Illustration of the variation in the content of flavanone rutinosides in various citrus germplasms from genetic and enzymatic perspectives

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Abstract
In citrus, 1,6-rhamnosytransferase (1,6RhaT) and 1,2-rhamnosytransferase (1,2RhaT) catalyze flavanone-7-O-glucosides to form nonbitter flavanone rutinosides (FRs) and bitter flavanone neohesperidosides (FNs), respectively. As revealed in this study of fruit peels from 36 citrus accessions, FRs varied from undetectable levels in pummelo and kumquat to being the dominant flavonoids in sweet orange and loose-skin mandarins. Furthermore, a previously annotated full-length 1,6RhaT-encoding gene by in vitro experiments. In total, 28 alleles of full-length 1,6RhaTs were isolated and classified into A, B and C types with only type A alleles encoding a functional protein. Coincidently, only the accessions that contained FRs harbored type A alleles, as was further verified in two F1 hybrid populations. Moreover, the inferior substrate conversion efficiency of 1,6RhaTs in comparison with that of 1,2RhaT in vitro might partly explain the lower proportions of FRs to total flavanone disaccharides in citrus hybrids harboring both functional rhamnosyltransferases. Our findings provide a better understanding of FR content variations among citrus and are meaningful for a mechanistic illustration of citrus flavonoid metabolism and fruit quality improvement practices.

Introduction
As the most abundant polyphenol compounds in citrus, flavonoids comprise a large family that includes flavanones, flavonols, flavones, isoflavones, anthocyanins, and flavanols [1, 2]. Flavonoids reportedly possess a wide range of biological activities, including antioxidant, anticancer, antimicrobial, anti-inflammatory, anti-obesity and cardiovascular and cerebrovascular disease-preventing activities [3–6]. Most flavonoids in citrus are present in the form of glycosyl derivatives, which increases their stability, solubility and bioavailability [7]. Flavanone rutinosides (FRs) and flavanone neohesperidosides (FNs), which are the predominant flavanone disaccharides in citrus, contain the α-L-rhamnosyl-β-D-glucoside disaccharide moiety bound to the flavanone aglycone and exhibit different glycosidic links (β1 → 2 of FRs, β1 → 6 of FNs) between the two sugar units. Recently, FRs have received increasing attention due to their wide range of features that are beneficial to human health [8–10]. For example, hesperidin is the main pharmacological component of Pericarpium Citri Reticulatae (chenpi), which can effectively relieve skin inflammation, muscle pain, coughing and breath difficulties [11].

Flavanone disaccharides are synthesized via the phenylpropanoid pathway in plants as stabilized end products resulting from two glycosylation steps [12]. The first glycosylation step involves the reaction of flavanone aglycones to flavanone-7-O-glucosides catalyzed by 7-O-glucosyltransferase (7-GlcT), and these products are further converted to flavanone disaccharides of FRs and FNs by 1,6-rhamnosytransferase (1,6RhaT) and 1,2-rhamnosytransferase (1,2RhaT), respectively [13] (Supporting Fig. S1). These two flavanone disaccharides contribute significantly to the flavor of citrus fruits by providing nonbitter FRs (eriocitrin, didymin, narirutin and hesperidin, etc.) and bitter FNs (neoerocitrin, poncirirol, neohesperidin and naringin, etc.), respectively [14]. It is worth noting that FRs and FNs are synthesized from the same substrate, and competition may exist within germplasms harboring both functional genes.

A considerable number of studies have investigated the specific accumulation of FRs and FNs in citrus...
germplasms and the changes in the contents of these compounds among different developmental stages or tissues [15–18]. High levels of FRs are present in orange, mandarin and lemon; low levels of FRs are present in grapefruit and bitter orange, which contain high levels of FNs; and FRs were not detected in pummelo [19]. The available evidence shows that the accumulation of FRs varies among citrus germplasms, but the mechanisms are not well understood. Fortunately, based on our previous elucidation of the molecular mechanism underlining the variations in the FN profiles among different citrus germplasms [20], revealing the mechanisms underlying the variations in the FR profiles in different citrus germplasms is feasible.

In this study, the FRs in various citrus accessions covering the main cultivated species, wild species and their relatives were investigated. Another 1,6RhaT gene was identified, which implies that there are multiple 1,6RhaT genes in citrus. The 1,6RhaT genes and their allelic variations were then characterized in vitro. Furthermore, the production of FNs and FRs in two hybrid cross populations harboring both functional 1,2RhaT and 1,6RhaT genes was further investigated. This study will contribute to a better understanding of the accumulation of flavanone disaccharides with different bioactive characteristics in various citrus germplasms and is thus meaningful for improving fruit quality.

Materials and methods

Plant materials and compounds

Thirty-four accessions were obtained from the National Citrus Breeding Centre (NCBC) at Huazhong Agricultural University, Wuhan, China, and two other accessions were obtained from Haohuahong Town, Huishui County, Guizhou Province, China (Table S1). For each accession, young leaves and 15–18 fruits were randomly collected from at least three healthy trees at their respective commercial maturation stages at approximately 230 d post anthesis (DPA).

Hybrid populations of "Hirado Butun" (HB) pummelo (C. maxima) × Fairchild tangelo (Citrus reticulata × Chrysopelea paradisi) and red tangerine (Citrus reticulata) × trifoliata orange (Poncirus trifoliata) were established in 2003, and fruits and leaves from 37 and 66 F1 plants were collected from these two populations, respectively.

All fruits and leaves were randomly divided into three biological replications. The fruit peel (flavedo and albedo) tissues were immediately separated, frozen in liquid nitrogen, ground into a fine powder and stored at −80°C. All operations were performed under dim light conditions, and long-term exposure to open air was avoided to prevent the degradation of flavonoids. Transgenic tobacco bright yellow (BY2) calli harboring Cm1,2RhaT were obtained as described by Chen et al. [17].

Authentic standard compounds of UDP-glucose, poncirin, didymin, neoeoeriocitrin, eriocitrin, naringin, narirutin, neohesperidin, hesperidin, naringenin, hesperetin, naringenin-7-O-glucoside, and hesperetin-7-O-glucoside were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China), with a high purity of 98%. UDP-rhamnose was purchased from Angfei Biological Technology Co., Ltd. (Guangdong, China).

Extraction of flavonoids and determination of FRs and FNs

The extraction and separation of the flvanone disaccharides were conducted as described previously [17]. Flavonoids were separated using a gradient elution program with a flow rate of 1 ml/min, a column temperature of 35°C and a Waters 1525 binary system equipped with a 2996 photodiode array detector, a 717 Plus autosampler (Waters Corp., Milford, MA, USA) and a C18 column (250 mm × 4.6 mm, 5 μm, Thermo Scientific, Waltham, MA, USA). Subsequently, 0.15% formic acid diluted in water was prepared as mobile phase A, and 0.15% formic acid-acetonitrile was prepared as mobile phase B. The data were analyzed using Empower Chromatography Manager software (Waters Co., Milford, MA, USA).

RNA isolations, cDNA synthesis and real-time PCR

Total RNA was isolated using an EASYspin Plus Plant RNA Kit (Aidlab Biotechnologies Co., Ltd.). HiScript II Reverse Transcriptase (Aidlab Biotechnologies Co., Ltd.) was used for the amplification of cDNA. qRT-PCR was performed using a Prism 7900HT (Applied Biosystems) with the SYBR Green system (Hieff qPCR SYBR Green Master Mix, Yeasen Biotech Co., Ltd.). All gene expression analyses were performed with three independent biological replicates. The primers used in the analysis are listed in Supporting Table S2. The actin gene was used as the reference control.

BLAST analysis, phylogenetic analysis and gene cloning

BLAST analysis was performed against the Phytozone 12 database (https://phytozone.jgi.doe.gov/pz/portal.html#!search?show=BLAST) and Citrus sinensis Genome Annotation Project database (http://citrus.hzau.edu.cn/cgi-bin/orange/blast) using Cs1,6RhaT (accession number: DQ119035) as the reference sequence. ClustalW (http://www.genome.jp/tools-bin/clustalw) was utilized for multiple alignments of the nucleotide sequences of the alleles in 36 accessions. Phylogenetic analysis was generated using MEGA 7.0 according to the maximum likelihood method. There were 1000 bootstrap replications to test the reliability.

Young leaves of 36 accessions and two F1 hybrid populations were collected for genomic DNA extraction according to the protocol described by Cheng et al. [21]. To amplify the complete coding sequence of 1,6RhaT genes from both gDNA and cDNA templates,
specific PCR primers were designed based on Cs1,6RhaT (Supporting Table S2). The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). To ensure accuracy, at least eight positive clones of each accession were sequenced by TSINGKE Biological Technology Co., Ltd. (Beijing, China).

Expression and biotransformation assay in tobacco BY2 cells

Seven 1,6RhaT alleles that were found at a higher frequency (A1, A5, A10, B2 and C4) during isolation or appeared in some unique accessions (A8 and C6) were cloned into the pH7WG2D vector under the control of a CAMV-35S promoter harboring green fluorescent protein (GFP) and transferred into Agrobacterium GV3101. The resulting construct was used to stably transform BY2 callus suspensions according to a published protocol [20, 22]. The transgenic calli were confirmed by fluorescence detection through epifluorescence stereomicroscopy at 488 nm, PCR amplification and sequencing. The positively transformed calli were subjected to subsequent substrate feeding analysis.

To carry out the biotransformation assays, a substrate feeding experiment using BY2 cells was performed according to the protocol described by Frydman et al. [13]. Briefly, transgenic and wild-type BY2 calli were suspended in Erlenmeyer flasks for one week at 28°C with shaking at 140 rpm. Subsequently, 1 g of callus was diluted in 20 mL of MS liquid media and incubated under the same conditions for 4 d. Flavonoid substrates (mg/ml) were added to the flask and then incubated for another 2 d. The resulting BY2 cells were harvested and used for extraction and determination of flavonoids.

Protein expression and purification

The expression and purification of Cme1,6RhaT-like was performed as described by Peng et al. [23]. Briefly, Cme1,6RhaT-like was cloned into the pMal-c2x expression vector with a maltose-binding protein tag. Recombinant proteins were expressed in BL21 (DE3) cells and induced with IPTG at 16°C. The harvested cells were lysed in phosphate-buffered saline pH 7.4 containing 400 mM NaCl by sonication at 4°C. The protein was transferred into a column containing dextrin beads 6FF and purified by washing buffer (20 mM Tris–HCl, 1 mM EDTA, and 1 mM DTT, pH 7.4) and elution buffer (20 mM Tris–HCl, 10 mM maltose, and 1 mM DTT, pH 7.4).

Cp1,2RhaT and Cp1,6RhaT were expressed and purified following a similar protocol.

Assay enzyme activity and kinetics

The enzyme assay was performed according to Peng et al. [23]. The glycosylation reaction was performed in a total volume of 100 μl containing 5 μg of purified glycosyltransferases, 5 μg of flavonoid substrates, 5 μg of UDP-rhamnose, and 0.25 mM MgCl₂ in phosphate buffered saline (50 mM, pH 7.4). The reaction was then incubated for 30 min at 35°C. The reaction was stopped by the addition of 300 μl of ice-cold methanol, and the resulting products were subjected to LC–MS analysis.

To determine the enzymatic kinetic constants of Cp1,2RhaT and Cp1,6RhaT, their activities were determined using 10 to 400 μM flavonoid aglycones with 5 μg UDP-rhamnose. All kinetic parameters were calculated using the Michaelis–Menten model (SigmaPlot, version 12.5).

Results

FR accumulation is species-dependent in various citrus germplasms

In our previous study, flavonoids were found to accumulate at higher levels in fruit peel (flavedo and albedo) than in the pulp of most citrus germplasms. Consequently, substantial variations in the contents of FRs and FNs were observed among the fruit peels of 36 accessions (Supplement Table S1). These accessions cover the main cultivated species, wild species and their relatives, including trifoliata orange (Poncirus trifoliata), kumquat (Citrus japonica), loose-skin mandarin (Citrus reticulata), sweet orange (Citrus sinensis), pummelo (Citrus maxima), grapefruit (Citrus paradisi), lemon (Citrus limon), citron (Citrus medica) and ichang papeda (Citrus ichangensis).

These citrus accessions could be divided into three groups according to their contents of FRs (Fig. 1). The accessions in Group 1, such as sweet orange, loose-skin mandarin and citrons, had high levels of FRs but undetectable levels of FNs. Group 2 included accessions with both FRs and FNs, such as ichang papeda, trifoliata orange and grapefruit. However, the proportions of FR among the total flavanone disaccharides were lower than those of FNs. Group 3 was comprised of pummelo and kumquat and exhibited undetectable levels of FRs; moreover, pummelo was enriched in FNs, whereas FNs were undetectable in kumquat. These results showed that the citrus FR profiles are species-dependent. The associations among the contents and proportions of the FRs and the genetic background in various citrus accessions was then investigated.

Identification of a new 1,6RhaT gene in citrus genomes

A 1,6RhaT-like gene with high nucleotide sequence homology to Cs1,6RhaT (GenBank accession No. AY048882) was identified in the genomes of various citrus species (Fig. 2). The 1,6RhaT-like gene was annotated in the genomes of various citrus species with the exception of sweet orange and loose-skin mandarin and it was located on different loci from those of 1,6RhaT. The genomic sequences revealed that 1,6RhaT-like shared high nucleotide sequence identities (approximately 96%) with Cs1,6RhaT, and various base deletions and insertions were found in Atalantia buxifolia, C. maxima...
and C. ichangensis, whereas an intact 1,6RhaT-like was only found in Citrus medica with an ORF of 1428 bp. Cme1,6RhaT-like from Eureka lemon was isolated, sequenced and subjected to an analysis of its catalytic activity. Cme1,6RhaT-like was expressed in Escherichia coli and characterized. After the application of naringenin-7-O-glucoside and UDP-rhamnose as substrates, a new peak was detected and identified as narirutin by comparing the retention time and the MS2 spectra of the corresponding product of Cs1,6RhaT (Fig. 3a). To ensure
Figure 3. HPLC chromatogram of reaction products of recombinant Cme16RhaT-like and Cs1,6RhaT and MS2 spectra of the products. (a) Naringenin-7-O-glucoside was used as the substrate. (b) Kaempferol-3-O-glucoside was used as the substrate. (c) Diosmetin-7-O-glucoside was used as the substrate.

its catalytic activity, we used other types of flavonoids, including flavone (kaempferol-3-O-glucoside) and flavonol (diosmetin-7-O-glucoside), as substrates (Fig. 3b-c). The results showed that Cme1,6RhaT-like also exhibited rhamnosylation activity toward these two types of flavonoids and converted these substrates into their corresponding flavanone disaccharides.

Thus, our results showed that Cme1,6RhaT-like is another 1,6RhaT gene, and multiple 1,6RhaT genes are found in citrus. Consequently, we defined 1,6RhaT and 1,6RhaT-like as 1,6RhaTs in the rest of the manuscript.

Allelic sequence variations of citrus 1,6RhaTs
To examine the correlation between the 1,6RhaT genotype and the rutinoside accumulation phenotype, 1,6RhaT was amplified from the DNA of 36 citrus accessions. A total of 28 different alleles were isolated with high similarity, and insertions, deletions and single nucleotide polymorphisms (SNPs) were also found. These alleles can be divided into three types, A, B and C, based on their sequences. Type A contains 18 alleles defined as A1 to A18, type B consists of B1 to B4, and C contains C1 to C6. Each accession investigated possessed two different
Table 1. 1,6RhaT alleles related to FR phenotypes in 36 citrus accessions

| Samples                  | Abbreviation | Alleles isolated | Length (bp) | Scientific name | Deduced amino acid (aa) | Phenotype |
|--------------------------|--------------|------------------|-------------|-----------------|-------------------------|-----------|
| “Cara Cara” navel orange | HRQC         | A1, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Hong Anliu sweet orange  | HAL          | A1, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Tarocco blood orange     | TLK          | A1, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Valencia orange          | FLX          | A1, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Seike navel range        | QQC          | A1, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Washington navel orange  | HSD          | A3, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Anliu sweet orange       | AL           | A1, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Newhall navel orange     | NHE          | A1, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Niu Rouhong tangerine    | NRH          | A5, A10          | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Nanfengmiju tangerine    | NF           | A1, A2           | 1428        | Citrus sinensis | Intact protein (475aa)  | +         |
| Fairchild tangelo        | FAIRC        | A5, A10          | 1428        | Citrus reticulata × | Intact protein (475aa) | +         |
| Zhu Hongju tangerine     | ZHJ          | A5, A10          | 1428        | Citrus reticulata | Intact protein (475aa)  | +         |
| “Guoqing No.1” satsuma mandarin | QG  | A1 | 1428 | Citrus reticulata | Intact protein (475aa)  | +         |
| Qingjiangponkan mandarin | QPG          | A1, A10          | 1428        | Citrus reticulata | Intact protein (475aa)  | +         |
| Bendizao tangerine       | BDZ          | A6               | 1428        | Citrus reticulata | Intact protein (475aa)  | +         |
| Clementine               | KLMD         | A5, A10          | 1428        | Citrus reticulata | Intact protein (475aa)  | +         |
| Red tangerine            | HJ           | A4, A10          | 1428        | Citrus reticulata | Intact protein (475aa)  | +         |
| Lime                     | LAIM         | A1, A17          | 1428        | Citrus aurantiifolia | Intact protein (475aa)  | +         |
| Limonia                  | LIM          | A12, A18         | 1428        | Citrus limonia  | Intact protein (475aa)  | +         |
| Eureka lemon             | YLK          | A11, A16         | 1428        | Citrus limon  | Intact protein (475aa)  | +         |
| Rough lemon               | CNM          | A16, B3          | 1428/1433   | Citrus jambhiri | Intact protein (475aa)/ Truncated protein (174aa) | +         |
| Fingered citrus          | FS           | A12, A16         | 1428        | Citrus medica  | Intact protein (475aa)  | +         |
| Ichang papeda            | YCC          | A6, A15          | 1428        | Citrus i changensis | Intact protein (475aa)  | +         |
| Trifoliata orange        | ZK           | A8, A9           | 1428        | Poncirus trifoliata | Intact protein (475aa)  | +         |
| Citrange                 | ZC           | A10, A14         | 1428        | Citrus sinensis × Poncirus trifoliata | Intact protein (475aa)  | +         |
| Citrumelo                | ZY           | A13, B1          | 1428/1433   | Poncirus trifoliata × Citrus paradiisi | Intact protein (475aa)/ Truncated protein (174aa) | +         |
| Star ruby grapefruit     | XNB          | A10, C2          | 1428/1433   | Citrus paradiisi | Intact protein (475aa)/ Truncated protein (68aa) | +         |
| Red marsh grapefruit     | HMX          | A10, C2          | 1428/1433   | Citrus paradiisi | Intact protein (475aa)/ Truncated protein (68aa) | +         |
| Marsh grapefruit          | MX           | A10, C2          | 1428/1433   | Citrus paradiisi | Intact protein (475aa)/ Truncated protein (68aa) | +         |
| Cocktail grapefruit      | JW           | A7, C2           | 1428/1433   | Citrus reticulata × Citrus maxima | Intact protein (475aa)/ Truncated protein (68aa) | +         |
| Feicui pummelo           | FC           | B2, C2           | 1433/1427   | Citrus maxima  | Truncated protein (9aa)/ Truncated protein (68aa) | −         |
| Hiradobutun pummelo      | HB           | B2, C4           | 1433/1420   | Citrus maxima  | Truncated protein (9aa)/ Truncated protein (68aa) | −         |
| Chuhong pummelo          | CH           | B2, C3           | 1433/1427   | Citrus maxima  | Truncated protein (9aa)/ Truncated protein (68aa) | −         |
| Guanxi pummelo           | GX           | B2, C4           | 1433/1420   | Citrus maxima  | Truncated protein (9aa)/ Truncated protein (68aa) | −         |
| Shatian pummelo          | ST           | B4, C1           | 1433/1427   | Citrus maxima  | Truncated protein (9aa)/ Truncated protein (68aa) | −         |
| Kumquat                  | JG           | C5, C6           | 1420/1420   | Citrus japonica | Truncated protein (339aa)/Truncated protein (339aa) | −         |

*“+” indicated accumulation of FRs; “−” indicated FRs were not detected.*

alleles, with the exceptions of Bendizao tangerine and Guoqing No.1 Satsuma mandarin, which contained two identical alleles (Table 1).

All type A alleles consisted of 1428 bp with 9–41 SNP differences compared with Cs1,6RhaT (Supporting Table S3), which encodes a polypeptide of 475 amino acid (aa) residues and is speculated to produce complete functional 1,6RhaT proteins. Type A alleles can be amplified from some species, such as sweet orange and loose-skin mandarin, which accumulate FRs. Type B and C alleles...
Citrus 1,6RhaT type a alleles encode proteins with 1,6RhaT function

As indicated above, premature stop codons in the type B and C alleles were hypothesized to produce truncated proteins without 1,6RhaT enzymatic function, which would lead to the unsuccessful accumulation of FRs. To validate this hypothesis, the catalytic activities of representative 1,6RhaT alleles were analyzed in suspensions of transgenic BY2 tobacco calli. Wild-type BY2 and transgenic cell lines for the A10 and B2 alleles were given hesperetin or naringenin substrate for 48 h, and the resulting biotransformation products were extracted and identified by HPLC (Fig. 4b-c). New peaks were detected only in the transgenic cell lines for the A10 allele but not in the B2 allele and BY2 control. The products in the A10 allele cell lines were identified as hesperedin (hesperetin-7-O-rutinoside) and narirutin (naringenin-7-O-rutinoside) by comparison with standards. Thus, the substrate could be transformed into FRs in cells transformed with the A10 allele but this failed in cells with B2 and in the wild type, which indicated that only type A alleles have 1,6RhaT function.

FR accumulation is correlated with the presence of type a alleles in citrus

The results from the 1,6RhaT allele analysis with the FR contents among the 36 accessions suggested a significant
The proportion of FRs among flavanone disaccharides is affected by the genotypes of both 1,6RhaT and 1,2RhaT in the hybrids

The crossing of “HB pummelo” and “Fairchild tangelo” resulted in extremely significantly lower levels of FRs in the F1 hybrid population, which ranged from 1.05 to 5.71 mg/g DW with an average content of 2.54 mg/g DW in peel. The same result was also found in natural hybrids of trifoliate orange and grapefruit among the Group 2 accessions (Fig. 4). Therefore, a question raised in this study is what is the cause of the relatively low contents of FRs in these hybrids.

The abovementioned progenies contain functional 1,2RhaT to produce FNs, which are a subgroup of flavanone disaccharides that compete with FRs converted from the same substrates of flavanone-7-O-glucosides (Supporting Fig. S2). Further determination of the contents of FNs (e.g. naringin and neohesperidin) showed that FRs were found at lower proportions, ranging from 5.53% to 30.20%, whereas FNs were found at proportions of 69.80%–94.47% (Fig. 5a), which implied that the synthesis of FNs might have negative influences on the accumulation of FRs.

To determine the above influences, F1 progenies resulting from another crossing of “red tangerine” and

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“trifoliata orange” were employed. Both the female and male parents in the crossing combination contained two type A 1,6RhaT alleles (red tangerine: A4 and A10, trifoliata orange: A8 and A9) (Supplementary Fig. S3). However, 1,2RhaT exists in the male parent “trifoliata orange” and 39 progenies but does not appear in the female parent “red tangerine” and 27 other progenies. High contents of FRs and undetectable levels of FNs were detected in the peel of “red tangerine”, and both FRs and FNs were detectable in the abovementioned 39 progenies harboring 1,2RhaT (Fig. 5b). However, the FR contents were lower than those of FNs in most of the 39 progenies (all except ZJ60, ZJ61, ZJ67, ZJ73 and ZJ77): FRs consisted of 12.43%–44.16% of all flavanone disaccharides and were found at levels of 2.17–5.58 mg/g DW with an average content of 3.29 mg/g DW, whereas FNs consisted of 55.84%–87.57% of all flavanone disaccharides and were found at contents of 2.98–22.21 mg/g DW with an average content of 8.40 mg/g DW (Supporting Table S6). These results showed that due to the involvement of FN biosynthesis, low FR contents were found in 39 of 66 F1 progenies from crossings of “red tangerine” and “trifoliata orange”, whereas high contents were detected in 27 other F1 progenies without FN biosynthesis. Therefore, it seems that the lower proportion of FRs among flavanone disaccharides was caused by the synthesis of FNs, which requires further in-depth research.

Enzymatic analyses of Cp1,6-RhaT (A10) and Cp1,2-RhaT

Furthermore, to clarify the influences of both 1,6RhaT and 1,2RhaT on FR production by considering substrate preference or competition, enzyme kinetic assays were performed in vitro. First, 1,6RhaT and 1,2RhaT were cloned from C. paradisi, which contains both genes. After the optimal temperature and pH for the reactions catalyzed by 1,2RhaT and 1,6RhaT were identified (Supporting Fig. S4), naringenin-7-O-glucoside (N7G) and hesperetin-7-O-glucoside (H7G) were used as substrates to determine the kinetic parameters because most citrus flavonoids are produced from these two substrates.

As shown in Table 2, the Km values of 1,6RhaT for N7G and H7G were 14.7 and 12.9 μM, respectively, whereas those of 1,2RhaT were 11.7 and 25.5 μM, respectively, which indicated that both enzymes exhibited strong binding affinity for these two compounds. The conversion efficiency (Kcat) of 1,2RhaT for N7G and H7G was approximately 1.5-fold higher and approximately 1.6-fold higher than that of 1,6RhaT, respectively. Both 1,2RhaT and 1,6RhaT consumed N7G more efficiently than H7G. Analysis of the ratio of the catalytic efficiency to specificity (Kcat/Km) for N7G showed that the ratio of 1,2RhaT was approximately 1.9-fold higher than that of 1,6RhaT. In contrast, the Kcat/Km ratio of 1,2RhaT for H7G was lower due to the weaker affinity between 1,2RhaT and H7G.

These results showed that the efficiency of 1,2RhaT to form FNs was higher than that of 1,6RhaT to form FRs, which might partly explain the lower FR contents found in hybrids containing both 1,2RhaT and 1,6RhaT.

### Discussion

In our study, 1,6RhaT-like genes were found in the genomes of relatively primitive species such as A. baxifolia and C. medica but were missing in model species such as loose-skin mandarin and sweet orange. Many reports have shown that plant flavanone disaccharides are related to resistance to environmental stress [24, 25]. For example, hesperidin accumulates in young leaves and fruits and plays an important role in defense against animal and microbial intrusion [26]. Naringin, hesperidin, and quercetin-3-O-rutinoside together with other active compounds can prevent Papilio machaon from laying eggs on young citrus leaves and can reduce the occurrence of citrus diseases and pests [27]. Identification of another 1,6RhaT (Cme1,6RhaT-like) shows that multiple 1,6RhaT genes work together to guarantee the synthesis of FRS in resisting the harsh environment of ancient times. As citrus began to be cultivated, 1,6RhaT-like might have gradually become redundant due to the improvement of cultivation conditions and is now absent in modern germplasms.

A phylogenetic tree was constructed based on the allelic variations among the 36 accessions, which not only successfully separated type A alleles from the other two types of alleles but also reflected complex genetic backgrounds among these accessions (Fig. 6). The results show that the type B and C alleles of hybrids such as grapefruits and “citrumelo” (ZY) were derived from pummelo, as these type B and C alleles clustered together. Type A alleles of grapefruits and citrange (ZC) were clustered with A10 of sweet orange, indicating that type A alleles of these accessions were derived from sweet orange. These results are consistent with the kinship of

### Table 2. Enzymatic kinetic parameters of Cs1,6RhaT and Cm1,2RhaT

| Substrate                  | Vmax (μM s⁻¹ mg⁻¹) | Km (μM) | Kcat (min⁻¹) | Kcat/Km (s⁻¹ mM⁻¹) |
|----------------------------|-------------------|---------|--------------|-------------------|
| 1,6RhaT Naringenin-7-O-glucoside | 22.2 ± 0.87       | 14.7 ± 0.21 | 7.02 ± 0.28  | 7.959             |
| 1,6RhaT Hesperetin-7-O-glucoside  | 0.70 ± 0.05       | 12.9 ± 0.39 | 2.23 ± 0.17  | 2.884             |
| 1,2RhaT Naringenin-7-O-glucoside  | 34.4 ± 1.28       | 11.7 ± 2.11 | 10.6 ± 0.39  | 15.03             |
| 1,2RhaT Hesperetin-7-O-glucoside  | 11.7 ± 0.83       | 25.5 ± 6.51 | 3.58 ± 0.26  | 2.346             |

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Type A alleles Type B and C alleles

Germplasms accumulate FRs except hybrids

Hybrids

Figure 6. Phylogenetic tree of 1,6RhaTs alleles in citrus accessions. Red lines represent type A alleles; blue lines represent type B and type C alleles; red circles represent accessions that accumulate FRs except hybrids; blue circles represent accessions that cannot accumulate FRs; green circles represent hybrids. Numbers indicate bootstrap replications. Abbreviations of the thirty-six citrus accessions are shown in Supplementary Table S1.

these species and accessions (grapefruit: sweet orange × pummelo; citrumelo: trifoliata orange × grapefruit; citrange: sweet orange × trifoliata orange). Interestingly, sweet orange results from a hybrid of pummelo and mandarin followed by a backcross with a mandarin [28] and therefore should have inherited the type B or C allele from the pummelo parent. Notably, we found that modern sweet orange does not contain type B or C alleles. We speculated that type B and C alleles existed in the initial hybrids of pummelo and mandarin; however, the hybrid not harboring type B and type C alleles that appeared during the backcross was selected for cultivation, which consequently led to the disappearance of type B and type C alleles in modern sweet orange.

The transcript levels of key genes are usually used to elucidate the accumulation of metabolites in various plants. However, the expression levels of 1,6RhaTs showed marked differences among the 36 citrus accessions but were not well associated with the FR contents (Supporting Fig. S5). For example, the expression of 1,6RhaT was detected in species such as pummelo and kumquat that do not accumulate FRs. This may be due to the simultaneous amplification of transcripts from nonfunctional 1,6RhaTs (type B and C alleles) in these citrus species. High expression levels of 1,6RhaT were detected in citrons that accumulate low levels of FRs, and previous research has shown that low enzyme levels of 1,6RhaT result in a low content of FRs in citron [19]. However, flavonoid-7-O-glucosides were not detected in citrus, indicating that the catalytic efficiency of 1,2RhaT and 1,6RhaT is very strong and that the supply of substrate is not saturated. Therefore, an insufficient substrate supply of 1,6RhaT may also be a cause of the low content of FR in citron.

The synthesis of citrus FNs and FRs involves two distinct but closely related competing subpathways during the rhamnosylation step that are catalyzed by 1,6RhaT and 1,2RhaT, respectively. Unlike 1,6RhaTs, 1,2RhaT has only one locus and it is directly related to the accumulation of FNs in citrus fruits, as demonstrated through an association analysis of 1,2RhaT with the FN accumulation phenotypes of 50 citrus accessions [20]. It is well known that FNs and FRs produce citrus fruits with nonbitter and bitter tastes, respectively, and these compounds have thus become important targets in citrus flavor-related breeding.

Our study found that the normal 1,6RhaT (type A alleles) genotype is correlated with high levels of FRs
in all investigated accessions, whereas accessions with early terminations of 1,6RhaT resulting from frameshift mutations (type B and C alleles) had undetectable levels of FRs. Frameshift mutations are widespread in kiwifruit [29], Brassicaceae [30], pear [31] and apricot [32] crops and result in nonfunctional truncated proteins. However, exceptions were also found in grapefruit, trifoliata orange and its citrulmoel hybrid harboring the type A allele and a lower FR content. These exceptions might be due to the influence of other genes related to the substrate supply, such as 7-O-glucosyltransferase, which catalyzes the formation of substrates of flavanone 7-O-glucosides, or to genes sharing the same substrates, such as 1,2RhaT, which would account for the accumulation of FRs. The accumulation characteristics of flavanone glycosides in the two artificial hybrid populations also indicated that other factors might affect the accumulation of FRs.

Previous studies have shown that low enzyme levels of 1,6RhaT and high enzyme levels of 1,6RhaT were detected by corresponding antibodies in grapefruit and bitter orange, and different enzyme levels in germlasms containing both 1,6RhaT and 1,2RhaT may be one of the causes of germlasms with type A alleles but with low FR content [13, 19]. Moreover, we think that the catalytic efficiency of 1,2RhaT and 1,6RhaT is also a factor that affects the flavonoid content. Our results showed that the efficiency of 1,2RhaT to form FNs was higher than that of 1,6RhaT. This result is consistent with the flavonoid content in grapefruit, which might explain the lower FR contents from another perspective.

It is worth noting that kumquat, which contains functional 1,2RhaT and nonfunctional type C 1,6RhaT alleles, harbors another flavanone accumulation mode but no detectable FNs. This finding might be due to the loss or nonfunction of upstream genes that results in a lack of flavanone-7-O-glucoside supply [20].

Collectively, our results obtained in this study could partially explain the FR metabolic characteristics of various citrus species and can provide a better understanding of the biosynthesis and regulation of FRs, which is meaningful in theory and practice for improving citrus flavor and the functional components of flavonoid production. Using two distinct but closely related competing subpathways to reduce the bitter taste of citrus, we hope to obtain a certain degree of freedom to modulate the biosynthesis of flavanone disaccharides between FRs and FNs in the future.

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Author contributions

W.Y.L. and G.L. performed the experiments and wrote the manuscript; M.L.T. established the hybrid populations; Z.Y.Y., M.Y.L., Y.H.M. and J.J.C. provided technical assistance and research guidance. J.X. designed the research and X.X.D. and J.X. revised the manuscript.

Data availability

The data used to support the findings of this study are included within the article.

Conflict of interest statement

The authors declare no competing interests.

Supplementary data

Supplementary data is available at Horticulture Research Journal online.

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