

RESEARCH

Open Access



# Molecular characterization of a flavanone 3-hydroxylase gene from citrus fruit reveals its crucial roles in anthocyanin accumulation

Gang Ma<sup>1,2</sup>, Lancui Zhang<sup>1</sup>, Risa Yamamoto<sup>2</sup>, Nami Kojima<sup>2</sup>, Masaki Yahata<sup>1,2</sup> and Masaya Kato<sup>1,2\*</sup>

## Abstract

**Background** Flavanone 3-hydroxylase (F3H), a key enzyme in the flavonoid biosynthetic pathway, plays an important role in the regulation of flavonols and anthocyanidins accumulation. Citrus fruit is a rich source of flavonoids with varied flavonoid compositions among different varieties. To date, the study on *F3H* is limited in citrus, and its roles in regulating flavonoid accumulation in citrus fruit are still unclear.

**Results** In this study, we isolated a *CitF3H* from three different citrus varieties, Satsuma mandarin (*Citrus unshiu* Marc.), Ponkan mandarin (*C. reticulata* Blanco) and blood orange 'Moro' (*C. sinensis* Osbeck). Functional analysis showed that *CitF3H* encoded a functional flavanone 3-hydroxylase. It catalyzed the hydroxylation of naringenin to yield dihydrokaempferol, which was a precursor of anthocyanins in flavonoid biosynthetic pathway. In the juice sacs, *CitF3H* was differentially expressed among the three citrus varieties, and its expression level was positively correlated with the accumulation of anthocyanins during the ripening process. In the juice sacs of Satsuma mandarin and Ponkan mandarin the expression of *CitF3H* kept constant at an extremely low level, and no anthocyanin was accumulated during the ripening process. In contrast, the expression of *CitF3H* increased rapidly along with the accumulation of anthocyanin in the juice sacs of blood orange 'Moro' during the ripening process. In addition, we found that blue light irradiation was effective to up-regulate the expression of *CitF3H* and improve anthocyanin accumulation in the juice sacs of blood orange 'Moro' in vitro.

**Conclusion** *CitF3H* was a key gene regulating anthocyanin accumulation in the juice sacs of citrus fruit. The results presented in this study will contribute to elucidating anthocyanin biosynthesis in citrus fruit, and provide new strategies to improve the nutritional and commercial values of citrus fruit.

**Keywords** Anthocyanin, Blue light, Citrus, Flavanone 3-hydroxylase, Juice sacs

\*Correspondence:

Masaya Kato  
kato.masaya@shizuoka.ac.jp

<sup>1</sup>Department of Bioresource Sciences, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga, Shizuoka 422-8529, Japan

<sup>2</sup>Graduate School of Integrated Science and Technology, Shizuoka University, 836 Ohya, Suruga, Shizuoka 422-8529, Japan



© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

## Background

Flavonoids are a group of polyphenolic compounds with diverse structures. In nature, more than 8,000 different types of flavonoids have been identified, which are widely distributed in fruit, vegetables, and cereals. Flavonoids fulfill a variety of important functions in plants. They protect plants from various biotic and abiotic stresses, and act as signal molecules, allopathic compounds, detoxifying agents, and antimicrobial defensive compounds [1–4]. In addition, flavonoids are well known for their pharmacological properties. Flavonoids are one of the important compositions of herbal drugs, and have long been used in traditional medicine with high antioxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic activities [5–10]. Due to the indispensable benefits in plants and human health, flavonoids drew more and more attention of researchers over the past decades, and considerable progress has been made in the elucidation of flavonoid biosynthesis in plants [11–14].

Flavanone 3-hydroxylase (F3H), a member of 2-oxoglutarate-dependent dioxygenase family, is a key enzyme in the flavonoid biosynthetic pathway. Li et al. (2020) reported that F3Hs of seed plant (i.e., gymnosperms and angiosperms) were evolutionarily connected to flavone synthase Is (FNS Is) of liverworts [15]. The early-evolved FNS I has gone through functional transition as FNS I/F2H and FNS I/F3H, and eventually shifted to the bona fide F3H. The F3H gene was first reported in the flowers of *Antirrhinum majus*, and the homologous genes were subsequently isolated in other plant species, such as *Arabidopsis*, maize, *torenia*, *Reaumuria trigyna*, tea, wolfberry, and soybean [16–22]. In plants, F3H catalyzes the 3-hydroxylation of flavanones to form dihydroflavonols, which are precursors of flavonols and anthocyanins. F3H is the key gene in the regulation of flavonoid accumulation at the bifurcation of the flavonol and anthocyanin branches [16–18, 20]. Overexpression of *Lycium chinense* F3H and tea F3H significantly enhanced the contents of flavanols in tobacco [20, 21]. In strawberry, the RNAi-mediated silencing of F3H led to reduced anthocyanin levels, producing colorless *torenia* flower and strawberry fruit [23]. In addition, F3H not only regulates the flavonoid composition, but also plays an important role in stress resistance in plants. Under the abiotic stresses, the expression of F3H was induced along with the increases of flavonoids contents, which contributed to protecting the plants from oxidative damage [21, 24–27].

Citrus is a rich source of flavonoids, which are accumulated in the root, leaf, seed, and fruit. Citrus flavonoids are primarily classified into four groups: flavanones, flavones, flavonols, and anthocyanins according to the structure. The flavonoid composition varied greatly among different tissues and citrus varieties [28–30]. In citrus fruit, flavanones are accumulated as the major

flavonoid, followed by flavones. In contrast, flavonols and anthocyanins are rarely accumulated in citrus fruit. In citrus, anthocyanins are specifically accumulated in blood oranges, such as ‘Moro’ and ‘Tarocco’, while most common citrus varieties are devoid of the ability to produce anthocyanins during the evolution [31, 32].

To date, the study on citrus F3H is limited, and its effects on the flavonoid accumulation in citrus fruit are still unclear [33–35]. In the present study, we isolated a F3H gene (*CitF3H*) from the two common citrus varieties, Satsuma mandarin and Ponkan mandarin, and one variety of blood orange, ‘Moro’. To elucidate the roles of F3H in citrus fruit, the function and expression of *CitF3H* were investigated in the juice sacs of three citrus varieties, and its regulation in response to blue and red light was characterized in vitro. The molecular characterization of *CitF3H* presented in this study contributed to elucidating flavonoid accumulation in citrus fruit.

## Results

### Isolation and sequence analysis of *CitF3H*

In this study, to isolate the citrus flavanone 3-hydroxylase gene (*CitF3H*), a blast search in the *Citrus clementina* v.10 genome databases (<http://www.phytozome.net/>) was performed using the sequence of *LcF3H* as a query, which has been reported to encode a functional flavanone 3-hydroxylase in *Lycium chinense*. In citrus, *CitF3H* gene (Ciclev10025931m.g) was identified in the *Citrus clementina* v.10 genome databases, which was located at scaffold\_7:1207140.1210820 (forward). The gene structure of *CitF3H* included a 5' untranslated region, a coding sequence, and a 3' untranslated region. In this study, *CitF3H* was isolated from three citrus varieties, Satsuma mandarin (Accession number: OQ148589), Ponkan mandarin (Accession number: OQ148590), and blood orange ‘Moro’ (Accession number: OQ148591). The nucleotide sequence of *CitF3H* contained 1,089 bp and encoded a putative protein of 362 amino acids with an estimated molecular mass of 40 kD. In the N-terminal region of the protein encoded by *CitF3H*, no characteristic transit peptide was predicted by TargetP-2.0 (<http://www.cbs.dtu.dk/services/TargetP/>). The deduced amino acid sequence of *CitF3H* showed more than 80% homology with F3Hs reported in other plant species (Table S1). A phylogenetic analysis showed that *CitF3H* was clustered with F3Hs from *Canarium album* (CaF3H), *Litchi chinensis* (LcF3H), *Gossypium barbadense* (GbF3H), *Theobroma cacao* (TcF3H), and *Dimocarpus longan* (DlF3H) (Fig. 1A). In addition, the amino acid sequence of *CitF3H* shared high identities (more than 97%) among the three varieties, Satsuma mandarin, Ponkan mandarin, and blood orange ‘Moro’ (Fig. 1B). Alignment of amino acid sequences of *CitF3H* with other plant F3H proteins showed that *CitF3H* contained five similar motifs for



2-oxoglutarate-dependent dioxygenase. Three prolines (Pro147, Pro203, and Pro206), which were responsible for polypeptide folding, were strictly conserved in the CitF3H of the three citrus varieties. Moreover, the amino acid residues (His217, Asp219, and His275) that were responsible for ferrous iron ligating, and the amino acid residues (Arg285 and Ser287) that were involved in the 2-oxoglutarate binding were also detected in the CitF3H of the three citrus varieties (Fig. 1B). The conserved motifs and amino acids residues detected in CitF3H suggested that CitF3H has potential functions in the biosynthesis of flavonoids in citrus fruit.

#### Function analysis of recombinant *CitF3H* protein in vitro

To investigate the function of *CitF3H*, the full-length cDNA of *CitF3H* isolated from Ponkan mandarin was cloned into the pCold GST vector. The recombinant protein expressed in *E.coli* cells was extracted and a single band with about 40 kDa molecular mass was confirmed by SDS-PAGE, which was in agreement with the prediction. In this study, to assay the enzyme activity of CitF3H, five flavanones (naringenin, 3'-hydroxyflavanone, 4'-hydroxyflavanone, narirutin, and hesperidin), four flavones (apigenin, 4',7-hydroxyflavone, 3',4'-dihydroxyflavone, and 3',4',5,7-tetrahydroxyflavone), and two flavanols (dihydrokaempferol and myricetin) were used as substrates. As shown in Fig. 2A, when naringenin was used as a substrate, a new product was detected. Whereas, no new product was observed when other flavonoids were used as substrates. In the case of naringenin, the new product was eluted at 9.7 min; the elution time and absorption maximum of the new product were identical to the standard of dihydrokaempferol (Fig. 2B–E). In addition, we also investigated the function of CitF3H by using in vivo feeding experiments. The *E.coli* cells harboring the pGEX-6P-1-CitF3H construct were fed with naringenin, and HPLC analysis of the reaction product confirmed that dihydrokaempferol was produced as in vitro reactions (Fig. S1). These results suggested that *CitF3H* encoded a functional flavanone 3-hydroxylase, and it catalyzed the formation of dihydrokaempferol from naringenin in flavonoid biosynthetic pathway.

#### Changes in the expression of *CitF3H* and flavonoid contents in the juice sacs of Satsuma mandarin, Ponkan mandarin, and blood orange 'Moro' during the ripening process

As shown in Fig. 3, *CitF3H* was differentially expressed in the juice sacs among Satsuma mandarin, Ponkan mandarin, and blood orange 'Moro'. During the ripening process, the expression of *CitF3H* kept constant at an extremely low level in the juice sacs of Satsuma mandarin and Ponkan mandarin (Fig. 3). In blood orange 'Moro', in contrast, the expression of *CitF3H* increased rapidly in

the juice sacs during the ripening process. The expression level of *CitF3H* in the juice sacs of blood orange 'Moro' was much higher than that in Satsuma mandarin and Ponkan mandarin during the ripening process.

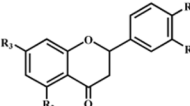
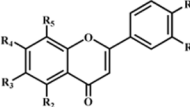
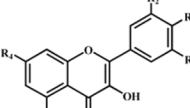
In this study, to elucidate the roles of *CitF3H* in the regulation of flavonoid accumulation in citrus fruit, the changes in the flavonoid content and composition were investigated in the juice sacs of the three citrus varieties during the ripening process. In Satsuma mandarin and Ponkan mandarin, three flavanones (narirutin, poncirin, and hesperidin) and two flavones (diosmin and rhoifolin) were accumulated in the juice sacs (Fig. 4). In blood orange 'Moro', the three flavanones were accumulated in the juice sacs, while flavones were not detected during the ripening process. In this study, we did not detect the flavonols in the juice sacs of Satsuma mandarin, Ponkan mandarin, or blood orange 'Moro' during the ripening process.

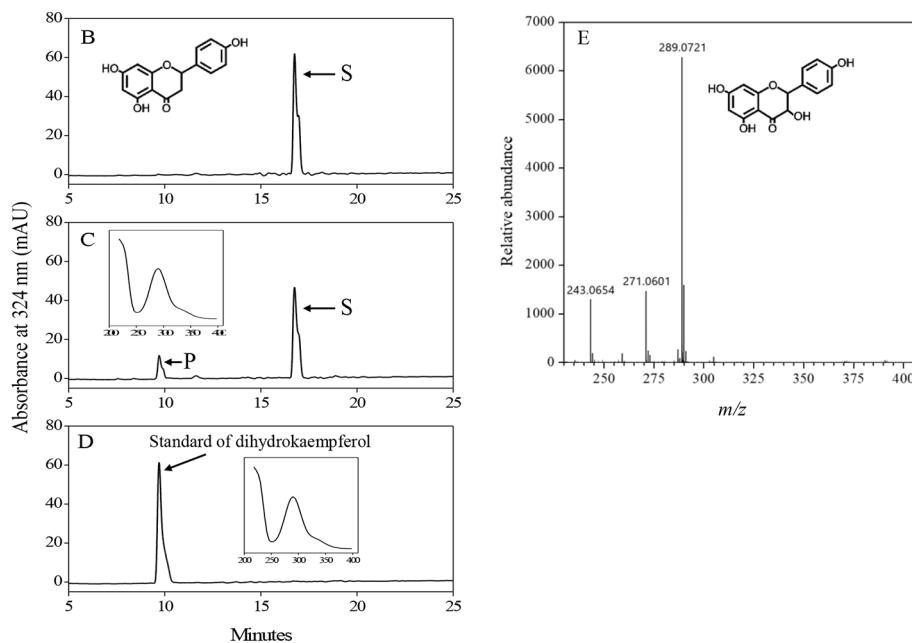
In addition, we found that a major difference in the flavonoid composition among the three citrus varieties was the accumulation of anthocyanins. In blood orange 'Moro', two anthocyanins, cyanidin-3-glucoside and cyanidin-3(6"-malonyl) glucoside, were accumulated in the juice sacs (Fig. 4). During the ripening process, the contents of cyanidin-3-glucoside and cyanidin-3(6"-malonyl) glucoside increased rapidly in the juice sacs of blood orange 'Moro', which was well consistent with the increase in the expression of *CitF3H* during the ripening process. In contrast, anthocyanins were undetectable in the juice sacs of Satsuma mandarin and Ponkan mandarin during the ripening process.

#### Changes in the expression of *CitF3H* and flavonoid content in the juice sacs of blood orange 'Moro' in response to blue and red LED lights irradiation

In this study, the effects of blue and red LED lights on the flavonoid content and the expression of *CitF3H* were investigated in the juice sacs of blood orange 'Moro' in vitro. Under the blue light, the contents of narirutin, poncirin, and hesperidin were not significantly affected, and the total flavonoid content in the blue light treatment was similar to that of the control (Fig. 5A). Under the red light, the content of hesperidin was decreased, and as a result the total flavonoid content in the red-light treatment was lower than that of the control (Fig. 5A). In addition, we found that the blue light treatment induced anthocyanin accumulation in the juice sacs of blood orange 'Moro' in vitro, which led the juices sacs showed a deeper red color (Fig. 5B and D). Moreover, in parallel with the increases in the anthocyanin content, the expression of *CitF3H* was significantly up-regulated by the blue light in the juice sacs of blood orange 'Moro' in vitro (Fig. 5C).

A

Group	Substrate	R	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	Product*
<b>Flavanone</b> 	Naringenin	H	OH	OH	OH	-	-	Detected
	3'-Hydroxyflavanone	OH	H	H	H	-	-	Not detected
	4'-Hydroxyflavanone	H	OH	H	H	-	-	Not detected
	Narirutin	H	H	OH	Rutinose	-	-	Not detected
	Hesperidin	OH	OCH <sub>3</sub>	OH	Rutinose	-	-	Not detected
<b>Flavone</b> 	Apigenin	H	OH	OH	H	OH	H	Not detected
	4', 7-Hydroxyflavone	H	OH	H	H	OH	H	Not detected
	3', 4'-Dihydroxyflavone	OH	OH	H	H	H	H	Not detected
	3', 4', 5, 7-Tetrahydroxyflavone	OH	OH	OH	H	OH	H	Not detected
<b>Flanonol</b> 	Dihydrokaempferol	H	OH	H	OH	OH	-	Not detected
	Myricetin	OH	OH	OH	OH	OH	-	Not detected



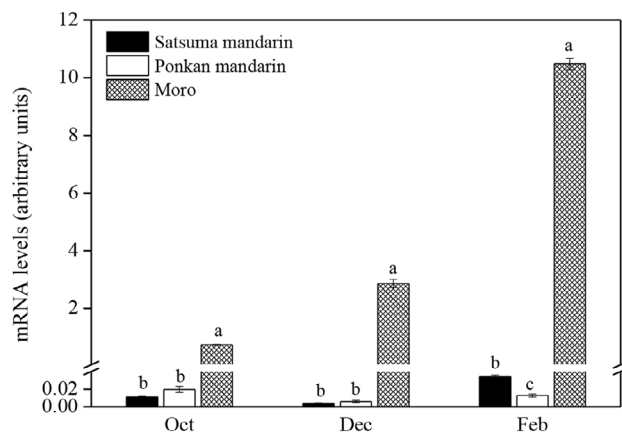
**Fig. 2** The enzyme activity of CitF3H in vitro. **(A)** Activities of CitF3H against different flavonoid substrates. **(B)** HPLC analysis of naringenin. **(C)** HPLC analysis of new hydroxylated product of naringenin catalyzed by CitF3H. **(D)** HPLC analysis of dihydrokaempferol standard. **(E)** Mass spectrum of the new hydroxylated product of naringenin catalyzed by CitF3H. S: naringenin, P: naringenin reaction product. \* "Detected" or "Not detected" indicates whether there was a new peak detected by HPLC analysis

## Discussion

### CitF3H encoded a functional flavanone 3-hydroxylase in citrus fruit

To date, although *F3H* has been cloned and characterized in several plant species the research on *F3H* in citrus is limited and its roles in the regulation of flavonoid accumulation in citrus fruit are still far from being elucidated [33–36]. In this study, we identified a citrus flavanone 3-hydroxylase gene (*CitF3H*) using the sequence of

*LcF3H* as a query in the *Citrus clementina* v.10 genome databases (<http://www.phytozome.net/>) and isolated it from three citrus varieties, Satsuma mandarin, Ponkan mandarin, and blood orange 'Moro'. The amino acid sequence analysis showed that CitF3H had more than 80% identity with *F3H*s reported in other plant species, which indicating that *F3H* was highly conserved in plants (Table S1). Moreover, the motifs for 2-oxoglutarate-dependent dioxygenase, and the amino acid residues



**Fig. 3** Gene expression analysis of *CitF3H* in the juice sacs of Satsuma mandarin, Ponkan mandarin, and blood orange 'Moro' during the ripening process. The mRNA levels were analyzed by TaqMan real-time quantitative PCR. 18 S ribosomal RNA was used to normalize the expression of gene in the same conditions. Columns and bars represent the means  $\pm$  SE ( $n=3$ ), respectively. Tukey's HSD test ( $P < 0.05$ ) was used to compare the different varieties. Different letters above each column indicate significant differences of the gene expression of *CitF3H* among the three citrus varieties in October, December, and February, respectively. Oct: October, Dec: December, Feb: February

that were responsible for polypeptide folding, ferrous iron ligating, and 2-oxoglutarate binding were strictly conserved in *CitF3H* of the three citrus varieties (Fig. 1). The further functional analysis confirmed that *CitF3H* catalyzed the formation of dihydrokaempferol from naringenin in vitro (Fig. 2). These results suggested that *CitF3H* isolated from the three citrus varieties encoded a functional flavanone 3-hydroxylase, which might be involved in the regulation of flavonoid biosynthesis in citrus.

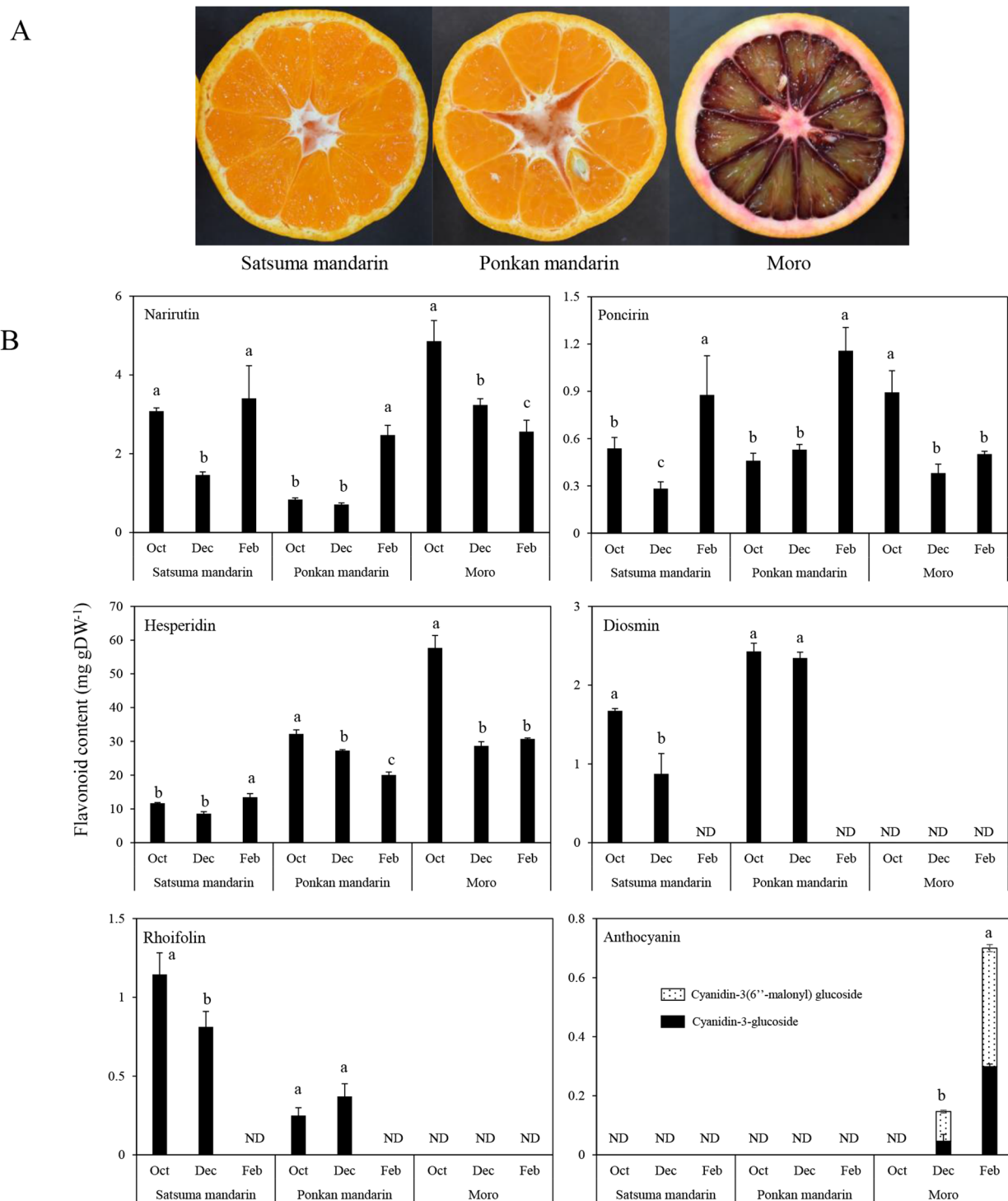
#### The expression of *CitF3H* was positively correlated with the anthocyanin accumulation in citrus juice sacs

In plants, *F3H* is a key structural gene in flavonoid biosynthetic pathway. It catalyzes the 3-hydroxylation of flavanone to form dihydrokaempferol, which is an intermediate for the biosynthesis of flavonols and anthocyanins. Previous studies suggested that *F3H* played an important role in the regulation of flavonols and anthocyanins accumulation in plants. In maize anthers, the expression of *F3H* was found to temporally coordinate with the appearance of flavonols [37]. Song et al. (2016) reported that overexpression of *LcF3H* in tobacco led to enhanced the accumulation of flavonols and flavan-3-ols [21]. In addition, *F3H* was also a rate-limiting enzyme in anthocyanin biosynthesis. The high expression of *F3H* led to the accumulation of anthocyanin in muscadine grapes and strawberry fruit [23, 38]. In this study, the results showed that the accumulation of anthocyanin was positively correlated with the expression of *CitF3H* in the juice sacs of the three citrus varieties. In blood orange

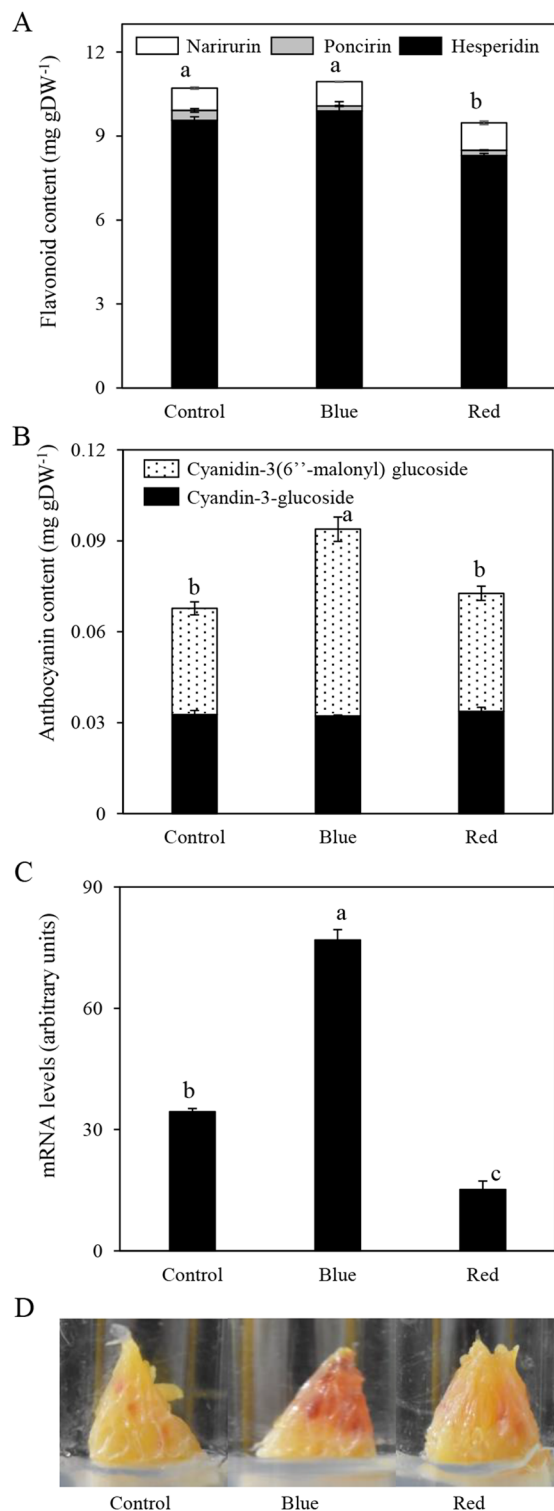
'Moro', the expression of *CitF3H* increased rapidly during the ripening process, which was well consistent with the accumulation of anthocyanin in the juice sacs. In Satsuma mandarin and Ponkan mandarin, in which anthocyanin was not accumulated, the expression of *CitF3H* kept constant at an extremely low level in the juice sacs during the ripening process. In citrus, flavonols are preferentially accumulated in leaves. In the fruit, however, a very small amount of flavonols is specifically accumulated in a few varieties, such as lemon and lime. In the present study, the analysis of flavonoid content and composition showed that flavonols were undetectable in the juice sacs of Satsuma mandarin, Ponkan mandarin, or blood orange 'Moro' during the ripening process. Although *CitF3H* was highly expressed, flavonols were not accumulated in the juice sacs of blood orange 'Moro', which indicating that *CitF3H* might be not the key gene controlling the flavonol accumulation in citrus fruit. Therefore, based on the results of flavonoid accumulation in the three different citrus varieties, it was suggested that *CitF3H* played crucial roles in the regulation of anthocyanin accumulation in the citrus juice sacs.

In this study, the functional analysis showed that *CitF3H* of Ponkan mandarin encoded a functional flavanone 3-hydroxylase; it catalyzed the formation of dihydrokaempferol from naringenin in vitro (Fig. 2). However, the expression of *CitF3H* in the juice sacs of Ponkan mandarin was extremely low and anthocyanin was not accumulated during the ripening process. Thus, these results indicated that the differential expression of *CitF3H* was a key molecular mechanism that regulated anthocyanin accumulation in the juice sacs of the three citrus varieties. In a previous study, the expression of *CitF3H* was investigated in *Citrus reticulata* and *Poncirus trifoliata* [34]. It was found that an indel variation in the promoter region of *CitF3H* led to different expression levels of *CitF3H* in the two citrus varieties. In the present study, to elucidate the different expression levels of *CitF3H* among Satsuma mandarin, Ponkan mandarin, and blood orange 'Moro', about 1000-bp promoter sequences of *CitF3H* were isolated and analyzed. The results showed that there was no genetic variation in the promoter of *CitF3H*, and promoter sequence of *CitF3H* shared 100% identity among the three citrus varieties. These results indicated that the differential expression of *CitF3H* might not be attributed to the promoter activity in Satsuma mandarin, Ponkan mandarin, and blood orange 'Moro'.

In plants, it is well recognized that the regulation of anthocyanin biosynthesis at the transcriptional level is controlled by the MYB transcription factors. To date, two MYB transcriptional factors, *Ruby1* and *Ruby2*, have been identified in citrus [31, 39]. The citrus *Ruby1* and *Ruby2* can bind to the promoters of anthocyanin biosynthetic genes and activate the anthocyanin biosynthesis. In



**Fig. 4** Flavonoid accumulation in the juice sacs of Satsuma mandarin, Ponkan mandarin, and blood orange ‘Moro’ during the ripening process. **(A)** The appearance of the juice sacs of Satsuma mandarin, Ponkan mandarin, and blood orange ‘Moro’; **(B)** Flavonoid content in the juice sacs of Satsuma mandarin, Ponkan mandarin, and blood orange ‘Moro’. Columns and bars represent the means ± SE (n = 3), respectively. Different letters above each column indicate significant differences at *P* < 0.05 by Tukey’s HSD test. ND, not detected. Oct: October, Dec: December, Feb: February



**Fig. 5** Effect of blue and red LED lights on flavonoid content (A), anthocyanin content (B), gene expression of *CitF3H* (C), and appearance of the juice sacs in blood orange 'Moro' in vitro (D). The mRNA levels were analyzed by TaqMan real-time quantitative PCR. 18 S ribosomal RNA was used to normalize the expression of gene in the same conditions. Columns and bars represent the means  $\pm$  SE (n = 3), respectively. Different letters above each column indicate significant differences at  $P < 0.05$  by Tukey's HSD test

addition, *Ruby1* was specifically expressed in citrus fruit, which was controlled by the retrotransposon. In blood orange, an insertion of a Copia-like retrotransposon in 254-bp upstream of promoter activated the expression of *Ruby1*, and led to the accumulation of anthocyanin in the juice sacs. However, *Ruby1* was not expressed in most common citrus varieties because of lack of retrotransposon. In this study, we analyzed the *cis*-acting elements in the promoter of *CitF3H* by PLACE database, and found that promoter sequence of *CitF3H* contained three MYB bounding sites, which indicating that the expression of *CitF3H* might be regulated by the MYB transcription factors in citrus fruit (Fig. S2). To confirm it, we also investigated the expression of *Ruby1* in the juice sacs of the three citrus varieties in the present study (Fig. S3). The results showed that *Ruby1* was highly expressed in the juice sacs of blood orange 'Moro', while its expression level was extremely low in the juice sacs of Satsuma mandarin and Ponkan mandarin during the ripening process. The expression patterns of *Ruby1* were well consistent with those of *CitF3H*, which indicating that the differential expression of *CitF3H* might be regulated by *Ruby1* in the three citrus varieties.

#### Blue light induced the expression of *CitF3H* and the accumulation of anthocyanin in the juice sacs of blood orange 'Moro'

Light is one of the most important environmental factors influencing anthocyanin accumulation in plants. In citrus fruit, it was reported that bagging treatment (without light) inhibited anthocyanin accumulation in blood orange, while the biosynthesis of anthocyanins was restored when the bags were removed [40]. In the present study, the analysis of the *cis*-acting elements in the *CitF3H* promoter showed that eleven light responsive elements including five G-box elements were present in the up-stream 1000-bp promoter sequence of *CitF3H*, which indicating that the expression of *CitF3H* was light inducible in citrus fruit (Fig. S2). The further experiments on the effects of light quality on the expression of *CitF3H* showed that the expression of *CitF3H* was induced by blue light, while it was not significantly affected by the red light in the juice sacs of blood orange 'Moro' in vitro. Under blue light, the increase in the expression of *CitF3H* was accompanied with the enhanced anthocyanins contents in the juice sacs of blood orange 'Moro' in vitro (Fig. 5). These results were consistent with previous studies, in which it was suggested that blue light was one of the most effective in inducing anthocyanin biosynthesis among the different light wavelengths.



## Conclusions

In this study, a *CitF3H* gene was isolated, and its molecular characterization was investigated in citrus fruit. The results showed that *CitF3H* was positively associated with the anthocyanin biosynthesis in the juice sacs of citrus fruit. In the juice sacs of two common citrus varieties of Satsuma mandarin and Ponkan mandarin, in which anthocyanin was not accumulated, the expression of *CitF3H* kept at an extremely low level during the ripening process. In contrast, *CitF3H* was highly expressed in the juice sacs of blood orange blood orange 'Moro'. The high expression of *CitF3H* was well consistent with the accumulation of anthocyanin in juice sacs of blood orange 'Moro'. In addition, blue light was an important factor for inducing the expression of *CitF3H* and accumulation of anthocyanin in the juice sacs of blood orange 'Moro'. These results suggested that *CitF3H* was a key gene involved in anthocyanin biosynthesis in citrus fruit. The results presented in this study contributed to a better understanding of flavonoid biosynthesis in citrus, and provided new insights into improving the nutritional and commercial values of citrus fruit.

## Methods

### Plant materials

Satsuma mandarin 'Miyagawa-wase' (*Citrus unshiu* Marc.), Ponkan mandarin 'Onta Ponkan' (*C. reticulata* Blanco) and blood orange 'Moro' (*C. sinensis* Osbeck) grown at the Fujieda Farm of Shizuoka University (Shizuoka, Japan) were used as plant materials. Fruit samples were harvested periodically from October to February. Juice sacs were separated from the sampled fruit, and then immediately frozen in liquid nitrogen and kept at  $-80\text{ }^{\circ}\text{C}$  until analysis.

To study the effects of blue and red lights on flavonoid accumulation, the juice sacs of blood orange 'Moro' (December) were excised and placed on 10 mL of MS medium supplemented 10% (w/v) sucrose and 1% (w/v) agar according to the method of Zhang et al. (2012) [41]. The juice sacs were irradiated with blue (470 nm,  $100\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ ) and red (660 nm,  $100\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ ) LED lights for 4 weeks at  $10\text{ }^{\circ}\text{C}$ . The juice sacs cultured in the dark were used as the control.

### Functional analysis of CitF3H enzyme *in vitro*

In this study, we conducted a Blast search in *Citrus clementina* v.10 genome databases (<http://www.phytozome.net/>) using the sequence of *LcF3H* (GenBank: KJ636468.1) as a query, which has been reported to encode a functional flavanone 3-hydroxylase in *Lycium chinese*. The cDNA of *CitF3H* isolated from Ponkan mandarin was cloned into the pCold GST vector (Takara Bio., Otsu, Japan). The recombinant plasmid was transformed into XL1-Blue *E. Coli* cells. After cultured overnight, 2

mL of culture of the transformants harboring the gene of *CitF3H* was inoculated in a 200 mL of 2×YT medium with carbenicillin ( $50\text{ }\mu\text{g mL}^{-1}$ ). Cultures were grown at  $37\text{ }^{\circ}\text{C}$  until an optical density at 600 nm of 0.8 was reached. The culture solution was maintained at  $15\text{ }^{\circ}\text{C}$  for 30 min. The expression of proteins was induced by the addition of IPTG (final concentration, 1 mM), and the cultures were grown at  $15\text{ }^{\circ}\text{C}$  for additional 24 h. After centrifugation, the cells were harvested and frozen in liquid nitrogen, and then resuspended in extraction buffer (20 mM Na-Pi buffer pH 8.0, 10% glycerol). The suspensions containing the *E. coli* cells were lysed by sonication, and then 1% (v/v) Triton X-100 was added and shaken on ice for 30 min. After centrifugation at  $5,000\times g$  for 90 min at  $4\text{ }^{\circ}\text{C}$ , recombinant protein of CitF3H was subjected to TALON metal affinity resin immobilization (Takara Bio., Otsu, Japan). The His-GST-tagged proteins were then purified according to the manufacturer's instructions, and imidazole was removed from the purified fraction using a PD-10 column (GE Healthcare, Chicago, IL, USA). The recombinant proteins were analyzed by SDS-PAGE with a 12.5% (w/v) polyacrylamide gel and WIDE-VIEW Prestained Protein Size Marker (Wako, Japan) using PhastSystem (Amersham Bioscience, US).

To investigate the enzymatic activity of CitF3H protein, the purified recombinant protein was reacted with flavanones (naringin, naringenin, 3'-hydroxyflavanone, 4'-hydroxyflavanone, and hesperidin), flavones (apigenin, 4',7'-hydroxyflavone, 3',4'-dihydroxyflavone, and 3',4',5,7'-tetrahydroxyflavone), flavanols (dihydrokaempferol and myricetin). Reaction mixtures consisted of 100 mM Tris-HCl buffer (pH 7.2), 250 mM 2-Oxo-glutaric acid, 30 mM sodium ascorbate, 50  $\mu\text{M}$  FeSO<sub>4</sub>, 10% (v/v) glycerol, 0.05% (v/v) Triton X-100, 500  $\mu\text{M}$  substrates, and 40  $\mu\text{g}$  of purified protein in a total volume of 100  $\mu\text{L}$ . Assays were incubated at  $37\text{ }^{\circ}\text{C}$  with shaking for 2 h. The reaction solution was analyzed by HPLC and the new product was purified and analyzed by DART MS according to the method described by Seoka et al. (2020) [42].

### Extraction and determination of flavonoids and anthocyanin

The identification and quantification of flavonoids were conducted according to the method described by Seoka et al. (2020) [41]. Flavonoids were extracted from the freeze-dried juice sacs using a DMSO:methanol (1:1, v/v) solution. After homogenization, ultrasonication and centrifugation, the supernatant was filtered through a TORAST disc syringe filter (pore size 0.22  $\mu\text{m}$ ; SHIMADZU GLC Ltd., Japan). Then, the samples were analyzed using a HPLC system fitted with a YMC-UltraHT Pro C18 column (100×3.0 mm i.d. S-2  $\mu\text{m}$ , 12 nm; YMC, Japan) at a flow rate of 0.6 mL min<sup>-1</sup>. The eluent was monitored at 274, 310, 324, 338, and 362 nm using a

MD4010 PDA detector. The flavonoid concentration was estimated by the standard curves and expressed as milligrams per gram dry weight. Flavonoid quantification was performed in three replicates.

Anthocyanins were extracted from the freeze-dried juice sacs using a HCl: methanol (1:99, v/v) solution. After homogenization, ultrasonication and centrifugation, the supernatant was filtered through a TORAST disc syringe filter (pore size 0.22  $\mu\text{m}$ ; SHIMADZU GLC Ltd., Japan). Then, the samples were analyzed using a HPLC system fitted with a YMC-UltraHT Pro C18 column (100 $\times$ 3.0 mm i.d. S-2  $\mu\text{m}$ , 12 nm; YMC, Japan) at a flow rate of 1.0 mL min<sup>-1</sup>. The eluent was monitored at 650 nm using a MD4010 PDA detector. A two-solvent gradient system of formic acid: milliQ water (1:9, v/v) (A) and formic acid: acetonitrile: methanol: milliQ water (4:9:9:16, v/v) (B) was used. The gradient program consisted four periods: (1) 0–35 min, 75%A, 25%B, (2) 35–45 min, 35%A, 65%B, (3) 45–50 min, 0%A, 100%B, (4) 40–69 min, 93%A, 7%B. The column temperature was operated at 30 °C. In this study, two anthocyanin peaks were detected, and peak 1 was identified as cyanidin-3-glucoside according to the standard. Peak 2 was identified as cyanidin-3(6"-malonyl) glucoside according to the MS analysis. The concentrations of all peaks were calculated with the standard curve of cyanidin-3-glucoside expressed as milligrams per gram dry weight. Anthocyanin quantification was performed in three replicates.

#### RNA extraction and gene expression analysis

Total RNA was extracted from the juice sacs according to the method described by Ma et al. (2022) [43]. The RNeasy Mini Kit (Qiagen, Germany) was used to clean the extracted total RNA using on-column DNase digestion. The cDNA was synthesized with 2  $\mu\text{g}$  of purified total RNA using a TaqMan Reverse Transcription Regents (Applied Biosystems, USA).

The gene expression was conducted by real-time quantitative PCR according to the method of Ma et al. (2022) [42]. As an endogenous control, the TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems) was used. The real-time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) on a StepOnePlus™ system (Applied Biosystems). Each reaction contained template cDNA, 900 nM primers, and a 250 nM probe (Table S2). The thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The data obtained from the StepOnePlus™ real-time PCR software (Applied Biosystems) was used to analyze the gene expression. The results were normalized with the results of 18 S ribosomal RNA. The Real-time quantitative RT-PCR was performed in three replicates for each sample.

#### Statistical analysis

All values are shown as the mean  $\pm$  SE for three replicates. The data were analyzed and Tukey's HSD test ( $P < 0.05$ ) was used to compare the different varieties or treatments.

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-023-04173-3>.

Supplementary Material 1

#### Acknowledgements

Not applicable.

#### Author contributions

Masaya Kato, Gang Ma and Lancui Zhang: conceived and designed the experiments. Gang Ma, Risa Yamamoto and Nami Kojima: carried out the experiments and analyzed the data. Masaki Yahata: contributed to sample collection. All authors approved the final revision to be published.

#### Funding

This work was supported by KAKENHI Grant Numbers JP20H02976 (to M.K.), JP22K05610 (to G.M.), and JP19K06030 (to G.M.) from Japan Society for the Promotion of Science (JSPS).

#### Data Availability

All data generated or analyzed during this study are included in this published article.

*CitF3Hs* of Satsuma mandarin, Ponkan mandarin, and blood orange 'Moró' have been submitted to NCBI (<https://www.ncbi.nlm.nih.gov/>) with accession numbers: OQ148589- OQ148591.

#### Declarations

##### Ethics approval and consent to participate

Plant samples used in the study were collected from Fujieda Farm of Shizuoka University (Shizuoka, Japan). All methods and materials were in compliance with relevant institutional, national, and international guidelines and legislation.

##### Consent for publication

Not applicable.

##### Competing interest

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

Received: 16 December 2022 / Accepted: 15 March 2023

Published online: 03 May 2023

#### References

- Falcone Ferreyra ML, Rius SP, Casati P. Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Front Plant Sci.* 2012;28:222.
- Iwashina T. Flavonoid function and activity to plants and other organisms. *Biol Sci Space.* 2003;17:24–44.
- Iwashina T. Contribution to flower colors of flavonoids including anthocyanins: a review. *Nat Prod Commun.* 2015;10:529–44.
- Samanta A, Das G, Das S. Roles of flavonoids in plants. *Int J Pharm Sci Tech.* 2011;6:12–35.
- Berk Ş, Kaya S, Akkol EK, Bardakçı H. A comprehensive and current review on the role of flavonoids in lung cancer-experimental and theoretical approaches. *Phytomedicine.* 2022;98:153938.

6. Bharti S, Rani N, Krishnamurthy B, Arya DS. Preclinical evidence for the pharmacological actions of naringin: a review. *Planta Med.* 2014;80:437–51.
7. Garg A, Garg S, Zaneveld LJ, Singla AK. Chemistry and pharmacology of the Citrus bioflavonoid hesperidin. *Phytother Res.* 2001;15:655–69.
8. Kaur R, Sood A, Lang DK, Bhatia S, Al-Harrasi A, Aleya L, Behl T. Potential of flavonoids as anti-Alzheimer's agents: bench to bedside. *Environ Sci Pollut Res Int.* 2022;29:26063–77.
9. Roohbakhsh A, Parhiz H, Soltani F, Rezaee R, Iranshahi M. Molecular mechanisms behind the biological effects of hesperidin and hesperetin for the prevention of cancer and cardiovascular diseases. *Life Sci.* 2015;124:64–74.
10. Slika H, Mansour H, Wehbe N, Nasser SA, Itratni R, Nasrallah G, Shaito A, Ghaddar T, Kobeissy F, Eid AH. Therapeutic potential of flavonoids in cancer: ROS-mediated mechanisms. *Biomed Pharmacother.* 2022;146:112442.
11. Davies KM, Jibran R, Zhou Y, Albert NW, Brummell DA, Jordan BR, Bowman JL, Schwinn KE. The evolution of flavonoid biosynthesis: a bryophyte perspective. *Front. Plant Sci.* 2020;11:7.
12. Liu W, Feng Y, Yu S, Fan Z, Li X, Li J, Yin H. The flavonoid biosynthesis network in plants. *Int J Mol Sci.* 2021;22:12824.
13. Naik J, Misra P, Trivedi PK, Pandey A. Molecular components associated with the regulation of flavonoid biosynthesis. *Plant Sci.* 2022;317:111196.
14. Winkel-Shirley B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* 2001;126(2):485–93.
15. Li D, Ni R, Wang P, Zhang X, Wang P, Zhu T, Sun C, Liu C, Lou H, Cheng A. Molecular basis for chemical evolution of flavones to flavonols and anthocyanins in land plants. *Plant Physiol.* 2020;184:1731–43.
16. Kim BG, Kim JH, Kim J, Lee C, Ahn JH. Accumulation of flavonols in response to ultraviolet-B irradiation in soybean is related to induction of flavanone 3-beta-hydroxylase and flavonol synthase. *Mol Cells.* 2008;25:247–52.
17. Liu Y, Qian J, Li J, Xing M, Grierson D, Sun C, Xu C, Li X, Chen K. Hydroxylation decoration patterns of flavonoids in horticultural crops: chemistry, bioactivity and biosynthesis. *Hortic Res.* 2022;9:uhab068.
18. Nishihara M, Yamada E, Saito M, Fujita K, Takahashi H, Nakatsuka T. Molecular characterization of mutations in white-flowered torenia plants. *BMC Plant Biol.* 2014;14:86.
19. Pelletier MK, Shirley BW. Analysis of flavanone 3-hydroxylase in Arabidopsis seedlings. *Plant Physiol.* 1996;111:339–45.
20. Singh K, Rani A, Kumar S, Sood P, Mahajan M, Yadav SK, Singh B, Ahuja PS. An early gene of the flavonoid pathway, flavanone 3-hydroxylase, exhibits a positive relationship with the concentration of catechins in tea (*Camellia sinensis*). *Tree Physiol.* 2008;28:1349–56.
21. Song X, Diao J, Ji J, Wang G, Guan C, Jin C, Wang Y. Molecular cloning and identification of a flavanone 3-hydroxylase gene from *Lycium chinense*, and its overexpression enhances drought stress in tobacco. *Plant Physiol Biochem.* 2016;98:89–100.
22. Zhang H, Zhao L, Wang J, Zheng L, Dang Z, Wang Y. Cloning and functional analysis of two flavanone-3-hydroxylase genes from *Reaumuria trigyna*. *Acta Physiol Plant.* 2014;36:1221–29.
23. Jiang F, Wang JY, Jia HF, Jia WS, Wang HQ, Xiao M. RNAi-mediated silencing of the flavanone 3-hydroxylase gene and its effect on flavonoid biosynthesis in strawberry fruit. *J Plant Growth Regul.* 2013;32:182–90.
24. Liu ML, Li XR, Liu YB, Cao B. Regulation of flavanone 3-hydroxylase gene involved in the flavonoid biosynthesis pathway in response to UV-B radiation and drought stress in the desert plant, *Reaumuria soongorica*. *Plant Physiol Biochem.* 2013;73:161–7.
25. Mahajan M, Yadav SK. Overexpression of a tea flavanone 3-hydroxylase gene confers tolerance to salt stress and *Alternaria solani* in transgenic tobacco. *Plant Mol Biol.* 2014;85:551–73.
26. Watkinson JI, Hendricks L, Sioson AA, Vasquez-Robinet C, Stromberg V, Heath LS, et al. Accessions of *Solanum tuberosum* ssp *andigena* show differences in photosynthetic recovery after drought stress as reflected in gene expression profiles. *Plant Sci.* 2006;171:745–58.
27. Zheng Y, Tian L, Liu H, Pan Q, Zhan J, Huang W. Sugars induce anthocyanin accumulation and flavanone 3-hydroxylase expression in grape berries. *Plant Growth Regul.* 2009;58:251–60.
28. Gattuso G, Barreca D, Gargiulli C, Leuzzi U, Caristi C. Flavonoid composition of citrus juices. *Molecules.* 2007;12:1641–73.
29. Nagata U, Sakamoto K, Shiratsuchi H, Ishi T, Yano M, Ohta H. Flavonoid composition of fruit tissues of citrus species. *Bio Bio Biochem.* 2006;70:178–92.
30. Tripoli E, Guardia ML, Giammanco S, Majo DD, Giammanco M. Citrus flavonoids: molecular structure, biological activity and nutritional properties: a review. *Food Chem.* 2007;104:466–79.
31. Butelli E, Licciardello C, Zhang Y, Liu J, Mackay S, Bailey P, Reforgiato-Recupero G, Martin C. Retrotransposons control fruit-specific, cold-dependent accumulation of anthocyanins in blood oranges. *Plant Cell.* 2012;24:1242–55.
32. Butelli E, Garcia-Lor A, Licciardello C, Las Casas G, Hill L, Reforgiato GR, Keremane ML, Ramadugu C, Krueger R, Xu Q, Deng X, Fanciullino AL, Froelicher Y, Navarro L, Martin C. Changes in anthocyanin production during domestication of citrus. *Plant Physiol.* 2017;173:2225–42.
33. Moriguchi T, Kita M, Tomono Y, Endo-Inagaki T, Omura M. Gene expression in flavonoid biosynthesis: correlation with flavonoid accumulation in developing citrus fruit. *Physiol Plant.* 2001;111:66–74.
34. Mou J, Zhang Z, Qiu H, Lu Y, Zhu X, Fan Z, Zhang Q, Ye J, Fernie AR, Cheng Y, Deng X, Wen W. Multiomics-based dissection of citrus flavonoid metabolism using a *Citrus reticulata* × *Poncirus trifoliata* population. *Hortic Res.* 2021;8:56.
35. Pelt JL, Downes WA, Schoborg RV, McIntosh CA. Flavanone 3-hydroxylase expression in *Citrus paradisi* and *Petunia hybrida* seedlings. *Phytochemistry.* 2003;64:435–44.
36. Pelletier MK, Shirley BW. Analysis of flavanone 3-hydroxylase in Arabidopsis seedlings. Coordinate regulation with chalcone synthase and chalcone isomerase. *Plant Physiol.* 1996;111(1):339–45.
37. Deboob GB, Albertsen MC, Taylor LP. Flavanone 3-hydroxylase transcripts and flavonol accumulation are tempo-rally coordinated in maize anthers. *Plant J.* 1995;7:703–13.
38. Hall J, Ananga A, Georgiev V, Ochieng J, Cebert E, Tsolova V. Molecular cloning, characterization, and expression analysis of flavanone 3-hydroxylase (F3H) gene during muscadine grape berry development. *J Biotechnol Biomater.* 2015;5:2.
39. Huang D, Wang Xia, Tang Z, Yuan Y, Xu Y, He J, Jiang X, Peng S, Li L, Butelli E, Deng X, Xu Q. Subfunctionalization of the *Ruby2-Ruby1* gene cluster during the domestication of citrus. *Nat Plants.* 2018;4:930–41.
40. Huang D, Yuan Y, Tang Z, Huang Y, Kang C, Deng X, Xu Q. Retrotransposon promoter of Ruby1 controls both light-and cold - induced accumulation of anthocyanins in blood orange. *Plant, cell & environ.* 2019; 42: 3092–3104.
41. Zhang L, Ma G, Kato M, Yamawaki K, Takagi T, Kiriiwa Y, Ikoma Y, Matsumoto H, Yoshioka T, Nesumi H. Regulation of carotenoid accumulation and the expression of carotenoid metabolic genes in citrus juice sacs *in vitro*. *J Exp Bot.* 2012;63:871–86.
42. Seoka M, Ma G, Zhang L, Yahata M, Yamawaki K, Kan T, Kato M. Expression and functional analysis of the nobiletin biosynthesis-related gene *CitOMT* in citrus fruit. *Sci Rep.* 2020;10:15288.
43. Ma G, Zhang L, Seoka M, Nakata A, Yahata M, Shimada T, Fujii H, Endo T, Yoshioka T, Kan T, Kato M. Characterization of a caffeic acid 8-O-methyltransferase from citrus and its function in nobiletin biosynthesis. *J Agric Food Chem.* 2022;70:543–53.

## Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.