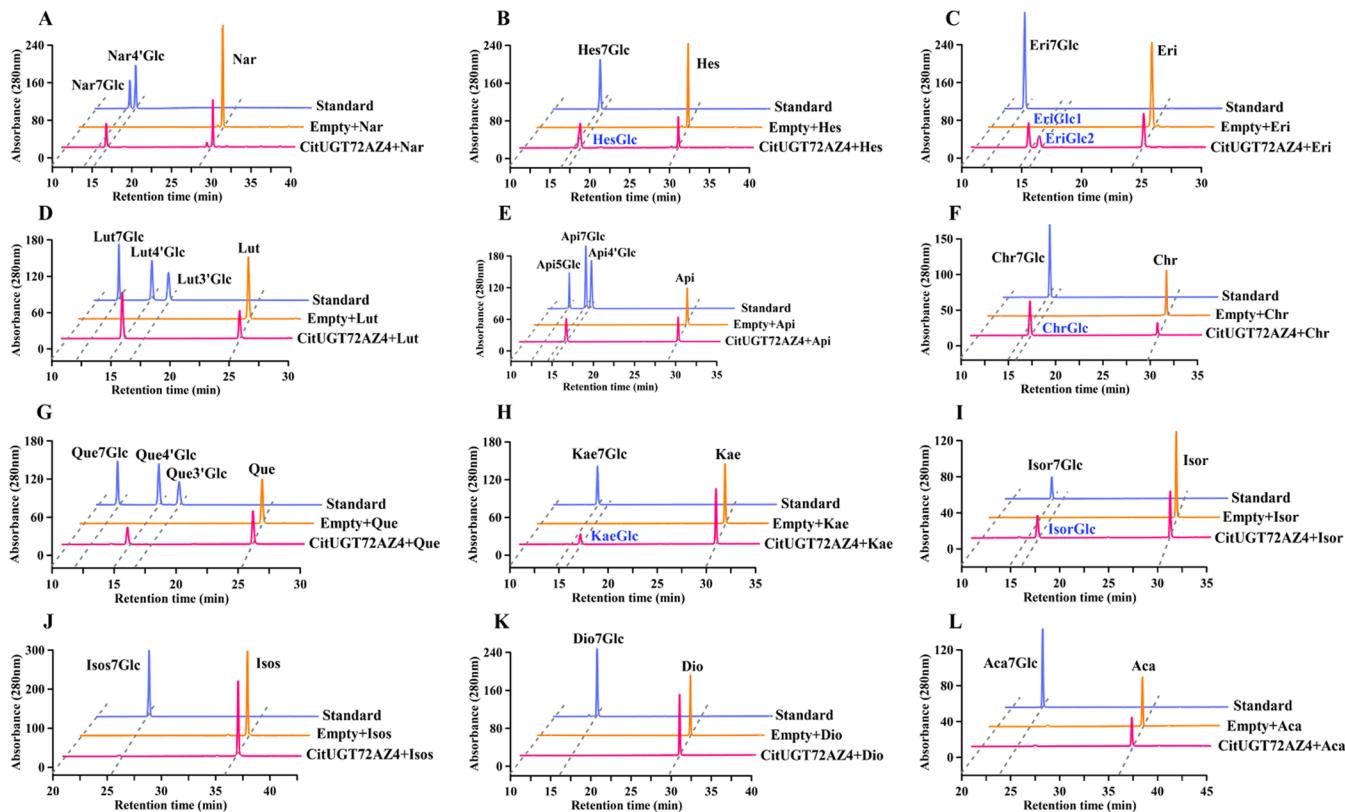


Figure 5. Enzymatic activity of recombinant CitUGT72AZ4. (A–L) HPLC chromatograms for the enzymatic product of recombinant CitUGT72AZ4 when incubated with substrates (naringenin, hesperetin, eriodictyol, luteolin, apigenin, chrysoeriol, quercetin, kaempferol, isorhamnetin, isosakuranetin, diosmetin, and acacetin) using UDP-glucose as the sugar donor. The bottom part shows substrates catalyzed by recombinant CitUGT72AZ4 in magenta, with predicted products in blue; the middle part shows substrates reacted with the empty vector in orange; the top part displays authentic standards in blue. (A–C) Flavanones employed as substrates: Nar, naringenin; Nar⁴Glc, naringenin 4'-O-glucoside; Hes, hesperetin; Hes⁷Glc, hesperetin 7-O-glucoside; EriGlc, hesperetin O-glucoside; Eri, eriodictyol; Eri⁷Glc, eriodictyol 7-O-glucoside; EriGlc, eriodictyol O-glucoside. (D–F) Flavones served as substrates: Lut, luteolin; Lut⁷Glc, luteolin 7-O-glucoside; Lut³Glc, luteolin 3'-O-glucoside; Lut⁴Glc, luteolin 4'-O-glucoside; Api, apigenin; Api⁷Glc, Api 7-O-glucoside; Api⁴Glc, apigenin 4'-O-glucoside; Chr, chrysoeriol; Chr⁷Glc, chrysoeriol 7-O-glucoside; ChrGlc, chrysoeriol O-glucoside. (G–I) Flavonols used as substrates: Que, quercetin; Que⁷Glc, quercetin 7-O-glucoside; Que³Glc, quercetin 3'-O-glucoside; Que⁴Glc, quercetin 4'-O-glucoside; Kae, kaempferol; KaeGlc, kaempferol 7-O-glucoside; KaeGlc, kaempferol O-glucoside; Isor, isorhamnetin; Isor⁷Glc, isorhamnetin 7-O-glucoside; IsorGlc, isorhamnetin O-glucoside. (J–L) Three substrates without a free hydroxyl group at their C⁴' position. Isos, isosakuranetin; Isos⁷Glc, isosakuranetin 7-O-glucoside; Dio, diosmetin; Dio⁷Glc, diosmetin 7-O-glucoside; Aca, acacetin; Aca⁷Glc, acacetin 7-O-glucoside. The MS/MS fragmentation information for glycosylated products catalyzed by CitUGT72AZ4 and their corresponding authentic standards can be found in Figure S6.

frames for CitUGT72AZ4 and CitUGT84J10 measured 1482 and 1479 bp, respectively, encoding proteins of 493 and 492 amino acids (Table S1). Sequence alignment analysis demonstrated that both CitUGT72AZ4 and CitUGT84J10 possessed a conserved PSPG box close to the C-terminal, and the final amino acid residue in these motifs was glutamine (Q). This finding implied that these proteins were likely to utilize UDP-glucose as the sugar donor (Figure S3). The proteins encoded by CitUGT72AZ4 and CitUGT84J10, fused with two histidine tags, were successfully expressed in *E. coli*. SDS-PAGE analysis confirmed that the purified recombinant proteins displayed a band at approximately 73 and 74 kDa, corresponding to their theoretical molecular weights of 72.58 and 74.40 kDa (Figure S4A).

To evaluate the enzymatic activity of CitUGT72AZ4 and CitUGT84J10, assays were performed using three representative types of flavonoid aglycones (flavanone, flavone, and flavonol aglycones) as potential substrates, with UDP-glucose serving as the sugar donor. The results showed that CitUGT72AZ4 exhibited strong catalytic activities for flavonoid substrates, whereas CitUGT84J10 displayed weak

catalytic activities for these substrates (Figures 5 and S5). For flavanone aglycones (e.g., naringenin, hesperetin, and eriodictyol), CitUGT72AZ4 was capable of transferring a glucose moiety from UDP-glucoside to naringenin, resulting in the formation of a new peak. This peak shared the identical retention time (RT) and fragmentation pattern as the authentic naringenin 4'-O-glucoside standard (Figures 5A and S6A). Using hesperetin as the substrate, CitUGT72AZ4 produced a new peak eluting at 17.8 min, which eluted later than hesperetin 7-O-glucoside standard (at RT of 16.6 min) (Figures 5B and S6B). Hesperetin has three hydroxyl groups at C5, C7, and C3' that can be O-glycosylated, leading to the formation of hesperetin O-glucosides (hesperetin 5-O-glucoside, hesperetin 7-O-glucoside, and hesperetin 3'-O-glucoside). Taking the knowledge that flavonoid 5-O-glucosides typically elute earlier than their corresponding 7-O-glucoside² led us to presume that this unknown glycosylated product may be hesperetin 3'-O-glucoside. Two new peaks were observed when CitUGT72AZ4 was incubated with eriodictyol as the substrate (Figures 5C and S6C). Compared with naringenin (C5, C7, and C3'), eriodictyol has an additional hydroxyl

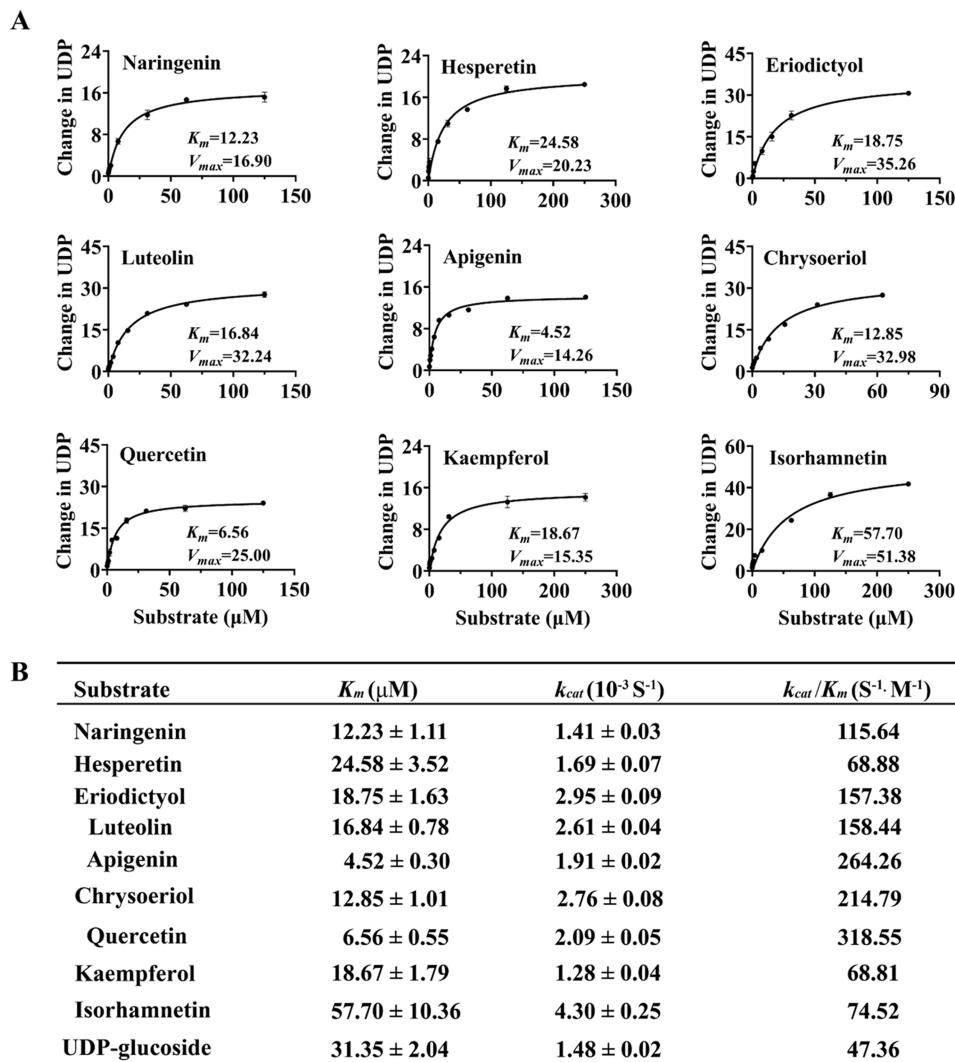


Figure 6. Kinetic parameters of CitUGT72AZ4 with different flavonoid substrates. (A) Michaelis–Menten curves of CitUGT72AZ4. The change in UDP represents the background-corrected UDP for each reaction. (B) Kinetic parameters of CitUGT72AZ4. K_m and k_{cat} were evaluated using a nonlinear curve fitting.

group at its C3' position, where it can be *O*-glycosylated, leading to the production of eriodictyol 3'-*O*-glucoside. Moreover, these two different eriodictyol *O*-glucosides eluted at RT of 14.9 and 15.8 min, respectively, and both of them eluted later than the eriodictyol 7-*O*-glucoside standard (at RT of 11.8 min). Thus, these two unknown eriodictyol *O*-glucosides were speculated to be eriodictyol 4'-*O*-glucoside and eriodictyol 3'-*O*-glucoside. These findings indicated that CitUGT72AZ4 could catalyze the glycosylation of flavanones at their 4'-OH and 3'-OH positions.

Additionally, CitUGT72AZ4 exhibited activity toward flavone aglycones (luteolin, apigenin, and chrysoeriol) and flavonol aglycones (quercetin, kaempferol, and isorhamnetin). When using luteolin and quercetin as substrates, each of their enzymatic assays showed a single new peak. Through comparison of the RT and fragmentation patterns with those of known standards, these products were determined to be luteolin 4'-*O*-glucoside and quercetin 4'-*O*-glucoside, respectively (Figures 5D,G and S6D,G). It is worth noting that although luteolin and quercetin have a free hydroxyl group at their C3' position where it can be *O*-glycosylated, neither luteolin 3'-*O*-glucoside nor quercetin 3'-*O*-glucoside were

yielded among their glucosylated products. This finding led us to hypothesize that CitUGT72AZ4 may preferentially catalyze the 4'-*O*-glycosylation of flavones and flavonols. To validate this hypothesis, enzymatic assays were further examined using substrates such as apigenin, chrysoeriol, kaempferol, and isorhamnetin, resulting in the formation of their respective *O*-glucosides (Figures 5E,F,H,I and S6E,F,H,I). These compounds were identified by comparing retention times, ultraviolet (UV) spectra, and fragmentation patterns with authentic standards and a previous study.⁴² Specifically, the apigenin *O*-glucoside and kaempferol *O*-glucoside were identified as apigenin 4'-*O*-glucoside and kaempferol 4'-*O*-glucoside, respectively. Meanwhile, chrysoeriol *O*-glucoside and isorhamnetin *O*-glucoside were speculated to be chrysoeriol 4'-*O*-glucoside and isorhamnetin 4'-*O*-glucoside, respectively.

3.7. Enzymatic Kinetics of Recombinant CitUGT72AZ4. To elucidate the optimal conditions for the enzymatic reaction of CitUGT72AZ4 *in vitro*, UDP-glucose was employed as the sugar donor. This enzyme achieved peak activity at a pH of 7.0 and a temperature close to 40 °C when using naringenin as the substrate (Figure S4B). Moreover, the

kinetic parameters for both sugar donors and flavonoid acceptors were evaluated in a 100 mM Tris-HCl buffer (pH 7.0). The results demonstrated that *CitUGT72AZ4* showed a relatively lower k_{cat}/K_m value ($47.36 \text{ M}^{-1} \text{ S}^{-1}$) for UDP-glucose than *CsUGT78A14* ($2.57 \times 10^5 \text{ M}^{-1} \text{ S}^{-1}$) in tea.¹⁴ In terms of substrate preference, *CitUGT72AZ4* displayed the highest catalytic efficiency with quercetin ($k_{cat}/K_m = 318.55 \text{ M}^{-1} \text{ S}^{-1}$), followed by apigenin, chrysoeriol, and luteolin with k_{cat}/K_m values of 264.26, 214.79, and 158.44 $\text{M}^{-1} \text{ S}^{-1}$, respectively (Figure 6A,B). In contrast, *CitUGT72AZ4* exhibited a lower catalytic efficiency with hesperetin ($k_{cat}/K_m = 68.88 \text{ M}^{-1} \text{ S}^{-1}$). Compared to hesperetin, *CitUGT72AZ4* displayed relatively higher catalytic efficiency toward eriodicetol ($k_{cat}/K_m = 157.38 \text{ M}^{-1} \text{ S}^{-1}$) and naringenin ($k_{cat}/K_m = 115.64 \text{ M}^{-1} \text{ S}^{-1}$), but still less than that of the three flavones (e.g., apigenin, chrysoeriol, and luteolin). Despite kaempferol and isorhamnetin sharing structural similarities with quercetin, *CitUGT72AZ4* exhibited relatively lower catalytic efficiency toward both kaempferol and isorhamnetin compared to quercetin. These results suggested that *CitUGT72AZ4* preferentially glycosylated the 4'-OH group of a diverse range of flavonoids, exhibiting higher catalytic efficiency with quercetin and three flavones (e.g., apigenin, chrysoeriol, and luteolin).

3.8. Virus-Induced Gene Silencing of *CitUGT72AZ4* in Citrus Seedlings. To further explore the role of *CitUGT72AZ4* in flavonoid glycoside biosynthesis, VIGS was applied to "Jincheng" seedlings. Compared to the control plants carrying with the TRV2 empty vector, all five VIGS-positive lines showed a marked decrease in the expression of *CitUGT72AZ4* (Figure 7A). Meanwhile, this decreased gene expression in the positive plants caused a considerable drop in the accumulation of flavonoid 4'-O-glucosides, including luteolin 4'-O-glucoside and quercetin 4'-O-glucoside (Figure 7B). In comparison with the average values observed in the CK group ($36.62 \mu\text{g} \cdot \text{g}^{-1} \text{ FW}$), the total content of flavonoid 4'-O-glucosides in VIGS-4 and VIGS-5 was decreased by 49.8% ($15.24 \mu\text{g} \cdot \text{g}^{-1} \text{ FW}$) and 35.8% ($14.73 \mu\text{g} \cdot \text{g}^{-1} \text{ FW}$), respectively. These results confirmed that *CitUGT72AZ4* participated in the biosynthesis of flavonoid 4'-O-glucosides in citrus.

4. DISCUSSION

Plant UGTs are integral to the diversity of flavonoids, with this multigene family having rapidly expanded and diversified throughout evolution. So far, the UGT gene family has been discovered in a variety of plant species, including crops and medicinal plants. In addition, several UGTs involved in the glycosylation of bioactive flavonoids have been identified in horticultural plants, such as *P. persica*,¹¹ *V. vinifera*,¹² and *M. domestica*.⁴² However, in citrus, few *CitUGTs* have been functionally characterized for their role in the glycosylation of bioactive flavonoids. Therefore, it is crucial to broaden the study of UGTs in citrus.

4.1. Characterization of *CitUGT* Gene Family in Citrus.

We identified 136 *CitUGTs* in the *C. clementina* genome, representing 0.4% of the whole genome. This number exceeded that in the model plant *A. thaliana* (107 members)¹⁰ but less than that in several other fruit trees, such as *M. rubra* (152 members),⁴² *P. persica* (168 members),¹¹ *V. vinifera* (181 members),¹² and *M. domestica* (229 members)⁴³ (Table S7). Unlike other fruit trees, the UGT gene family in citrus did not exhibit notable expansion, which may be associated with the

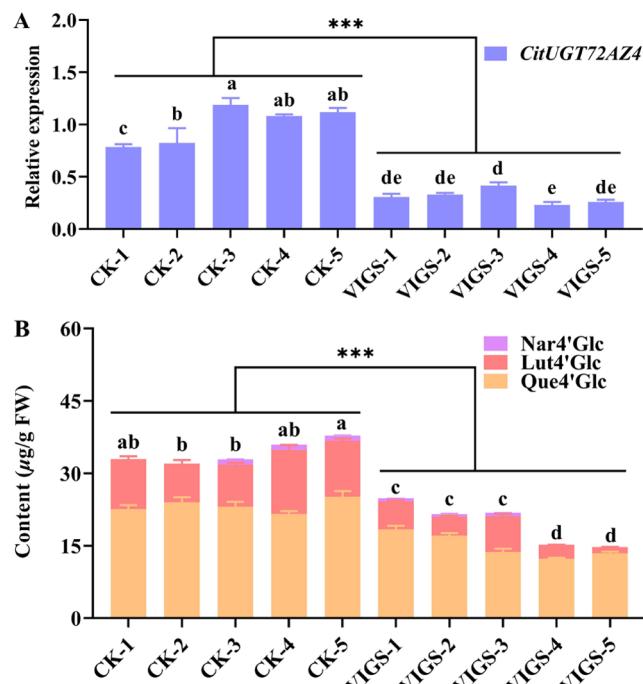


Figure 7. Virus-induced silencing (VIGS) of *CitUGT72AZ4* reduced the accumulation of flavonoid 4'-O-glucosides in "Jincheng" seedlings. (A) Expression levels of *CitUGT72AZ4* in the *CitUGT72AZ4*-VIGS lines compared to the CK group. (B) Total contents of 4'-O-glycosylated flavonoid in the *CitUGT72AZ4*-VIGS lines and the CK group. Nar4'Glc, naringenin 4'-O-glucoside; Lut4'Glc, luteolin 4'-O-glucoside; Que4'Glc, quercetin 4'-O-glucoside. Error bars illustrate SE calculated from three technical replicates. One-way ANOVA and Tukey's test were applied to assess statistical significance, and bars labeled with various letters indicate significant differences ($P < 0.05$). CK, seedlings infiltrated with empty TRV2 vector; VIGS, seedlings infiltrated with *CitUGT72AZ4* silencing. Data are shown as mean \pm SEM ($n = 3$). Student's *t*-test; * $P < 0.05$; ** $P < 0.01$, and *** $P < 0.001$.

absence of recent whole-genome duplication events (WGDs) in citrus.⁴⁴

Gene duplication is essential for gene family expansion, and the UGT gene family in plants expands mainly through tandem and segmental duplication. In citrus, tandem duplication events (46 pairs) were more prevalent than segmental duplication events (9 pairs), which indicated that tandem duplications acted as the primary driving force behind the expansion of this superfamily (Tables S3 and S4). These findings aligned with previous studies in *A. thaliana*,¹⁰ *V. vinifera*,¹² and *B. papyrifera*.⁴⁵ In contrast, the UGT gene family in *A. hypogaea*⁴⁶ and *Gossypium hirsutum*⁴⁷ expanded mainly through segmental duplications, implying diverse evolutionary patterns among species.

Similar to several horticultural plants,^{42,43} *CitUGT* members displayed consistent exon–intron structures and motif patterns within each family, indicating that they might perform analogous functions (Figure 2 and Table S2). Among the 14 conserved motifs, motif 1 was highly conserved and present in almost all *CitUGTs*. This motif included the PSPG box that is essential for the catalytic activity of the UGT enzyme (Figure S2). Moreover, previous studies have revealed that the final glutamine in the PSPG motif was vital for the specificity of UDP-glucose as the sugar donor.⁴⁸ These specific amino acids

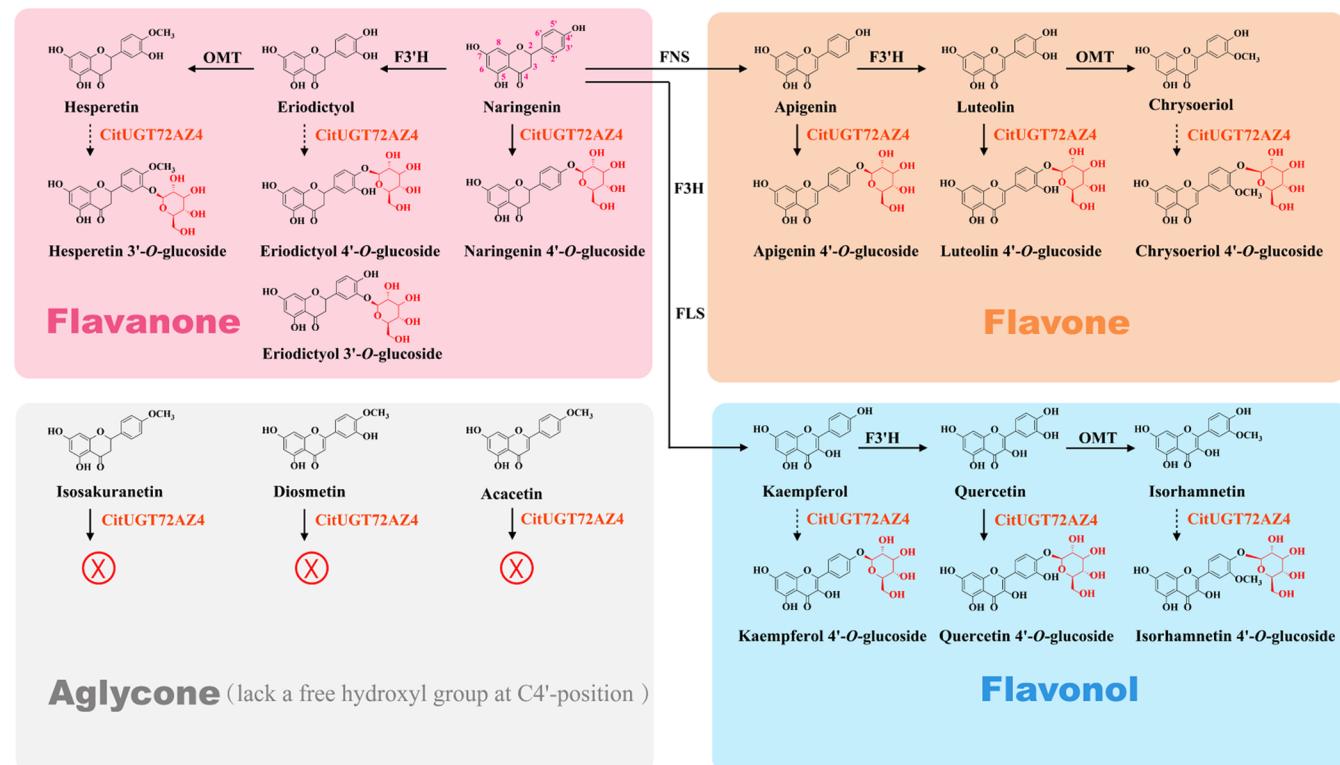


Figure 8. Proposed model for *CitUGT72AZ4* participated in the biosynthesis of flavonoid glucosides in citrus. F3'H, 3'-hydroxylase; FNS, flavone synthase; OMT, O-methyltransferase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase.

provide critical functional insights that could be valuable for future UGT enzyme research and discovery.

4.2. Phylogenetic Analysis of *CitUGT* Gene Family in Citrus. Our phylogenetic analysis classified 136 *CitUGT*s into 18 groups (A–R), with groups Q and R newly identified in citrus (Figures 1, S1 and Table S1). This finding was different from those reported in pomelo (*C. maxima*), where the 145 CgUGTs were categorized into 16 groups (A–P), lacking group Q and R (Table S7).⁴⁹ This discrepancy was likely due to the different phylogenetic analysis methods used for the UGT gene family. Compared to the previous analysis in pomelo, our study provides a more detailed examination of the evolutionary relationship of the *CitUGT* gene family in citrus. Several UGT members from Q and R were included in our phylogenetic tree construction, such as PgUGT95B2 (*Punica granatum*)⁵⁰ and FcCGT.⁴⁰ Group Q was once considered to be present exclusively in monocots like rice⁹ and maize.¹³ However, recent evidence has shown the presence of group Q in dicot plants, such as *M. domestica*⁴² and *Epimedium pubescens*,⁵¹ suggesting that group Q was not confined to monocots. Group R was composed of the UGT708 family, and many plant UGTs from this family were identified as C-glycosyltransferases (CGTs), which were responsible for the biosynthesis of C-glycosylflavonoids.⁵² In *C. clementina*, the *CitUGT* member (Ciclev10031419m) was assigned to group R, and its two hypothetical paralogous genes (FcCGT and CuCGT) have been characterized as CGTs.⁴⁰ This finding provided more solid evidence of the presence of group R in citrus (Figure 1).

During the evolutionary process of higher plants, groups A, D, E, G, and L were found to expand more extensively than other groups, typically comprising a higher number of UGT members.¹² Similarly, in citrus, a higher number of *CitUGT*s

were observed in groups A, D, E, H, I, and L, implying these six groups underwent rapid expansion (Figure S1 and Table S7). Among them, group E had the most number of *CitUGT*s with 28 members, followed by group L with 19 members, representing 16.91 and 13.97% of the total *CitUGT*s, respectively. Additionally, several UGTs from groups E and L have been characterized as participating in the biosynthesis of terpenes, flavonoids, and benzoates.^{49,53} These findings indicated that *CitUGT* members from groups E and L were crucial for the biosynthesis of secondary metabolites.⁵³

4.3. *CitUGT72AZ4* Participated in the Biosynthesis of Flavonoid Glycoside. Citrus is a rich source of bioactive flavonoids, particularly flavonoid glycosides, which accumulate differently across various tissues. In “Ponkan”, a total of 180 glycosylated flavonoids were identified, including 134 O-glycosylflavonoids, 24 C-glycosylflavonoids, and 19 C, O-glycosylflavonoids (Table S6). Most of these flavonoid glycosides were abundant in the leaf and flavedo, which was consistent with previous reports that flavonoids were highly accumulated in these tissues, whereas coumarins were predominantly found in the root³² (Figure 3). Moreover, 44 *CitUGT*s were found to be highly expressed in either the leaf or flavedo, consistent with the accumulated patterns of flavonoid glycosides. Notably, *CitUGT72AZ4* showed the highest expression levels in the leaf, indicating its potential role in the biosynthesis of flavonoid glycosides. However, the correlation analysis revealed a weak positive correlation between the expression levels of *CitUGT72AZ4* and the accumulation of flavonoid 4'-O-glucosides, such as naringenin 4'-O-glucoside ($R = 0.0971$, $P > 0.05$) and quercentin 7-O-rutinoside-4'-O-glucoside ($R = 0.0492$, $P > 0.05$), across various tissues of “Ponkan” (Table S7). These findings suggest that multiple *CitUGT*s might participate in the biosynthesis of

flavonoid 4'-O-glycoside, with *CitUGT72AZ4* specifically active in leaf, while other *CitUGTs* may function in other tissues. Plant UGTs could catalyze the glycosylation of flavonoids at one or more of the 3-OH, 5-OH, 7-OH, 3'-OH, or 4'-OH positions.⁴³ In this work, we identified *CitUGT72AZ4* as flavonoid 4'-O-glucosyltransferase for the first time in citrus. Unlike *CgUGT89AK1*, which primarily glycosylated the 7-OH group of flavonoids,²³ *CitUGT72AZ4* preferentially glycosylated the 4'-OH group of flavonoids *in vitro*, exhibiting higher catalytic efficiency toward quercetin and flavones (e.g., apigenin, chrysoeriol, and luteolin) (Figures 5, 6, and 8). Although *CitUGT72AZ4* could glycosylate the 3'-OH group of flavanone like hesperetin, its catalytic efficiency was lower than that of the 4'-OH group of flavonoids, suggesting that *CitUGT72AZ4* preferred to glycosylate the 4'-OH group of flavonoids. This regiospecificity was similar to that of *NmF4'GT* from *N. menziesii*, which specifically catalyzed the glycosylation of flavones at their 4'-OH position.¹⁵ Sequence alignment analysis revealed a 32.58% identity in amino acid sequences between *CitUGT72AZ4* and *NmF4'GT*, both containing the conserved PSPG box at their C-terminal regions (Table S3). Nevertheless, the catalytic mechanisms underlying the regioselectivity of *CitUGT72AZ4* remain unclear and require further structural investigation. Despite *CitUGT72AZ4* and *NmF4'GT* sharing high regioselectivity toward flavonoids, their substrate preferences differ. *NmF4'GT* displayed a strong preference for apigenin and had a limited range of substrates,¹⁵ whereas *CitUGT72AZ4* showed a strong preference for quercetin and catalyzed 4'-O-glucosylation of a wider range of flavonoids (Figure 6). Moreover, *CitUGT72AZ4* exhibited higher catalytic efficiency toward apigenin ($k_{cat}/K_m = 264.26 \text{ M}^{-1} \text{ S}^{-1}$) compared to *CgUGT89AK1* ($k_{cat}/K_m = 107.06 \text{ M}^{-1} \text{ S}^{-1}$), but its efficiency was lower than that of *CsUGT75L12* ($k_{cat}/K_m = 4.54 \times 10^3 \text{ M}^{-1} \text{ S}^{-1}$)⁵⁴ and *NmF4'GT* ($k_{cat}/K_m = 1.0 \times 10^5 \text{ M}^{-1} \text{ S}^{-1}$).¹⁵ Silencing of *CitUGT72AZ4* caused a notable decrease in the accumulation of flavonoid 4'-O-glucosides in "Jincheng" seedlings, confirming its role in the biosynthesis of flavonoid 4'-O-glucosides in citrus (Figure 7). Taken together, *CitUGT72AZ4* is an efficient and regioselective flavonoid 4'-O-glucosyltransferase involved in the biosynthesis of flavonoid 4'-O-glucosides in citrus.

ASSOCIATED CONTENT

Data Availability Statement

The transcriptome data presented in this study was acquired from NCBI BioProject (Accession Number PRJNA954191) and additional information is detailed in Supporting Tables.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.4c07454>.

Distribution of 136 *CitUGTs* in different phylogenetic groups (Figure S1); weblogos of the 14 motifs identified in *CitUGT* proteins (Figure S2); multiple sequence alignment of *CitUGT72AZ4* and *CitUGT84J10* with other known UGTs (Figure S3); characterization of *CitUGT72AZ4* and *CitUGT84J10* recombinase (Figure S4); enzymatic activity analysis of recombinant *CitUGT84J10* (Figure S5); MS/MS fragmentation information for glycosylated products catalyzed by *CitUGT72AZ4* and their corresponding authentic standards (Figure S6) (PDF)

comprehensive information on the *CitUGT* family genes in citrus (Table S1); number of introns and exons in *CitUGT* family members (Table S2); analysis of gene duplication events in *CitUGT* gene family (Table S3); tandem or segmental duplicated pairs of *CitUGTs* in citrus (Table S4); FPKM values of *CitUGTs* in various tissues of "Ponkan" (Table S5); distribution of flavonoid glycosides in various tissues of "Ponkan" (Table S6); number of plant UGTs in various phylogenetic groups (Table S7); list of accession numbers for UGTs from other plant species (Table S8); and primers utilized for recombinant constructs and qRT-PCR analysis (Table S9) (XLSX)

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B.L. and X.L. contributed equally to this work. C.D.S., X.J.L., X.L., and C.N.Z. designed the experiment; B.L. performed analyses and experiments with the help of X.J.L., Y.J.L., Y.F.G., X.L., Z.K.L., and H.X.W.; Z.W.G., and X.D.W. provided technical support for the experiments; D.L.W. provided plant materials for the experiment; and C.D.S., X.J.L., Z.K.L., C.N.Z., C.J.P., Y.W., and S.J.L reviewed and revised the manuscript.

Notes

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ABBREVIATIONS

UGT, UDP glycosyltransferase; PSPG box, plant secondary product glycosyltransferase box; 1,2RhaT, 1,2-rhamnosyltransferase; 1,6-rhamnosyltransferase, 1,6RhaT; IPTG, isopropyl β -D-thiogalactoside; OD, optical density; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; VIGS, virus-induced gene silencing; LB, Luria Broth; MES, 2-morpholinoethanesulfonic acid; LC-MS/MS, liquid chromatograph/mass spectrometer; qRT-PCR, quantitative real-time PCR; FPKM, fragments per kilobase of transcript per million mapped reads; SE, standard error; HMM, hidden Markov model; K_a , nonsynonymous substitution rate; K_s , synonymous substitution rate; λ , substitution rate; MYA, million years ago; K_m , Michaelis constant; k_{cat} , catalytic constant

REFERENCES

- (1) Li, P.; Li, Y.; Zhang, F.; Zhang, G.; Jiang, X.; Yu, H.; Hou, B. The *Arabidopsis* UDP-glycosyltransferases UGT79B2 and UGT79B3, contribute to cold, salt and drought stress tolerance via modulating anthocyanin accumulation. *Plant J.* **2017**, *89*, 85–103.
- (2) Peng, M.; Shahzad, R.; Gul, A.; Subhani, H.; Shen, S.; Lei, L.; Zheng, Z.; Zhou, J.; Lu, D.; Wang, S.; et al. Differentially evolved glucosyltransferases determine natural variation of rice flavone accumulation and UV-tolerance. *Nat. Commun.* **2017**, *8*, No. 1975.
- (3) Dai, X.; Shi, X.; Yang, C.; Zhao, X.; Zhuang, J.; Liu, Y.; Gao, L.; Xia, T. Two UDP-glycosyltransferases catalyze the biosynthesis of bitter flavonoid 7-O-neohesperidoside through sequential glycosylation in tea plants. *J. Agric. Food Chem.* **2022**, *70*, 2354–2365.
- (4) Rauter, A. P.; Ennis, M.; Hellwich, K. H.; Herold, B. J.; Horton, D.; Moss, G. P.; Schomburg, I. Nomenclature of flavonoids (IUPAC Recommendations 2017). *Pure Appl. Chem.* **2018**, *90*, 1429–1486.
- (5) Naeem, A.; Yang, M.; Hu, P.; K, Y.; Liu, Y.; Hai, Z.; Xiao, S.; Li, W.; Wu, L.; Zhang, M.; Liu, S.; Zheng, Q. The fate of flavonoids after oral administration: a comprehensive overview of its bioavailability. *Crit. Rev. Food Sci. Nutr.* **2022**, *62*, 6169–6186.
- (6) Yang, B.; Liu, H.; Yang, J.; Gupta, V. K.; Jiang, Y. New insights on bioactivities and biosynthesis of flavonoid glycosides. *Trends Food Sci. Technol.* **2018**, *79*, 116–124.
- (7) Yonekura-Sakakibara, K.; Hanada, K. An evolutionary view of functional diversity in family 1 glycosyltransferases. *Plant J.* **2011**, *66*, 182–193.
- (8) Vogt, T.; Jones, P. Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. *Trends Plant Sci.* **2000**, *5*, 380–386.
- (9) Wilson, A. E.; Tian, L. Phylogenomic analysis of UDP-dependent glycosyltransferases provides insights into the evolutionary landscape of glycosylation in plant metabolism. *Plant J.* **2019**, *100*, 1273–1288.
- (10) Li, Y.; Baldauf, S.; Lim, E.-K.; Bowles, D. J. Phylogenetic analysis of the UDP-glycosyltransferase multigene family of *Arabidopsis thaliana** 210. *J. Biol. Chem.* **2001**, *276*, 4338–4343.
- (11) Wu, B.; Gao, L.; Gao, J.; Xu, Y.; Liu, H.; Cao, X.; Zhang, B.; Chen, K. Genome-wide identification, expression patterns, and functional analysis of UDP glycosyltransferase family in peach (*Prunus persica* L. Batsch). *Front. Plant Sci.* **2017**, *8*, No. 251763.
- (12) Caputi, L.; Malnoy, M.; Goremykin, V.; Nikiforova, S.; Martens, S. A genome-wide phylogenetic reconstruction of family 1

- UDP-glycosyltransferases revealed the expansion of the family during the adaptation of plants to life on land. *Plant J.* **2012**, *69*, 1030–1042.
- (13) Li, Y.; Li, P.; Wang, Y.; Dong, R.; Yu, H.; Hou, B. Genome-wide identification and phylogenetic analysis of Family-1 UDP glycosyltransferases in maize (*Zea mays*). *Planta* **2014**, *239*, 1265–1279.
- (14) Cui, L.; Yao, S.; Dai, X.; Yin, Q.; Liu, Y.; Jiang, X.; Wu, Y.; Qian, Y.; Pang, Y.; Gao, L.; Xia, T. Identification of UDP-glycosyltransferases involved in the biosynthesis of astringent taste compounds in tea (*Camellia sinensis*). *J. Exp. Bot.* **2016**, *67*, 2285–2297.
- (15) Okitsu, N.; Matsui, K.; Horikawa, M.; Sugahara, K.; Tanaka, Y. Identification and characterization of novel *Nemophila menziesii* flavone glucosyltransferases that catalyze biosynthesis of flavone 7, 4'-O-diglucoside, a key component of blue metalloanthocyanins. *Plant Cell Physiol.* **2018**, *59*, 2075–2085.
- (16) Griesser, M.; Vitzthum, F.; Fink, B.; Bellido, M. L.; Raasch, C.; Munoz-Blanco, J.; Schwab, W. Multi-substrate flavonol O-glucosyltransferases from strawberry (*Fragaria × ananassa*) achene and receptacle. *J. Exp. Bot.* **2008**, *59*, 2611–2625.
- (17) Barreca, D.; Bellocchio, E.; Caristi, C.; Leuzzi, U.; Gattuso, G. Flavonoid composition and antioxidant activity of juices from chinotto (*Citrus × myrtifolia* Raf.) fruits at different ripening stages. *J. Agric. Food Chem.* **2010**, *58*, 3031–3036.
- (18) Wang, Y.; Liu, X.; Chen, J.; Cao, J.; Li, X.; Sun, C. Citrus flavonoids and their antioxidant evaluation. *Crit. Rev. Food Sci. Nutr.* **2022**, *62*, 3833–3854.
- (19) del Río, J. A.; Gómez, P.; Baídez, A. G.; Arcas, M. C.; Botía, J.; Ortúño, A. Changes in the Levels of polymethoxyflavones and flavanones as part of the defense mechanism of *Citrus sinensis* (Cv. Valencia Late) fruits against *Phytophthora citrophthora*. *J. Agric. Food Chem.* **2004**, *52*, 1913–1917.
- (20) Ramli, S. Total phenolic content and antioxidant activity of flavonoids isolated from leaves of selected citrus species. M.S. Thesis, Universiti Putra Malaysia, 2006. <http://psasir.upm.edu.my/id/eprint/4969/>.
- (21) Barreca, D.; Bisignano, C.; Ginestra, G.; Bisignano, G.; Bellocchio, E.; Leuzzi, U.; Gattuso, G. Polymethoxylated, C-and O-glycosyl flavonoids in tangelo (*Citrus reticulata* × *Citrus paradisi*) juice and their influence on antioxidant properties. *Food Chem.* **2013**, *141*, 1481–1488.
- (22) Chen, J.; Yuan, Z.; Zhang, H.; Li, W.; Shi, M.; Peng, Z.; Li, M.; Tian, J.; Deng, X.; Cheng, Y.; et al. *Cit1,2RhaT* and two novel *CitdGlcTs* participate in flavor-related flavonoid metabolism during citrus fruit development. *J. Exp. Bot.* **2019**, *70*, 2759–2771.
- (23) Yuan, Z.; Li, G.; Zhang, H.; Peng, Z.; Ding, W.; Wen, H.; Zhou, H.; Zeng, J.; Chen, J.; Xu, J. Four novel *Cit7GlcTs* functional in flavonoid 7-O-glucoside biosynthesis are vital to flavonoid biosynthesis shunting in citrus. *Hortic. Res.* **2024**, *11*, No. uhae098. DOI: [10.1093/h/uhae098](https://doi.org/10.1093/h/uhae098).
- (24) Frydman, A.; Liberman, R.; Huhman, D. V.; Carmeli-Weissberg, M.; Sapir-Mir, M.; Ophir, R.; W Sumner, L.; Eyal, Y. The molecular and enzymatic basis of bitter/non-bitter flavor of citrus fruit: evolution of branch-forming rhamnosyltransferases under domestication. *Plant J.* **2013**, *73*, 166–178.
- (25) Frydman, A.; Weisshaus, O.; Bar-Peled, M.; Huhman, D. V.; Sumner, L. W.; Marin, F. R.; Lewinsohn, E.; Fluhr, R.; Gressel, J.; Eyal, Y. Citrus fruit bitter flavors: isolation and functional characterization of the gene *Cm1,2RhaT* encoding a 1,2 rhamnosyltransferase, a key enzyme in the biosynthesis of the bitter flavonoids of citrus. *Plant J.* **2004**, *40*, 88–100.
- (26) Shen, S.; Wang, S.; Yang, C.; Wang, C.; Zhou, Q.; Zhou, S.; Zhang, R.; Li, Y.; Wang, Z.; Dai, L.; et al. Elucidation of the melitinidin biosynthesis pathway in pummelo. *J. Integr. Plant Biol.* **2023**, *65*, 2505–2518.
- (27) Chen, C.; Wu, Y.; Li, J.; Wang, X.; Zeng, Z.; Xu, J.; Liu, Y.; Feng, J.; Chen, H.; He, Y.; Xia, R. TBtools-II: A "One for All, All for One" bioinformatics platform for biological big-data mining. *Mol. Plant* **2023**, *16*, 1733–1742.
- (28) Tamura, K.; Stecher, G.; Kumar, S. MEGA11: molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027.
- (29) Wang, Y.; Tang, H.; DeBarry, J. D.; Tan, X.; Li, J.; Wang, X.; Lee, Th.; Jin, H.; Marler, B.; Guo, H.; et al. MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res.* **2012**, *40*, No. e49.
- (30) Chen, C.; Wu, Y.; Xia, R. A painless way to customize Circos plot: From data preparation to visualization using TBtools. *iMeta* **2022**, *1*, No. e35.
- (31) Carbonell-Caballero, J.; Alonso, R.; Ibanez, V.; Terol, J.; Talon, M.; Dopazo, J. A phylogenetic analysis of 34 chloroplast genomes elucidates the relationships between wild and domestic species within the genus *Citrus*. *Mol. Biol. Evol.* **2015**, *32*, 2015–2035.
- (32) Liang, X.; Wang, H.; Xu, W.; Liu, X.; Zhao, C.; Chen, J.; Wang, D.; Xu, S.; Cao, J.; Sun, C.; Wang, Y. metabolome and transcriptome analysis revealed the basis of the difference in antioxidant capacity in different tissues of *Citrus reticulata* 'Ponkan'. *Antioxidants* **2024**, *13*, No. 243.
- (33) Ren, C.; Guo, Y.; Xie, L.; Zhao, Z.; Xing, M.; Cao, Y.; Liu, Y.; Lin, J.; Grierson, D.; Zhang, B.; et al. Identification of UDP-rhamnosyltransferases and UDP-galactosyltransferase involved in flavonol glycosylation in *Morella rubra*. *Hortic. Res.* **2022**, *9*, No. uha138.
- (34) Xie, L.; Guo, Y.; Ren, C.; Cao, Y.; Li, J.; Lin, J.; Grierson, D.; Zhao, X.; Zhang, B.; Sun, C.; et al. Unravelling the consecutive glycosylation and methylation of flavonols in peach in response to UV-B irradiation. *Plant Cell Environ.* **2022**, *45*, 2158–2175.
- (35) Liu, X.; Zhao, C.; Gong, Q.; Wang, Y.; Cao, J.; Li, X.; Grierson, D.; Sun, C. Characterization of a caffeoyl-CoA O-methyltransferase-like enzyme involved in biosynthesis of polymethoxylated flavones in *Citrus reticulata*. *J. Exp. Bot.* **2020**, *71*, 3066–3079.
- (36) Sheikh, M. O.; Halmo, S. M.; Patel, S.; Middleton, D.; Takeuchi, H.; Schafer, C. M.; West, C. M.; Haltiwanger, R. S.; Avci, F. Y.; Moremen, K. W.; Wells, L. Rapid screening of sugar-nucleotide donor specificities of putative glycosyltransferases. *Glycobiology* **2017**, *27*, 206–212.
- (37) Zhao, C.; Liu, X.; Gong, Q.; Cao, J.; Shen, W.; Yin, X.; Grierson, D.; Zhang, B.; Xu, C.; Li, X.; et al. Three AP2/ERF family members modulate flavonoid synthesis by regulating type IV chalcone isomerase in citrus. *Plant Biotechnol. J.* **2021**, *19*, 671–688.
- (38) Liu, X.; Gong, Q.; Zhao, C.; Wang, D.; Ye, X.; Zheng, G.; Wang, Y.; Cao, J.; Sun, C. Genome-wide analysis of cytochrome P450 genes in *Citrus clementina* and characterization of a CYP gene encoding flavonoid 3'-hydroxylase. *Hortic. Res.* **2023**, *10*, No. uha283.
- (39) Owens, D. K.; McIntosh, C. A. Identification, recombinant expression, and biochemical characterization of a flavonol 3-O-glycosyltransferase clone from *Citrus paradisi*. *Phytochemistry* **2009**, *70*, 1382–1391.
- (40) Ito, T.; Fujimoto, S.; Suito, F.; Shimosaka, M.; Taguchi, G. C-glycosyltransferases catalyzing the formation of di-C-glucosyl flavonoids in citrus plants. *Plant J.* **2017**, *91*, 187–198.
- (41) Xie, L.; Cao, Y.; Zhao, Z.; Ren, C.; Xing, M.; Wu, B.; Zhang, B.; Xu, C.; Chen, K.; Li, X. Involvement of *MdUGT75B1* and *MdUGT71B1* in flavonol galactoside/glucoside biosynthesis in apple fruit. *Food Chem.* **2020**, *312*, No. 126124.
- (42) Ren, C.; Cao, Y.; Xing, M.; Guo, Y.; Li, J.; Xue, L.; Sun, C.; Xu, C.; Chen, K.; Li, X. Genome-wide analysis of UDP-glycosyltransferase gene family and identification of members involved in flavonoid glucosylation in Chinese bayberry (*Morella rubra*). *Front. Plant Sci.* **2022**, *13*, No. 998985.
- (43) Li, Y.; Li, P.; Zhang, L.; Shu, J.; Court, M. H.; Sun, Z.; Jiang, L.; Zheng, C.; Shu, H.; Ji, L.; Zhang, S. Genome-wide analysis of the apple family 1 glycosyltransferases identified a flavonoid-modifying UGT, *MdUGT83L3*, which is targeted by *MdMYB88* and contributes to stress adaptation. *Plant Sci.* **2022**, *321*, No. 111314.
- (44) Xu, Q.; Chen, L. L.; Ruan, X.; Chen, D.; Zhu, A.; Chen, C.; Bertrand, D.; Jiao, W. B.; Hao, B.; Lyon, M. The draft genome of sweet orange (*Citrus sinensis*). *Nat. Genet.* **2013**, *45*, 59–66.

- (45) Wang, F.; Su, Y.; Chen, N.; Shen, S. Genome-wide analysis of the UGT gene family and identification of flavonoids in *Broussonetia papyrifera*. *Molecules* **2021**, *26*, No. 3449.
- (46) Ouyang, L.; Liu, Y.; Yao, R.; He, D.; Yan, L.; Chen, Y.; Huai, D.; Wang, Z.; Yu, B.; Kang, Y.; et al. Genome-wide analysis of UDP-glycosyltransferase gene family and identification of a flavonoid 7-O-UGT (*AhUGT75A*) enhancing abiotic stress in peanut (*Arachis hypogaea* L.). *BMC Plant Biol.* **2023**, *23*, No. 626.
- (47) Chen, Y.; Fu, M.; Li, H.; Wang, L.; Liu, R.; Liu, Z. Genome-wide characterization of the UDP-glycosyltransferase gene family reveals their potential roles in leaf senescence in cotton. *Int. J. Biol. Macromol.* **2022**, *222*, 2648–2660.
- (48) Kubo, A.; Arai, Y.; Nagashima, S.; Yoshikawa, T. Alteration of sugar donor specificities of plant glycosyltransferases by a single point mutation. *Arch. Biochem. Biophys.* **2004**, *429*, 198–203.
- (49) Wu, B.; Liu, X.; Xu, K.; Zhang, B. Genome-wide characterization, evolution and expression profiling of UDP-glycosyltransferase family in pomelo (*Citrus grandis*) fruit. *BMC Plant Biol.* **2020**, *20*, 1–12.
- (50) Wilson, A. E.; Wu, S.; Tian, L. J. P. PgUGT95B2 preferentially metabolizes flavones/flavonols and has evolved independently from flavone/flavonol UGTs identified in *Arabidopsis thaliana*. *Phytochemistry* **2019**, *157*, 184–193.
- (51) Yao, Y.; Gu, J.; Luo, Y.; Wang, Y.; Pang, Y.; Shen, G.; Guo, B. Genome-wide analysis of UGT gene family identified key gene for the biosynthesis of bioactive flavonol glycosides in *Epimedium pubescens* Maxim. *Synth. Syst. Biotechnol.* **2022**, *7*, 1095–1107.
- (52) Dai, L.; Hu, Y.; Chen, C. C.; Ma, L.; Guo, R. T. Flavonoid C-glycosyltransferases: Function, evolutionary relationship, catalytic mechanism and protein engineering. *ChemBioEng Rev.* **2021**, *8*, 15–26.
- (53) Liu, X.; Lin, C.; Ma, X.; Tan, Y.; Wang, J.; Zeng, M. Functional characterization of a flavonoid glycosyltransferase in sweet orange (*Citrus sinensis*). *Front. Plant Sci.* **2018**, *9*, 166.
- (54) Dai, X.; Zhuang, J.; Wu, Y.; Wang, P.; Zhao, G.; Liu, Y.; Jiang, X.; Gao, L.; Xia, T. Identification of a flavonoid glucosyltransferase involved in 7-OH site glycosylation in tea plants (*Camellia sinensis*). *Sci. Rep.* **2017**, *7*, No. 5926.